Toluene Diisocyanate Enhances Substance P in Sensory Neurons Innervating the Nasal Mucosa

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Inhalation of irritants, such as toluene diisocyanate (TDI), stimulates substance P (SP) release from peripheral processes of sensory neurons innervating the airways. The purpose of this study was to determine if TDI inhalation affects intraneuronal levels of SP and preprotachykinin (PPT) messenger RNA (mRNA) in the sensory neurons of the trigeminal ganglion (TG) which innervate the nasal epithelium. The nasal cavity of Fisher-344 rats was instilled with rhodamine-labeled latex microspheres. Ten days later, the rats were exposed to 60 ppb of 2,4-2,6-TDI vapor for 2 h. The TG were removed 1, 12, 24, 48, 72, and 96 h after TDI treatment and prepared for SP immunocytochemistry and PPT *in situ* hybridization. SP nerve fiber density in nasal epithelium was significantly increased 12, 24, and 48 h after TDI exposure. The proportion of microsphere-labeled cell bodies expressing high levels of SP immunoreactivity was decreased at 24 h but was increased above controls at 48 and 72 h. The proportion of microsphere-labeled cell bodies expressing high levels of PPT mRNA was increased above control levels at 24 and 48 h. The percentage of leukocytes observed in nasal lavage fluid was significantly increased 12, 24, 48, and 72 h after inhalation. These studies indicate that SP production in TG neurons projecting to the nasal epithelium is transiently increased after TDI exposure, suggesting that TDI inhalation not only causes SP release but also increased intraneuronal neuropeptide levels. Increased neuronal SP levels may be involved in maintaining neurogenic inflammation or the development of airway hyperresponsiveness. Hunter DD, Satterfield BE, Huang J, Fedan JS, Dey RD. Toluene diisocyanate enhances substance P in sensory neurons innervating the nasal mucosa.

AM J RESPIR CRIT CARE MED 2000;161:543–549.

Toluene diisocyanate (TDI)-induced asthma occurs in approximately 10% of workers involved in the production of polyurethane polymers and in the manufacture of plastics, foams, adhesives, and surface coatings (1). Inhalation of TDI vapor in the workplace induces occupational asthma (2). In addition to asthma, TDI exposure also produces rhinitis and nasal irritation (3). The increasing and widespread application of isocyanates in the workplace has contributed to more exposed workers and greater prevalence of pulmonary and nasal disorders (1, 4).

Airway responses to irritant inhalation result in part from pulmonary reflexes mediated by sensory and autonomic nerve fibers in the airways. Sensory nerve fibers in the airways and the mediators they release may be involved in regulating normal and abnormal responses to occupational chemical irritants (5). In the upper airways, the nasal mucosa is provided with sensory innervation from the trigeminal ganglia (6). The 11 amino acid residue peptide substance P (SP) has been strongly implicated in sensory regulation of upper airway responses (7, 8). SP is localized within cell bodies of the trigeminal ganglia and in the peripheral terminals of sensory nerves associated with arteries, veins, mucous glands, and epithelium (9, 10). The SP-containing nerve fibers projecting to the nasal mucosa are classified as nociceptive C-fibers: extensively branched, small diameter, unmyelinated nerve fibers that convey painful sensation after mechanical, thermal, or chemical stimulation (11). Neurogenic inflammation is mediated by the release of SP from capsaicin sensitive C fibers in the rodent airway mucosa and is characterized by increased vascular permeability, plasma extravasation, glandular secretion, and neutrophil chemotaxis (12). Stimulation of sensory nerves innervating the vascular beds of the nasal or tracheobronchial mucosa causes an increased release of SP resulting in vasodilation (13).

Recent studies suggest that airway irritants affect neuropeptide levels in sensory neurons. For instance, allergen provocation in the nasal cavity increases SP content and release resulting in neurogenic inflammation that is reduced after treatment with SP antibodies (14). Similarly, the messenger RNA (mRNA) message for SP and SP immunoreactivity in sensory cell bodies in the nodose ganglion were increased in antigen-challenged sensitized guinea pigs (15).

Airway responses to TDI are mediated largely by C-fiber activation and SP release (16, 17). TDI also causes decreased nerve fiber density, suggesting SP release, in the airway mucosa in both sensitized and nonsensitized guinea pigs (18). TDI exposure in guinea pigs causes the release of SP from peripheral nerve endings in the nasal mucosa as well, and in-

(*Received in original form December 10, 1998 and in revised form July 29, 1999*)

Supported by NIH-NHLBI HL 35812 and NIOSH.

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creases preprotachykinin (PPT) mRNA message levels and SP immunoreactivity in sensory nerve cell bodies of the trigeminal ganglia after direct application of TDI to the nasal mucosa over a 7-d period (19). These studies raise the possibility that neuronal responses to inhaled irritants, including TDI, involve not only release of SP from peripheral processes of sensory nerves, but also increased PPT gene expression and SP production in the nerve cell body in order to maintain adequate neuronal SP levels for continued neuropeptide release.

In the present study, we investigated SP and PPT gene product levels in sensory neurons innervating the nasal epithelium in rats exposed by inhalation to TDI. We hypothesized that acute exposure to TDI would increase SP message and peptide levels selectively in neurons projecting to the nasal epithelium, because these fibers constitute the afferent limb of the axon reflex. We were particularly interested in the time course of the response, especially during the recovery phase and return to control values.

METHODS

General Protocol

A total of 54 Fischer-344 male rats, 175 to 200 g, were used to evaluate responses in trigeminal ganglia sensory neurons innervating the nasal epithelium after inhalation exposure to TDI. The nasal cavities of all rats were bilaterally instilled on two consecutive days with 8 μl of rhodamine-labeled latex microspheres, a retrograde neuronal tracer to label trigeminal cell bodies projecting specifically to the trigeminal ganglion (20). The rats were randomly assigned to one of two groups, a control group of 18 rats exposed to filtered ambient air, and an experimental group of 36 rats exposed to 60 parts per billion (ppb) of TDI for 2 h in inhalation chambers. Rats from both groups were euthanized after postexposure survival time periods of 1, 12, 24, 48, 72, or 96-h exposure by intraperitoneal injection of sodium pentobarbital (90 mg/kg; Nembutal; Abbott Laboratories, Chicago, IL). Nasal lavage was performed, 25 ml of nasal lavage fluid was collected, and cellular differentials were analyzed as an indication of inflammation.

The right and left trigeminal ganglia were dissected out and prepared for SP immunocytochemistry and PPT *in situ* hybridization respectively. The nasal mucosa, containing mostly respiratory and some transitional epithelium, was removed and prepared to evaluate SP nerve fiber density. This protocol was approved by the West Virginia University Animal Care and Use Committee (ACUC) (9708-07) and the National Institute for Occupational Safety and Heath ACUC (FED-GP-19).

Rhodamine-labeled Latex Microsphere Instillation

We have described and validated the use of rhodamine-labeled latex microspheres to label trigeminal neurons projecting to the nasal epithelium in a previous report (20). Briefly, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). Using a 10-μl Hamilton microsyringe with plastic tubing connected to the needle, two lengths of plastic tubing (0.8 cm or 1.3 cm) were used to deposit microspheres into the anterior and posterior regions of both nasal cavities. The rats were then rotated in a circular pattern around the anterior–posterior axis five times to ensure that the tracer was evenly distributed over the entire nasal mucosa. A transport period of 10 d was used to ensure consistent and complete labeling of trigeminal neurons innervating the nasal epithelium.

TDI Inhalation

Rats were divided into two groups and placed in a chamber constructed for the delivery of TDI as described previously (21). Rats in one chamber were exposed for 2 h to filtered air containing 60 ppb of 80% 2,4-TDI/20% 2,6-TDI mixture. TDI vapor was generated by passing fresh, filtered air through a glass impinger containing TDI solution kept at 37° C. Incoming air was passed from the air inflow valve to the desiccant filter system. The vapor was fed into a 3:1 mixing chamber kept at 37° C and was diluted with fresh air to achieve the desired final concentration of 60 ppb. The TDI level in the mixing chamber was monitored and regulated continuously by pumping vapor from the mixing chamber to the TDI detector, a fixed point ion mobility spectrometer (Environmental Technologies Group, Baltimore, MD), and adjusting the flow rate of dilutant air to the mixing chamber to regulate the TDI concentration at the desired level. A display of the level was given by the instrument every second, providing the opportunity for moment-to-moment adjustments in dilutant air flow, which actually were not necessary once the detector became stabilized at the desired endpoint before delivery to the rats. The other group of rats was exposed in identical chambers to ambient air as a control.

Nasal Lavage

The rats were anesthetized and a midline incision was made from the sternum to the mandible and the subcutaneous tissue and strap muscles were removed. The trachea was cross-clamped as far rostral as possible isolating the nasal cavities and nasal pharynx. Using a disposable polyethylene transfer pipette, 5-ml aliquots of phosphate-buffered saline (PBS) were instilled alternately into each nostril and collected from the opposite nostril. The nasal lavage was continued until a total of 25 ml was collected.

The nasal lavage fluid was centrifuged at 1,500 rpm for 10 min. The supernatant was removed and the resultant cellular pellet was resuspended in 1.0 ml of PBS. The cells were deposited on slides at a density of 1×10^6 cells/ml by centrifugation at 440 rpm for 4 min using a Cytospin (Shandon Scientific, Ltd., Cheshire, UK), fixed in methanol and stained with Wright's stain for differential cell counts. One hundred cells were characterized as neutrophils, eosinophils, basophils, lymphocytes, or macrophages and the percentage of neutrophils was calculated as an indicator of inflammation after TDI exposure. Red blood cells and epithelial cells were also observed in the Cytospin samples but were not included for differential analysis.

SP Immunocytochemistry

The right trigeminal ganglia 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 from the ganglion. The nasal mucosa was also removed from the nasal cavity of the animals. All tissues were fixed by immersion in picric acid–formaldehyde (22) fixative for 3 h and rinsed overnight in 0.1 M PBS containing 0.3% Triton-X-100 (PBS-Tx).

For cryostat sectioning, the ganglia were oriented on corks so the first section would be taken from the ventral surface. The nasal mucosa was wrapped around skeletal muscle to enable uniform sectioning. Then, the tissues were frozen in isopentane, cooled with liquid nitrogen, and stored in airtight bags at −60° C.

Every fifth section (12-μm thickness) of the entire right trigeminal ganglia was collected on gelatin-coated coverslips. Cryostat sections of the nasal mucosa were cut at 12 μm thickness and picked up on subbed slides. Depending on the thickness of the mucosa, enough sections were cut to adequately survey the entire tissue.

Immunocytochemical procedures for localizing SP-immunoreactive neurons were identical to those described previously (23). Briefly, cryostat sections on coated coverslips were covered with rabbit anti-SP antiserum (Peninsula, Belmont, CA) diluted 1:100, incubated in a humid chamber at 37° C for 30 min, were rinsed with a 1% bovine serum albumin–phosphate saline buffer (pH 7.8) containing triton-X solution (PBS-Tx+BSA), covered with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (ICN Immunobiologicals, Inc., Costa Mesa, CA) diluted 1:100, incubated at 37° C for 30 min, rinsed again in PBS-Tx+BSA, and mounted with Fluoromount G (Southern Biotechnology, Birmingham, AL). 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, 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 (i.e., SP-IR) or SP-like immunoreactivity (i.e., SP-LI) are used.

SP-IR of the cell bodies in the trigeminal ganglia containing rhodamine-labeled latex microspheres was evaluated by digital image analysis. First, all cell bodies containing rhodamine-labeled latex microspheres were identified using an Olympus BH-2 (Lake Success, NY) fluorescence microscope equipped with a rhodamine filter (exci-

tation 540 nm and emission 580 nm). Then SP-IR of the identified cell bodies was observed by the same microscope equipped with a fluorescein filter (excitation wavelengths from 455 to 500 nm and emission wavelengths > 500 nm). Both filters were mounted in a slider on the microscope so that FITC and rhodamine could be individually visualized in the same tissue section simply by changing the position of the slider. The intensity of FITC immunofluorescence was measured using the Optimas Image Analysis System (Bioscan Inc., Redmond, WA). The system was calibrated by selecting the brightest cell body in the tissue section and setting the fluorescence intensity value at 100%, and then selecting a negative cell body (no fluorescence) and setting the intensity at 0%. The individual cell bodies innervating the nasal epithelium were encircled and immunofluorescence intensity was computed for each neuron. Neurons with less than 25% intensity were considered negative, whereas the fluorescence intensity greater than 25% was considered SP-immunoreactive.

SP-IR of the nerve fibers in the nasal respiratory epithelium was evaluated by digital image analysis. The sections were observed using an Olympus BH2 fluorescence microscope equipped with a fluorescein filter (excitation wavelengths from 455 to 500 nm and emission wavelengths > 500 nm) and nerve fiber density was measured. A threshold was performed each time on the nerve fibers in a given area. The percent area of SP-IR fibers was quantitated by dividing the SP-IR nerve fiber area by the total area of the field. The entire length of the epithelium present in 10 to 20 sections was measured.

PPT *In Situ* Hybridization

Fresh, unfixed, left trigeminal ganglia were excised, frozen on cork, and sectioned as described previously. The sections were placed on gelatin-coated slides and gently dried using a hair dryer. Continuous serial sections were collected on five slides. The first slide was used for the identification of trigeminal ganglia neurons labeled with rhodamine-labeled latex microspheres. The second two slides were used for PPT anti-sense (PPT-AS) probe hybridization, and the final two slides were utilized for PPT-AS probe hybridization + ribonuclease (RNase) treatment for control slides. Approximately 100 cell bodies per ganglion were identified with rhodamine-labeled latex microspheres and were measured for SP mRNA grain density.

The rat SP complementary RNA (cRNA) probes used in this study were generated from a plasmid containing a rat α-PPT complementary DNA (cDNA) (24). The plasmid pG1α-PPT was linearized by using the restriction enzyme *Hin*dIII. Next a radiolabeled probe complementary to the coding sequence of rat α-PPT (PPT-AS probe) was transcribed from the plasmid template by using ^{35}S-uridine triphosphate (UTP) (1,000 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.

In situ hybridization procedures for localizing PPT mRNA are similar to those described previously (25). The slides were removed from the desiccator 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 1.0 ml triethanolamine, 0.25 ml acetic anhydride, and 0.9 g NaCl. The slides were dehydrated with ethanol and then delipidated with chloroform. For negative controls, a few slides were treated with ribonuclease A (RNase A; 100 mg/ml) and RNase T_1 (5 mg/ml) in Tris–EDTA–saline (TES) buffer 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 saline sodium citrate (SSC) twice for 10 min at room temperature. The SSC was a mixture of 87.65 g NaCl and 44.10 g citric acid in 400 ml of DEPC-treated water. 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 mM dithiothreitol, and the ^{35}S-radiolabeled α-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 and processed using Kodak D19 developer.

The cell bodies containing rhodamine-labeled latex microspheres were identified using a fluorescence microscope equipped with a rhodamine filter. Image analysis was used to quantify α-PPT mRNA expression in the identified cell bodies. The sections were examined under dark field with a Zeiss Axiovert Microscope at ×20 magnification and a Dage MT 1 CCD72 camera (Michigan City, ID). Autoradiographic grain density was determined in microsphere-containing cell bodies only. The grain density was quantitated using the Optimas Image Analysis System (Bioscan, Inc., Redmond, WA). The individual neurons were circled and the percent area of grains in each neuron was computed. The percent of grain density was calculated by dividing the grain density area by the total cell body area.

Statistical Analysis

The means and standard errors were calculated for percentage of SP-immunoreactive neurons and of neurons with PPT mRNA-positive grain density in the trigeminal ganglia. Nerve fiber density in epithelium, and percent neutrophils in lavage fluid were calculated for TDI-exposed groups at each recovery time period and a single air-exposed control group. All slide were coded prior to data collection to avoid possible evaluator bias. For statistical analysis, a one-way analysis of variance (ANOVA) using recovery time as the single variable was performed using a single nonexposed group as the control. Significance was set at $p \leq 0.05$. Upon obtaining significant F values, the individual means were compared with the unexposed control group using the least significant difference test. All analyses were done using Sigma Stat 2.0 (Jandel Scientific, San Rafael, CA).

RESULTS

Percentages of Neutrophils Observed in Nasal Lavage Fluid after TDI Inhalation Exposure

The percentage of neutrophils in the nasal lavage fluid was significantly increased above controls at 1, 12, 24, 48, and 72 h after TDI inhalation in comparison to control values (Figure 1). In the control animals, the percentage was 22.67 ± 1.38%. Neutrophils increased as early as 1 h after TDI exposure (51.00 ± 3.56%) and the greatest increase in neutrophil differential was observed 12 h after TDI inhalation (77.00 ± 3.46%). Thereafter, the percentage decreased 24 (51.83 ± 4.46%), 48 (50.17 ± 3.89%), and 72 (50.00 ± 3.19%) h after

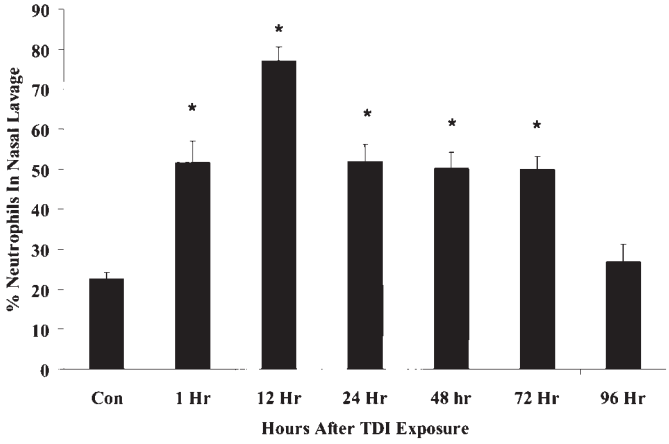

Figure 1. The percentage of neutrophils observed in the nasal lavage fluid at 1, 12, 24, 48, 72, and 96 h survival time after TDI exposure (TDI) or in ambient air control (CON). *Asterisk* denotes significant TDI-induced change relative to controls ($p \leq 0.05$). n = 6 for each survival time period.

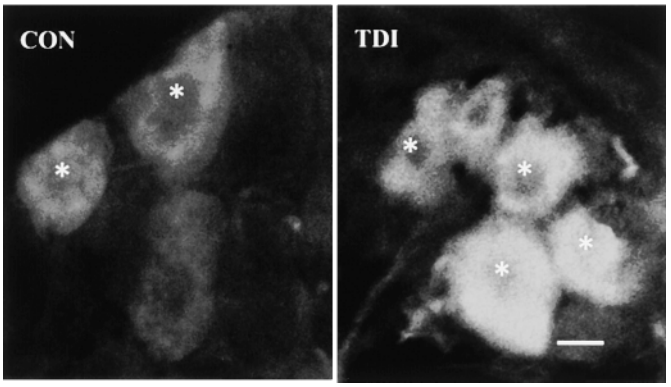

Figure 2. Photomicrographs showing the localization of SP-IR in microsphere-labeled (*) cell bodies of the trigeminal ganglia from CON and TDI-exposed rats for the 48-h postexposure time period. Note increased fluorescence intensity in cell bodies from TDI-treated group. Original magnification: ×400; bar = 20 μm.

TDI exposure; however, the percentages were still increased significantly above the control values. The differential returned to control levels 96 h after the cessation of TDI inhalation (26.83 ± 4.35%). Eosinophils were not prominent in the lavage fluid and most cells were macrophages or lymphocytes.

TDI-induced Changes in SP Immunofluorescence in the Cell Bodies of Trigeminal Ganglia Innervating the Nasal Epithelium

Changes in SP-IR of trigeminal ganglia cell bodies innervating the nasal epithelium depended on the length of time after TDI exposure (Figures 2 and 3). Initially, the frequency of SP-containing cell bodies significantly decreased from 82.50 ± 2.20% in control rats to 58.40 ± 3.80% in TDI-exposed rats 24 h after exposure. Then, the proportion of positive SP-immunoreactive cell bodies markedly increased from 72.60 ± 4.40% in control rats to 94.30 ± 4.70% after TDI exposure and from 78.30 ± 6.10 in control rats to 92.40 ± 5.30% after TDI exposure at 48 h and 72 h postexposure, respectively. SP-IR returned to control levels 96 h after cessation of TDI inhalation.

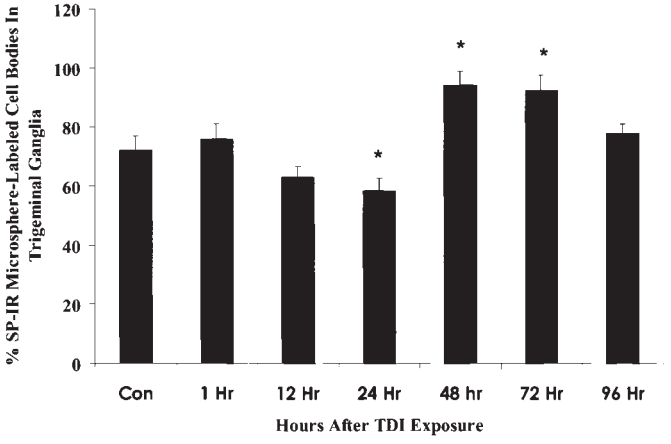

Figure 3. The percentage of microsphere-labeled cell bodies in the trigeminal ganglia demonstrating high SP-IR at 1, 12, 24, 48, 72, and 96-h survival time periods after TDI and ambient air CON. *Asterisk* denotes significant TDI-induced changes relative to controls ($p \leq 0.05$). n = 6.

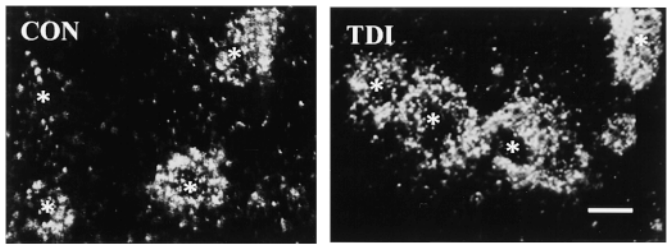

Figure 4. Photomicrographs showing autoradiographic localization of PPT mRNA in microsphere-labeled (*) cell bodies of trigeminal ganglia from CON and TDI-exposed rats at the 48-h survival time period. Autoradiographic signal from PPT mRNA is increased in cell bodies of TDI-exposed group. Original magnification: ×400; bar = 20 μm.

TDI-induced Changes in PPT mRNA in the Cell Bodies of Trigeminal Ganglia Neurons Innervating the Nasal Epithelium

The PPT mRNA expression in trigeminal ganglia cell bodies innervating the nasal epithelium was upregulated after TDI exposure (Figures 4 and 5). At 24 and 48 h after TDI exposure, the grain density in microsphere-labeled cell bodies was increased in comparison to controls. At the 24-h recovery period, 69.20 ± 2.00% of cell bodies in control rats were positive for PPT mRNA in comparison to 93.00 ± 6.10% in the TDI-exposed rats. In the 48-h recovery period, 71.50 ± 5.10% of the cell bodies expressed PPT mRNA in the control group and 95.70 ± 5.20% were positive in the TDI group. TDI-induced changes in SP mRNA expression in trigeminal cell bodies had returned to preexposure levels 72 h after TDI inhalation.

TDI-induced Changes in SP–Nerve Fiber Density in the Nasal Epithelium

The percent area of SP-immunoreactive nerve fibers in the nasal epithelium significantly changed over the 96-h time course after TDI inhalation (Figures 6 and 7). Initially, the percent area

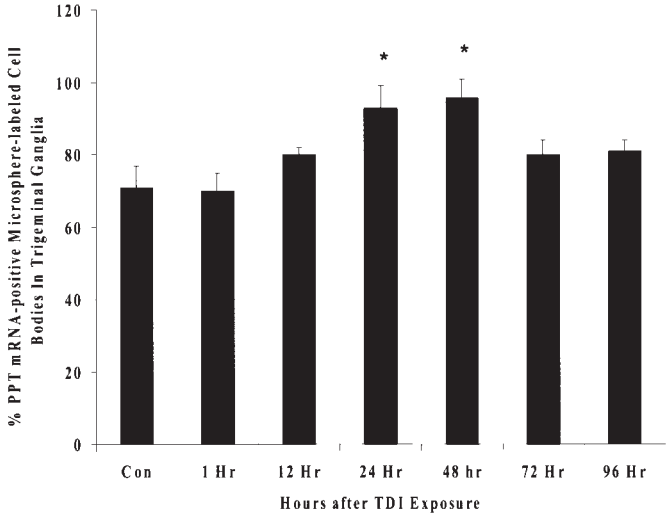

Figure 5. The percentage of microsphere-labeled cell bodies in the trigeminal ganglia that were PPT mRNA–positive at 1, 12, 24, 48, 72, and 96-h survival time periods after TDI and CON. *Asterisk* denotes a significant TDI-induced change relative to CON ($p \leq 0.05$). n = 6.

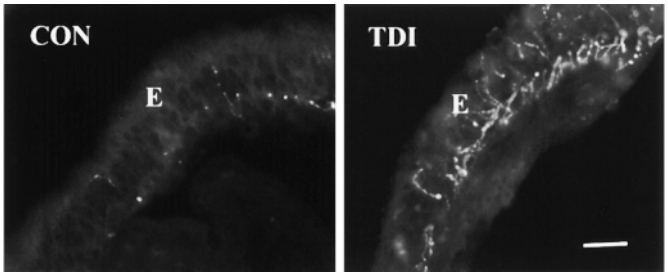

Figure 6. Photomicrographs showing the localization of SP-immunoreactive nerve fibers in the nasal epithelium (E) in CON and TDI-exposed rats at the 24-h survival time period. Innervation density is clearly greater after TDI exposure. Original magnification: ×200; bar = 20 μm.

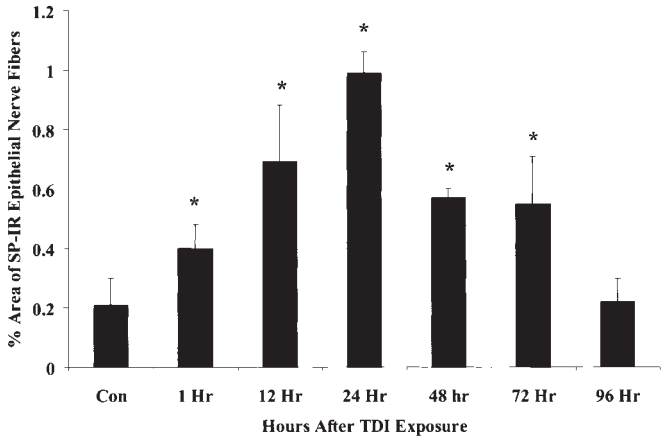

Figure 7. The percent area of SP-IR nerve fibers in the nasal epithelium of control or 1, 12, 24, 48, 72, and 96-h survival time periods after TDI inhalation. *Asterisk* denotes a significant TDI-induced change relative to CON ($p \leq 0.05$). n = 6 for TDI and n = 3 for CON at each time period.

of epithelial nerve fibers containing SP-IR was significantly increased from 0.21 ± 0.09 in the control group to 0.69 ± 0.19% in the TDI-exposed group 12 h after exposure. The 24-h time point demonstrated the maximal response in SP–nerve fiber density of 0.99 ± 0.07% and was still elevated at the 48-h postexposure (0.57 ± 0.03%). Thereafter, the SP-IR in the nerve fibers of the nasal epithelium began to recover to control levels (0.55 ± 0.16 at 72 h and 0.22 ± 0.08 at 96 h).

DISCUSSION

In the present study, sensory neurons in the trigeminal ganglion innervating the nasal epithelium were identified by retrograde transport of rhodamine-labeled latex microspheres from the nasal cavity (20). Using this procedure, it was possible to evaluate SP and PPT mRNA levels in sensory neurons innervating the nasal mucosa in rats exposed to the airway irritant TDI. A unique aspect of this study is that the labeling procedure specifically identifies nerve cell bodies of neurons with fiber projections to the nasal epithelium. Thus, assuming that collateral fibers of the same axon innervate blood vessels and glands in the nasal submucosa, it is likely that the identified neurons are mediating axon reflexes.

Previous studies have demonstrated that a single TDI exposure is sufficient to produce airway epithelial damage, inflammation, and bronchial smooth muscle hyperresponsiveness (17, 26). TDI-induced airway hyperresponsiveness is mediated in part by the release of SP from capsaicin-sensitive sensory nerve fibers in airway wall (16, 27). The present study demonstrates that acute TDI inhalation significantly alters SP and PPT mRNA levels in sensory neurons innervating the nasal epithelium. The findings suggest that, in addition to release of SP from sensory nerve endings, TDI exposure also activates a more generalized neuronal response involving increased SP synthesis in the nerve cell body.

Our study revealed that the neuronal response to TDI consisted of several different and seemingly coordinated events. First, SP nerve fiber density in the epithelium was increased 12 h after TDI inhalation and persisted through the 48-h postexposure time point. Increased nerve fiber density probably indicates increased SP levels in nerve fibers in which SP was previously lacking or undetectable. The early and rapid increase in SP levels in epithelial nerve terminals suggests enhanced axonal transport of neuropeptides. Previous studies have shown that inflammatory stimuli produce increased axonal transport of SP in dorsal root ganglion neurons (28). Increased levels of SP in epithelial fibers could mean that the amount of SP available for release from collateral fibers projecting to targets in the nasal submucosa is similarly increased. Coincident with the increased nerve fiber density, the percent of SP-immunoreactive trigeminal cell bodies was decreased, suggesting a reduction in SP levels in the cell bodies. At the same time point, the percent of PPT mRNA–positive cell bodies was increased, suggesting that the neurons were synthesizing new PPT message for enhanced SP synthesis. The close association in the rise (24, 48, and 72 h) and subsequent resolution (96 h) of nerve fiber density and the intrasomal SP and PPT mRNA levels supports the concept that the acute TDI exposure induces a neuronal response which includes initial SP release followed shortly by increased axonal transport and synthesis of SP and eventually a return to baseline values. The mechanisms responsible for reestablishing normal SP levels in the neuron may be an important aspect of disease processes such as rhinitis or asthma. In one study, SP levels in bronchoalveolar lavage fluid of asthmatics exposed to pollen were elevated even during periods outside of the pollen season (14), suggesting that elevated SP production may persist beyond the period of irritant inhalation. Failure to reestablish normal levels of SP in the nerve terminal may lead to higher than normal SP release and cause sustained hyperreactivity and inflammation.

A few studies have examined changes in SP and PPT mRNA levels in sensory nerve cell bodies. Most notably, direct application of TDI to the nasal mucosa on several consecutive days caused an increase SP nerve fiber density in the mucosa that coincided with a decrease in SP fluorescence intensity and an increase in PPT mRNA expression in trigeminal cell bodies (19). In a guinea pig model of ovalbumin sensitization with antigen challenge, the number of PPT mRNA–positive neurons in nodose ganglia was elevated 12 h after challenge and the SP-IR and calcitonin gene-related peptide (CGRP)-immunoreactivity in nerve cell bodies and SP levels in lung and airway extracts were all elevated 24 h after challenge (15). These studies support the view that SP release from sensory nerve fibers after exposure to inflammatory agents or allergens elicits a neuronal response characterized by enhanced gene expression and SP synthesis.

It is generally thought that SP release in the nasal cavity mediates or promotes protective responses including mucosal edema, mucous secretion, and inflammatory cell influx. As

shown in the present study, the enhanced SP production in sensory neurons projecting to the nasal cavity is protracted over days after an acute exposure, and may persist for weeks or longer in response to continuous exposures (19). Although released SP is actively metabolized by neutral endopeptidase (NEP) in the airways (27), recent evidence has shown that NEP is not extensively inhibited by TDI in the concentration range of 20 to 100 ppb (29), suggesting that increased SP release may account for enhanced capsaicin-sensitive hyperresponsiveness after TDI exposure. The ability of neurons to maintain or upregulate the SP levels in order to provide adequate supplies of SP at the nerve terminal appears essential. Reuptake of SP is not an important factor in either termination of action or maintaining neuronal SP levels (30). Thus, increased synthesis in the nerve cell body and enhanced axonal transport is probably a primary mechanism to replace and maintain SP levels in nerve terminals.

This study raises the question of how irritants initiate and upregulate neuropeptide synthesis in airway neurons. Previous studies have shown that TDI-induced sensory nerve activation occurs through the release of prostanoids (31), and a variety of mediators, including bradykinin, activate capsaicin-sensitive nerve fibers (6). The data show that neutrophil influx correlates with the timing of SP and mRNA increases and subsequent return to baseline, indicating that inflammation and neuronal SP responses are occurring in parallel. The early increase in neutrophil influx (at 1 h) suggests that the enhanced SP production may result from inflammatory mediators. Mediators associated with inflammation, such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) (32), are known to enhance SP synthesis. Another possible mechanism that may mediate upregulation of SP synthesis involves the release of neurotrophic agents such as nerve growth factor (NGF). NGF is synthesized in epithelial tissues (33). Recent studies demonstrated increased SP levels and SP-containing nerve fibers in airways of transgenic mice overexpressing the NGF gene (34) and increased SP levels both in airway tissues and in the nodose ganglion when NGF was instilled into the airway (35). Thus, although not fully defined, part of the neural response to inhaled irritants in the nasal cavity may be mediated through the actions of inflammatory mediators and growth factors.

In summary, this study showed that acute inhalation exposure of adult rats to 60 ppb TDI induces a response in trigeminal sensory neurons projecting to the nasal epithelium characterized by increased nerve fiber density in the nasal epithelium and increased SP and PPT mRNA levels in cell bodies. The responses generally increased for up to 48 h and returned to control levels 96 h after exposure. The findings support the concept that, in addition to SP release, inhaled airway irritants such as TDI initiate neuronal responses involving increased SP synthesis in sensory neurons. This process is probably essential to sustain airway responses to inhaled irritants and may be involved in the pathogenesis of prolonged airway inflammation and hyperresponsiveness seen in various forms of asthma.

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