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The role of nerve growth factor in neuropeptide up-regulation in trigeminal ganglia neurons following irritant exposure.

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ABSTRACT

The Role of Nerve Growth Factor in Neuropeptide Up-Regulation in Trigeminal Ganglia Neurons Following Irritant Exposure

Erin R. Wilfong

Workers exposed to various occupational irritants often develop symptoms ranging from nasal irritation to asthma. The mechanisms involved in the development of airway inflammation are unclear. In laboratory animals, occupational irritants such as silica, toluene diisocyanate, acrolein and ozone activate sensory nerves located in airway walls. The sensory nerves respond by increasing the production and release of neuropeptides. The main neuropeptide in the upper airway, substance P (SP), is produced in sensory nerve cell bodies located in the trigeminal ganglia (TG) and released from corresponding nerve endings in the nasal mucosa. Neurogenic inflammation results from the release of neuropeptides, especially SP, and is characterized by vasodilation, plasma extravasation, mucous secretion and neutrophil chemotaxis. The purpose of these studies was to characterize the response and regulation of neuropeptides in the upper airway after exposure to irritants.

Nasal irritation to asphalt fumes is a commonly reported symptom among road crew workers. In the first study, neuropeptide responses were characterized in the upper airway after asphalt fume exposure. Rats chronically exposed to asphalt fumes had increased levels of neuropeptides in TG neurons innervating the nasal epithelium and increased inflammatory cells in their nasal cavities. The enhanced neuropeptide production may produce neurogenic inflammation, which manifests as symptoms of nasal irritation in road construction workers exposed to asphalt fumes.

Since occupational exposure to airway irritants has health implications that may be related to increased neuropeptide production, the purpose of the remaining studies was to investigate mechanisms regulating SP production. Nerve growth factor secretion in the nasal cavity is believed to mediate irritant induced up-regulation of SP. Increased NGF levels in the nasal cavity preceded the up-regulation of SP in TG neurons innervating the nasal epithelium following TDI exposure. NGF is known to bind receptors on sensory nerve terminals and travel to cell bodies where it regulates SP production. This temporal relationship suggests that irritant-induced SP expression in sensory neurons maybe mediated by NGF.

To prove that NGF mediates SP production following irritant exposure, compounds were administered prior to TDI exposure to block NGF activity. The first compound, an antibody raised against NGF, failed to prevent the SP increase in airway sensory nerves and the inflammatory cell influx into the nasal mucosa. However, increased SP levels were also detected in the airway sensory nerves of control animals, indicating that the antibody initiated an inflammatory response. The second compound, K252a, a non-specific tyrosine-kinase inhibitor, prevented the increase in SP production and the inflammatory cell influx. In addition to blocking NGF receptor activation on sensory nerve terminals, K252a inhibits the phosphorylation of other neurotrophin

receptors and kinases. The final study used an *in vivo* and *ex vivo* approach to demonstrate that NGF is synthesized and released from the nasal mucosa and the release of NGF increases following TDI exposure. Therefore, these studies support the concept that NGF release from the nasal mucosa following TDI correlates with SP production in TG cell bodies.

PREFACE

This dissertation begins with a review of pertinent scientific literature followed by a series of five studies and a general unified discussion of the findings.

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GLOSSARY OF ABBREVIATIONS

AHR	Airway Hyperresponsiveness
ACGIH	American Conference of Governmental Industrial Hygienists
ANOVA	Analysis of Variance
BCA	Bicinchroninic Acid
BSA	Bovine Serum Albumin
BDNF	Brain Derived Neurotrophic Factor
CGRP	Calcitonin Gene Related Peptide
cDNA	Complimentary DNA
Ct	Threshold Cycle
DRG	Dorsal Root Ganglia
ELISA	Enzyme Linked Immunosorbent Assay
EA	Ethyl Acetate
IgG	Immunoglobin G
IR	Immunoreactivity
MGV	Mean Gray Value
mRNA	Messenger RNA
MAPK	Mitogen Activated Protein Kinase
NFD	Nerve Fiber Density
NGF	Nerve Growth Factor
NKA	Neurokinin A
NK-1	Neurokinin-1 Receptor
NT-3	Neurotrophin-3

NT-4/5	Neurotrophin 4/5
PBS	Phosphate Buffered Saline
PBS-Tx	Phosphate Buffered Saline and Triton X-100
PI3K	Phosphatidylinositol 3-Kinase
PLC- γ	Phospholipase C-γ
PPT	Preprotachykinin
RT-PCR	Real Time Polymerase Chain Reaction
RSV	Respiratory Syncytial Virus
RPM	Rotations Per Minute
SP	Substance P
SCG	Superior Cervical Ganglion
TLV	Threshold Limit Value
TWA	Time Weighted Average
TRPV1	Transient Receptor Potential Ion Channel
trkA	Tropomyosin Related Kinase A

INTRODUCTION

Structure of the Upper Airway

The upper respiratory tract consists of the nose, larynx and trachea. Functions of the upper airway include monitoring, filtering and humidifying inspired air. On an average day, humans inhale approximately 10,000 L of air. The majority of inhaled air enters the respiratory tract through the nasal cavity, which in humans has an average volume of 25 cm^3 (Montgomery *et al.*, 1979). Depending on the environment, inhaled air may contain particulate matter of varying toxicity or biological activity. Despite obvious anatomical differences in size and shape, the nasal passages of humans and mammals have similar physiological properties, which aids in the removal of soluble and insoluble particles from inhaled air. Although small in size relative to the lower airways, the anatomical and histological design of the nasal cavity provides the first line of defense against inhaled irritants.

The nasal airway extends from the external nares at the nostril to the nasopharynx (Morgan & Monticello, 1990). The nasal vestibule, which is located posterior to the external nasal opening, is lined with stratified squamous epithelium and vibrasse. The hairs trap and filter larger particulate matter (>3μm) from inspired air. However, the main site of filtration occurs in the inferior, middle and superior turbinates. The turbinates are bony shelves projecting from the lateral nasal wall into the nasal cavity. Large-diameter inspired particles collide with the turbinates, a process termed impaction. The mucociliary apparatus subsequently clears the trapped particles. The convoluted passages formed by the turbinates not only define airflow direction and rate but also

increase the luminal surface area. The large surface area allows inhaled air to maintain close contact with the respiratory epithelium covering the turbinates, which facilitates humidification (near 100% relative humidity) and temperature regulation (37 ˚C) (Harkema, 1990; Keck *et al.*, 2000).

The complex design of the nasal cavity is also apparent at the microscopic level. Approximately 46.5% of the Fischer 344/N rat nasal cavity is lined by respiratory epithelium (Gross *et al.*, 1982). The mucous-coated respiratory epithelium forms a protective barrier along most of the upper airway. Generally, particles smaller than 3 μm and larger than 0.5 μm are filtered by the nasal mucosa and transported by ciliary propulsion to the nasopharynx (Schwab & Zenkel, 1998). The rat respiratory epithelium is comprised of six morphologically distinct and unequally distributed cell types including ciliated cells, mucous goblet cells, non-ciliated columnar cells, cuboidal cells, brush cells and basal cells. Where cilia are present there are 50-100 per cell and the ciliated cells increase in number from the anterior to the posterior areas of the nasal cavity (Popp & Martin, 1984). The complexity and diversity of the epithelium in rats and other species may correlate directly with susceptibility to irritant injury.

Lying beneath the basement membrane, the nasal submucosa is comprised of resident inflammatory cells in addition to glands, nerves, blood and lymph vessels and connective tissue. The predominant resident cells have been identified from human nasal biopsies and include lymphocytes, monocytes (Lim *et al.*, 1995) and mast cells (Bradding *et al.*, 1993). Small numbers of neutrophils and eosinophils have also been detected (Lim *et al.*, 1995). The epithelial and mast cells of the nasal mucosa also contain a number of pro-inflammatory cytokines. Interleukin-8 (IL-8) appears to be confined to

the cytoplasm of epithelial cells while IL-4, IL-5 and IL-6 are present in mast cells (Bradding *et al.*, 1993). In addition to various inflammatory cells, the submucosa has a number of seromucous glands. The main duct of the glands are lined with simple cuboidal epithelium and divide into two side ducts that collect secretions from several tubules lined with either serous or mucous cells. The respiratory epithelium also contains intraepithelial glands that consist of 20-50 mucous cells arranged radially around a small lumen. Compared to seromucous glands, the intraepithelial glands and goblet cells produce only a small amount of mucous. The glandular secretions form a protective coating throughout the nasal cavity. In addition to glands, the submucosal blood supply, which originates from the sphenopalatine artery, also plays a key role in maintaining a homeostatic environment in the nasal cavity. Arterial branches ascend perpendicularly toward the surface before forming fenestrated capillary networks next to the respiratory epithelium and around the glandular tissue (Dawes & Prichard, 1953). The fenestrae always face the respiratory epithelium and are believed to be one of the sources of fluid for humidification and inflammation (Cauna & Hinderer, 1969). The rat nasal mucosa also receives extensive innervation from sympathetic, parasympathetic and sensory ganglia (Grunditz *et al.*, 1994). Innervation plays a central role in defense and homeostatic maintenance.

Autonomic Innervation of the Nasal Cavity

The autonomic nervous system controls blood flow and glandular secretion in the nasal cavity. The parasympathetic branch of the autonomic system is responsible for increased glandular secretion and vasodilation. Preganglionic parasympathetic fibers

destined for the upper airways originate in the superior salivary nucleus of the midbrain and synapse in the sphenopalatine ganglion (Goldiing-Wood, 1961; Nomura & Matsuura, 1972). Preganglionic parasympathetic nerves release acetylcholine, which binds to nicotinic receptors on postganglionic neurons in airway walls. The postganglionic parasympathetic nerve fibers supplying the nasal mucosa primarily contain acetylcholine and vasoactive intestinal peptide (VIP) and innervate epithelium, glands and some vessels (Wells & Widdicombe, 1986; Baraniuk & Kaliner, 1995). Nasal mucous secretions are predominately controlled by acetylcholine acting on M_3 muscarinic receptors. In animals, electrical stimulation of parasympathetic nerves induces glandular secretions that are blocked by atropine, a muscarinic receptor antagonist (Wells & Widdicombe, 1986).

Few studies have focused on sympathetic reflexes in the nose. Preganglionic sympathetic nerves originate in the thoracolumbar region of the spinal cord and synapse with postganglionic cells in the superior cervical ganglion (Franke & Bramante, 1964; Wang *et al.*, 1987). Postganglionic sympathetic fibers primarily innervate arteries, arterioles and veins and release either norepinephrine or a combination of norepinephrine and neuropeptide Y. Binding of these transmitters to α_1 and α_2 adrenergic receptors and neuropeptide Y receptors on the smooth muscle of blood vessels causes potent vasoconstriction (Malm, 1973; Uddman & Sundler, 1966). Agonists at α_1 and α_2 adrenergic receptors are successfully used as nasal decongestants in humans (Tai & Baraniuk, 2002). In addition to autonomic nerves, sensory nerves also innervate the nasal cavity.

Sensory Innervation of the Nasal Cavity

The fifth cranial nerve, the trigeminal, provides sensory innervation to the nasal cavity (Grunditz *et al.*, 1994). The cell bodies of the trigeminal ganglia (TG) are located in a dural recess ventral to the pons. The peripheral processes of the trigeminal cell bodies form three main branches: the ophthalmic (V1), the maxillary (V2) and the mandibular (V3). Only the ophthalmic and the maxillary divisions have peripheral processes innervating the nasal mucosa (Allen, 1924).

The afferent fibers projecting to the nose are extensively branched, small diameter, unmyelinated C-fibers (Lundblad *et al*., 1983; Lundblad, 1984). Afferent fibers in the airways are classified based on conduction velocity, and nerves that conduct action potentials less than 2 m/s are termed C-fibers (Erlanger & Gasser, 1924). In the feline nasal cavity, a small percentage of faster conducting (3 m/s) Aδ fibers are also detectible (Wallois *et al.*, 1993).

Unlike autonomic nerves, which terminate near targets in the submucosa, sensory nerves run perpendicular to the basal lamina and can ascend between epithelial cells reaching within 1 μm of the surface (Finger *et al.*, 1990). In addition to epithelium, sensory nerves also innervate mucous glands and blood vessels. Innervation is dense near small arteries and arterioles (Baraniuk & Kaliner, 1990). Activation of these nerves initiates central and peripheral reflexes.

Activation of Afferent Nerves

C-fibers can be activated by noxious chemical, mechanical or thermal stimuli (Anton & Peppel, 1991; Dubner & Benett, 1983). The resulting depolarization generates

action potentials that are conducted orthodromically toward the central nervous system and antidromically down collateral branches. The peripheral component of this process is termed an axon reflex (Barnes, 1986).

Nociceptive information is carried from trigeminal primary afferents in the nasal cavity to corresponding central projections in the subnucleus caudalis and subnucleus interpolaris, which are areas located in the trigeminal brainstem nuclear complex and known to be involved in processing of nociceptive information (Anton & Peppel, 1991). Projections have also been detected in the nucleus tractus solitarius, which is involved in respiratory control (Anton & Peppel, 1991). Central reflexes producing symptoms of pain (Kobal & Hummel, 1988), sneezing and apnea are involved in protecting the airways and can be initiated by the application of sensory nerve irritants such as capsaicin to the human nasal mucosa (Geppetti *et al.,* 1988; Lundblad, 1984).

Locally, sensory nerve activation stimulates the release of neuropeptides from sensory nerve terminals leading to neurogenic inflammation in the airways (Lee *et al.*, 1985a). Sensory nerves and the neuropeptides they contain mediate a number of functions in the nasal mucosa.

Neuropeptides in the Nasal Cavity

The first neuropeptide to be studied, substance P (SP), was originally discovered in 1931 by von Euler and Gaddum. SP is an 11-amino-acid peptide (Chang *et al*., 1971) localized within cell bodies of the TG and in peripheral terminals of sensory nerves located near arteries, veins, mucous glands and epithelium (Lee *et al.*, 1985a). SP is synthesized in cell bodies of the TG from a preprotachykinin (PPT) gene and undergoes

post-translational modification during peptide storage and axonal transport (Helke *et al.*, 1990). Once released from nerve terminals, SP primarily binds neurokinin 1 (NK-1) receptors, although it may also exert effects through the NK-2 and NK-3 receptors (Piedimonte, 1995). These receptors are localized within epithelium, around arteries and veins and on mucous glands in the human nasal mucosa (Baraniuk *et al.*, 1991). Upon binding to NK-1, SP launches a receptor-mediated inflammatory response characterized by mucus secretion, plasma extravasation and vasodilation (Jancso *et al*., 1967; Lundberg & Saria, 1983; Baluk *et al.*, 1992). After release, SP is rapidly destroyed by tissue peptidases neutral endopeptidase and angiotensin-conveting enzyme (Katayama *et al.*, 1991). The effect of SP on nasal mucosal secretions was demonstrated with the topical application of SP at concentrations as low as 10^{-7} M, to the guinea pig nasal mucosa. Within 10 minutes, albumin and total protein, the markers for plasma extravasation and glandular secretion, respectively, were elevated in the nasal lavage fluid (Gawin *et al.*, 1993). In the lower airways, SP receptors are also found in high density on airway smooth muscle from the trachea down to the small bronchioles (Lundberg *et al.*, 1984) and mediate smooth muscle contraction (Lundberg *et al.*, 1983).

Although SP has been implicated as the primary contributor in upper airway sensory responses (Baraniuk *et al.*, 1991), the neuropeptide, calcitonin-gene-related peptide (CGRP) is co-stored and co-released with SP (Lee *et al*., 1985b; Lundberg *et al.*, 1985). CGRP, a 37-amino-acid peptide occurs in two forms, α and β, which differ by three amino acids, and CGRP is synthesized from a calcitonin gene in cell bodies of the TG (Williams *et al.*, 1988). Nerve endings containing CGRP are extensively located along the walls of arterioles and venules. Upon release and binding to specific G protein

coupled receptors, CGRP causes long lasting vasodilatory effects (Stjarne *et al.*, 1989); (Zhao & Tao, 1994). Another neuropeptide found in the upper airways is neurokinin A (NKA). In addition to SP, the PPT gene also codes for NKA (Nakanishi, 1991). Although SP and NKA are co-localized in the same sensory nerves, NK-2 receptors for NKA are only found on arterial vessels.

Neurogenic Inflammation in the Airways

The term neurogenic inflammation is used to describe the inflammatory response to an irritant mediated locally by sensory nerves (Jancso *et al.*, 1967). The hallmarks of neurogenic inflammation in the nasal cavity include vasodilation, plasma extravasation and mucus secretion and result, in part, from the release of neuropeptides, especially SP from activated sensory nerves (Lundberg & Saria, 1983; Jancso *et al.*, 1967). Jancso first demonstrated this concept in the rat skin and eyes, which are both innervated by sensory nerves. Rats received an intravenous injection of Evan's blue, a solution that binds serum albumin, followed by instillation of capsaicin, the pungent ingredient in red peppers, in the eye. Within 15 minutes, the capsaicin-treated area turned dark blue indicating extravasation from blood vessels, a marker of inflammation. When the sensory nerve supplying the eye was transected, the eye failed to turn blue following application of capsaicin (Jancso *et al.*, 1967). Similarly, application of capsaicin to the guinea pig nasal mucosa causes SP to be released from sensory nerve endings but has no effect on parasympathetic or sympathetic nerves (Lundblad *et al.*, 1983). These studies were the first to demonstrate that irritants could cause inflammation solely by activating sensory nerves.

Neurogenic inflammation also occurs in human airways and is evident by significant mucus secretion 10 seconds after nasal application of capsaicin (Philip *et al.*, 1994). SP is detectible in nasal lavage specimens obtained from healthy volunteers in concentrations between 2 and 15 fmol/ml (Schultz *et al.*, 1996). There was a significant difference between the amount of SP (fmol/ml) detected in the bronchoalveolar lavages of healthy controls (30.0$\pm$7.0) and asthmatic patients (185.0$\pm$22.0) (Nieber *et al.*, 1992). The concentration of SP in the sputum of patients with chronic bronchitis was also significantly higher compared to healthy volunteers, suggesting a relationship between neurogenic inflammation and airway disease (Tomaki *et al.*, 1995). Neurogenic inflammation has been implicated in the pathology of a number of disease states, including rhinitis and asthma (Sanico *et al*., 1998; Barnes, 1986).

In addition to capsaicin, irritants such as cigarette smoke, formalin, histamine and sulfur dioxide cause airway inflammation that is mediated by the release of SP from sensory nerve endings. The increased vascular permeability normally observed 5 minutes following cigarette smoke exposure was either markedly reduced or abolished if the airways were pretreated with capsaicin to desensitize the sensory nerves (Lundberg & Saria, 1983). Similarly, rats exposed to acetone developed nasal vasodilation, increased blood flow and plasma extravasation during a 50-minute exposure. Capsaicin or NK-1 receptor antagonist N-acetyltrifluoromethyl tryptophan benzyl ester pre-treatment inhibited the responses, which suggests SP mediates inflammation (Morris *et al.*, 1999). Evidence of neuropeptide release and inflammation following irritant exposure has also been obtained from humans. Following short-term (2 hr) ozone exposure (0.2 ppm), the percentage of SP immunoreactive nerve fibers per unit area of submucosa was

significantly lower in the ozone-exposed patients, 0.15 (0.08-0.18) compared to control patients, 0.6 (0.05-1.2). The release of SP not only explains the decrease in SP levels in nerve endings but may also contribute to the influx of neutrophils observed following ozone exposure (Krishna *et al.*, 1997).

Neuropeptide Up-Regulation

Immediate airway responses mediated by neuropeptides released from sensory nerve terminals following irritant exposure have been clearly demonstrated. However, a second hallmark of neuronal activation is increased neuropeptide synthesis. This delayed phenomenon also contributes to neurogenic inflammation and has been demonstrated following inhalation of airway irritants. Twenty-four hours following toluene diisocyanate (TDI) exposure, SP immunoreactivity in TG neurons innervating the nasal epithelium (94.30$\pm$9.70%) was significantly increased over controls (72.60$\pm$4.40%) (Hunter *et al.*, 2000a). The increased SP content was attributed to enhanced SP synthesis. PPT mRNA, which codes for SP, was increased in the same subset of TG neurons 24 hr after TDI. The SP content of epithelial nerve fibers was also significantly increased 24 hr following TDI, indicating enhanced axonal transport following synthesis. The increased synthesis and release of SP from sensory nerves may contribute to the prolonged airway inflammation, which persisted 72 hr after TDI. In a six-month silica exposure study, SP-IR and PPT mRNA were significantly increased in TG neurons innervating the rodent nasal epithelium (Hunter *et al.*, 1998). Neuropeptide up-regulation may contribute to nasal irritation and rhinitis, two frequently reported symptoms following occupational exposure to silica dust (Chan-Yeung *et al.*, 1978) and TDI (Brugsch & Elkin, 1963).

Similar increases in neuropeptide levels were reported following viral infection and allergen challenge. Inoculation of guinea pigs with Sendai virus, a known cause of respiratory viral infections, results in airway inflammation within 3-4 days. Four days after inoculation 20% of nodose cell bodies with receptive fields in the trachea stained positive for SP/NKA compared to only 3% in the vehicle-inoculated animals (Carr *et al.*, 2002). Neuropeptide up-regulation is also present in allergic airway disease. Guinea pigs sensitized to ovalbumin and challenged had a 25% increase in the number of neuropeptide-immunoreactive nodose neurons innervating the airways and a four-fold increase in neuropeptide levels in lung tissue 24 hr after allergen challenge. This suggests that increased neuropeptide synthesis is accompanied by increased transport to peripheral nerve endings (Fischer *et al.*, 1996).

The alteration or increase in neuropeptide synthesis following irritants, viral infection and allergen exposure is an example of neuronal plasticity and is probably part of a defense mechanism in the airway to environmental conditions. However, the mechanism by which irritant, virus or allergen exposure leads to neuropeptide up-regulation in the centrally located ganglia remains unclear. To date, studies have focused primarily on endpoints establishing a link between irritant inhalation, neuronal neuropeptide increases and neurogenic inflammation. Few studies have focused on the events preceding neuropeptide up-regulation, which in theory extrapolated from developmental studies, requires a signaling molecule to relay communication between the activated nerve ending and the remotely-located cell body. During development, the neurotrophin, nerve growth factor (NGF), is a key neuronal signaling molecule, which is transported from the nerve ending to the cell body (Levi-Montalcini & Angeletti, 1968).

Role of NGF in Development

Described initially in mouse sarcomas (Cohen-Cory *et al.*, 1952), NGF was the first neurotrophin identified in a family that also includes brain-derived neurotrophin factor (BDNF), neurotrophin-3 (NT3) and neurotrophin 4/5 (NT4/5). The neurotrophins are small, basic, 13 kDa proteins involved in the differentiation, growth and survival of central and peripheral neurons, including sensory neurons (Levi-Montalcini, 1987 ; Levi-Montalcini & Angeletti, 1968). During development, excess neurons are generated and selective survival is accomplished by competition of innervating-processes for limited quantities of NGF released from surrounding tissue. NGF is selectively internalized and retrogradely transported to cell bodies where transcriptional changes occur (Yankner & Shooter, 1982). In mammals, 70-80% of sensory neurons in the dorsal root ganglia (DRG) and TG are dependent on NGF during development (Johnson *et al.*, 1980). Initially implicated only in development, NGF is now known to play a continuous role in the control of neuropeptide synthesis in mature sensory neurons (Goedert *et al.*, 1981).

NGF Structure and Receptors

All neurotrophins, including NGF, are initially synthesized as a pro-neurotrophins and intracellular cleavage by endopeptidases or pro-convertases produces the active neurotrophin (Mowla *et al.*, 2001). Pro-NGF consists of three subunits, $\alpha 2\beta\gamma 2$, and is commonly referred to by its sedimentation coefficient, 7S. The α and γ subunits dissociate revealing the β subunit, which is the active subunit of 7S and exhibits the biological properties ascribed to NGF. The β subunit, commonly called β NGF, loses

several additional amino acid residues to form 2.5S NGF, but the activity remains identical to β NGF (Shao *et al*., 1993; Silverman & Bradshaw, 1982).

Biologically active NGF binds two different receptors, p75 and trkA. The pan-neurotrophin p75 is a low-affinity-transmembrane receptor, which binds all neurotrophins, including NGF (Johnson *et al.*, 1986). A member of the tumor necrosis receptor family, p75 is expressed in adult and neonate neuronal and non-neuronal tissue (Yan & Johnson, Jr., 1988). The extracellular portion of p75 contains four cysteine-rich repeats, and the intracellular portion contains a death domain. Neurotrophin binding mediates survival, cell migration and myelination through several signaling pathways (Roux & Barker, 2002). Studies have also implicated the p75 receptor in apoptotic cell death during inflammatory conditions (Hempstead, 2002). The exact role of the p75 receptor in NGF signaling remains unclear; however, recent evidence suggests that the p75 receptor serves primarily as a co-receptor for the trkA receptor. When both receptors are present p75 forms a high affinity-binding site, which increases the binding affinity of trkA for NGF 100-fold. The ability of p75 to control ligand affinity may play an important role in neuronal responsiveness (Hempstead *et al.*, 1991).

Considered the central effector for NGF action, the tropomyosin-related kinase A (trkA), is a high-affinity-transmembrane-tyrosine-kinase receptor (K_d=2x10^{-11}M) located primarily in the nervous system (Kaplan *et al.*, 1991). Trk receptors contain extracellular immunoglobulin G domains for ligand binding and a catalytic tyrosine kinase sequence in the intracellular domain. NGF binding to trkA receptors located on peripheral terminals of sensory neurons results in receptor dimerization and kinase activation. (McMahon *et al.*, 1994). Phosphorylation of the tyrosine residues in the cytoplasmic domain of the

receptor creates docking sites for adaptor proteins coupling the receptor to intracellular signaling cascades (Patapoutian & Reichardt, 2001).

Intracellular Signaling and Retrograde Transport of NGF

In vitro studies using the PC12 neuronal cell line have been extensively used to study the signal transduction cascades, including mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and phospholipase C-γ (PLC-γ) pathways activated by trkA receptors (Greene & Tischler, 1976; Kaplan & Miller, 2000). NGF-bound trkA receptors activate various signaling cascades locally in the nerve terminal and at the remotely located cell body, leading to a variety of physiological responses.

At the nerve terminal, NGF potentiates the response of isolated sensory neurons to capsaicin, suggesting a potential role for NGF in neuronal sensitization (Mendell *et al.*, 1999). Capsaicin binds to the transient receptor potential ion channel (TRPV1), a capsaicin, proton and heat-sensitive non-selective cation channel, leading to receptor activation. Co-immunoprecipitation studies have found that trkA activation of PLC-γ increases the excitability of TRPV1 (Caterina *et al.*, 1997). TrkA-mediated signaling cascades are also responsible for the endocytosis and retrograde transport of the NGF-trkA complex (Vedder *et al.*, 1993). PI3K activation is required for the clathrin-mediated endocytosis of the ligand-receptor complex into a signaling endosome (Reynolds *et al.*, 1998). Phosphatidylinositol 4-kinase and the actin cytoskeleton participate in the retrograde axonal transport of the endosome to the cell body (Reynolds *et al.*, 1999). A number of studies have demonstrated axonal transport of endogenous NGF from both autonomic and sensory nerve terminals to distant cell bodies. ^{125}I-NGF injected into the

anterior eye-chamber is transported from adrenergic nerve terminals to cell bodies of postganglionic sympathetic neurons in the superior cervical ganglion (SCG) (Hendry *et al.*, 1974) and from sensory nerve terminals to cell bodies in the TG (Sandow *et al.*, 2000). ^{125}I-NGF can be detected in the SCG as early as 4 hr post injection and continues to increase until 16 hr. The rate of ^{125}I-NGF retrograde axonal transport is estimated to be 2.5 mm/hr (Hendry *et al.*, 1974). The specificity of retrograde axonal transport for NGF was tested following injection into the anterior eye of various radiolabeled proteins similar to NGF in respect to molecular weight and electrical charge. After 14 hr, ^{125}I-NGF was the only one detected in SCG cell bodies, demonstrating specificity of the retrograde axonal transport of NGF (Stockel *et al.*, 1974). In a separate study, a 50-fold excess of NGF completely inhibited the retrograde transport of ^{125}I-NGF, confirming that transport of NGF is receptor mediated (Sandow *et al.*, 2000).

At the nerve cell body, the tyrosine-phosphorylated trkA becomes a scaffolding structure recruiting several adaptor proteins and enzymes including Shc and PLC (Stephens *et al.*, 1994). These proteins are involved in the activation of various kinase cascades, which ultimately translocate to the nucleus and regulate expression of various genes, including neuropeptides (Lindsay & Harmar, 1989). The ability to undergo retrograde axonal transport and stimulate transcriptional changes at the nuclear level is a feature unique to NGF and is the basis for studying NGF in relation to neurogenic up-regulation in the airways.

NGF and Neuropeptide Regulation

The results published by Lindsay and Harmar in 1989 provided the first direct evidence that NGF regulated neuropeptide expression in adult sensory neurons. They isolated and cultured DRG neurons from adult Sprague-Dawley rats in the absence of NGF. After five days, levels of SP mRNA and CGRP mRNA declined progressively. When the culture media was supplemented with NGF, PPT mRNA and CGRP mRNA levels were elevated 4-6 fold after 24-48 hours. After five days in NGF-supplemented culture, the concentration of SP and CGRP increased 15-fold (Lindsay & Harmar, 1989). This study demonstrated that NGF increases the transcription and translation of neuropeptide genes in sensory neurons. Schwartz found similar results *in vivo* using guinea pigs, which received long-term immunization against mouse NGF. NGF deprivation did not affect the total number of neurons or normal neuronal function, but it significantly decreased the SP content in rat dorsal root sensory ganglia (Schwartz *et al.*, 1982).

NGF, Inflammation and Neuropeptide Up-regulation (Pain studies)

Studies using rat models of adjuvant arthritis were some of the first to investigate whether NGF was also responsible for the neuropeptide up-regulation during inflammation. Similar to airway inflammation, chronically-inflamed tissue, as in the case of arthritis, also has increased neuropeptide levels in the innervating sensory nerves. SP and CGRP both increased by 30-40% in the DRG and by 70% in the sciatic nerve supplying an inflamed paw five days following injection of Freund's adjuvant. After just one day, increases in NGF of 136% were detected in the sciatic nerve supplying the inflamed paw. In the same study, systemic pre-treatment with an anti-NGF serum prior

to the induction of arthritis with Freund's adjuvant prevented the increase of neuropeptides typically observed on the fifth day. Local injections of NGF for 5 days into a noninflammed paw increased SP and CGRP in the innervating sciatic nerve (Donnerer *et al.*, 1992). The agonist and antagonist approach used in this study directly illustrated the regulatory role of NGF in neuropeptide up-regulation during inflammation.

Similar findings were reported in inflamed oral mucosa, which is innervated by neurons of the TG. SP increases in TG neurons innervating inflamed oral tissue 48 hr after injury (Neubert *et al.*, 2000). However, injury to the occlusional surfaces of rat molar cusps induces a localized increase in NGF in the dental pulp within 4 hrs (Wheeler *et al.*, 1998). The local release of NGF observed in tooth inflammation may mediate the up-regulation of neuropeptides. Based on results from models of adjuvant arthritis and tooth injury NGF may also be responsible for the up-regulation of neuropeptides in sensory neurons during airway inflammation.

Sources of NGF in the Upper Airway

In the nasal mucosa, a number of cell types in the vicinity of sensory nerve terminals are known to synthesize and release NGF. Superficial nasal mucosal cells obtained from the inferior turbinates of healthy human subjects were found to constitutively express mRNA for NGF. The same subjects also had baseline levels of NGF protein in their nasal lavage fluid (Sanico *et al.*, 2000). NGF is also detectable in the human alveolar epithelial A549 cell line (Fox *et al.*, 1997). Mast cells (Leon *et al.*, 1994), lymphocytes (Barouch *et al.*, 2000), eosinophils (Solomon *et al.*, 1998), and macrophages (Braun *et al.*, 1998) are capable of synthesizing and releasing NGF, and the

inflammatory mediators IL-1, IL-4, IL-5, TNFα and interferon-γ promote the release of NGF from certain inflammatory cells (Yoshida *et al.*, 1992). Additional sources of NGF in the lungs include fibroblasts (Olgart & Frossard, 2001) and smooth muscle cells (Freund *et al.*, 2002). More than 80% of rat TG neurons express mRNA for NGF (Jacobs & Miller, 1999); however, it is unclear whether NGF synthesized in TG neurons is released at central or peripheral nerve terminals.

The actions of NGF are not limited to the nervous system. The high-affinity receptor, trkA is expressed on mast cells (Tam *et al.*, 1997), basophils (Burgi *et al.*, 1996) and macrophages (Garaci *et al.*, 1999) and NGF binding promotes the release of inflammatory mediators. NGF also affects the activity of T cells and B cells (Otten *et al*., 1989; Lambiase *et al.*, 1997). In the airways, NGF appears to affect both neuronal and immune cell activity (Braun *et al*., 1999)

NGF and Neuropeptide Regulation in the Airways

A number of studies have also demonstrated the effect of NGF on neuropeptide expression in the airways. Retrograde labeling techniques were able to characterize the direct effect of NGF on airway sensory neurons innervating the guinea pig trachea. Following injection of NGF into the tracheal wall, 10% of the large diameter nodose neurons projecting fibers to the trachea became SP-positive. Not only did NGF increase neuropeptide expression in airway neurons but it also changed the neuronal phenotype, since the large diameter Aδ neurons do not normally express SP (Hunter *et al.*, 2000b). Genetically manipulating the levels of NGF in the airway has provided further evidence that NGF regulates neuropeptides. Lung homogenates from transgenic mice

overexpressing NGF in Clara cells contained 5-fold more SP than lungs from non-transgenic mice. Mice overexpressing NGF also had significantly more tachykinin-containing sensory nerve fibers throughout the airway wall compared to wild-type mice. The increase in pulmonary SP correlated directly with the increase in tachykinin-containing nerve fibers throughout the airway wall (Hoyle *et al.*, 1998). The nasal mucosa of transgenic mice overexpressing NGF in keratin-producing cells, which includes respiratory epithelium, are hyperinnervated with CGRP-immunoreactive nerve fibers (Takami *et al.*, 1995). These transgenic studies demonstrated that NGF plays a continuous dynamic role in neuropeptide levels in the airways.

NGF and Airway Inflammation

In 1996, Bonini found that individuals with asthma had significantly higher NGF serum levels (87.6$\pm$59.8 pg/ml) than non-asthmatics (3.8$\pm$1.7 pg/ml) (Bonini *et al.*, 1996). In addition to asthmatics, the nasal lavage fluid from patients with allergic rhinitis had significantly increased levels of NGF compared to controls following allergen challenge (Sanico *et al.*, 2000). Since then a number of studies have tried to elucidate the role of NGF in airway inflammation. In a murine model of allergic airway inflammation, increased NGF production coexists with airway inflammation and airway hyperresponsiveness (AHR). The AHR commonly observed in these animals was prevented with anti-NGF treatment (Braun *et al.*, 1998). AHR, similar to what is observed in allergen-sensitized mice, could be provoked in normal mice following intranasal instillation of NGF (Braun *et al.*, 2001). Pre-treating guinea pigs with an NK-1 receptor antagonist completely blocked the NGF-induced AHR (de Vries *et al.*, 1999).

The neuropeptides SP and NKA have high affinity for the NK-1 receptor, suggesting that NGF induced AHR is mediated by a sensory neuronal mechanism. Intraperitoneal injection of anti-NGF three hours prior to inoculation with respiratory syncytial virus (RSV) reduced NK-1 receptor expression and capsaicin stimulated plasma extravasation (Hu *et al.*, 2002). In the murine model of allergic bronchial asthma, mice devoid of the p75 receptor did not develop inflammation-induced neuronal hyperreactivity to capsaicin, suggesting that the actions of NGF are receptor-mediated (Kerzel *et al.*, 2003).

These studies demonstrated a role for NGF in sensory neuronal mechanisms following RSV innoculation and allergen challenge. However, changes in neuropeptides and NGF were not examined simultaneously in the inflamed airways. To date, the effects of irritant exposure have not been investigated.

SPECIFIC AIMS

The goal of this study was to characterize the response and regulation of neuropeptides in the upper airway after exposure to irritants. Irritant inhalation results in inflammation of the nasal cavity. The hallmarks of inflammation in the nasal cavity include vasodilation, plasma extravasation and mucus secretion. Inflammation results, in part, from neuropeptides released from sensory nerves (Jancso *et al.*, 1967). When irritants are inhaled, nerve fibers in the lining of the nasal cavity are activated. In turn, the sensory nerve fibers produce signals, which are relayed to corresponding cell bodies located in the TG. Following irritant inhalation, increased synthesis and release of SP has been observed in the TG cell bodies innervating the nasal epithelium. The neuropeptide SP also increases in the sensory nerve fibers located in the epithelial lining of the nasal cavity (Hunter *et al.*, 2000). In these studies, the increase in SP also correlated with an increase in inflammatory cells and serum protein, two markers of tissue injury. Occupational exposure to airway irritants has clear health implications. Various symptoms of airway inflammation have been reported in workers exposed to silica (Chan-Yeung *et al.*, 1978), asphalt fumes (Hanley & Miller, 1996) and TDI (Brugsch & Elkin, 1963).

The increase in neuropeptides following irritant exposure may have occupational relevance; therefore, it is important to understand the mechanism leading to enhanced SP production. Studies suggest that nerve growth factor (NGF), a neurotrophin important during development, may be involved since it has been shown to increase neuropeptide gene expression in sensory neurons. Known sources of NGF in the nasal cavity include epithelial and inflammatory cells. Upon release, NGF can bind receptors on sensory

nerve endings. The NGF-receptor complex enters the nerve terminal and is transported along the axon to the TG cell body. Once at the cell body, NGF can increase the synthesis of neuropeptides including SP. The levels of NGF have been found to increase during inflammation. Injury to either skin or dental pulp causes local increases in NGF within 4hr (Wheeler *et al.*, 1998). Patients with rhinitis, a chronic inflammatory disease of the upper airway have higher levels of NGF in their nasal cavities compared to non-diseased control subjects (Sanico *et al.*, 2000). Not only has NGF been shown to increase during inflammation but NGF can also alter neuropeptide expression.

The hypotheses of this project are: 1) Irritants up-regulate neuropeptides, like SP and CGRP in the upper airways; and 2) NGF secretion from the nasal mucosa mediates the irritant-induced up-regulation of SP synthesis and release from TG sensory neurons supplying the nasal epithelium.

Aims to investigate these hypothese:

Specific Aim #1 Determine the effect of inhalation of asphalt fumes, an occupational irritant, on the synthesis and release of SP from TG neurons innervating the nasal epithelium. Changes in SP levels will be measured in TG neurons supplying the nasal epithelium and nerve fibers located in the nasal epithelium following inhalation of asphalt fumes.

Specific Aim #2 Evaluate NGF and SP production in the upper airways following irritant exposure. Rat nasal cavities will be exposed to TDI, an established airway

irritant. The levels of NGF and SP in the nasal-lavage fluid, epithelial nerve fibers and TG cell bodies innervating the nasal mucosa will be measured at various time points using enzyme linked immunosorbent assay (ELISA) and immunocytochemistry. If the overall hypothesis is correct, these studies will show that the increase in NGF levels precedes the increase in SP levels.

Specific Aim #3 Determine the effect of NGF on the synthesis and the release of SP from TG neurons innervating nasal epithelium. Rat nasal cavities will be instilled with either an anti-NGF antibody or a receptor tyrosine kinase inhibitor prior to irritant exposure. Changes in SP, NGF and NGF receptor immunoreactivity will be measured in TG neurons supplying the nasal epithelium using immunocytochemistry. The SP nerve fiber density in the nasal epithelium will also be measured using immunocytochemistry.

Specific Aim #4

Determine if NGF is released from epithelial cells or resident cells of the nasal submucosa following irritant exposure.

STUDY 1

Asphalt Exposure Enhances Neuropeptide Levels in Sensory Neurons Projecting to the Rat Nasal Epithelium

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ABSTRACT

Asphalt fumes have been reported to produce nasal irritation in road workers. Since inhaled irritants can increase substance P (SP) production in airway neurons, the effects of asphalt fumes on SP production in trigeminal ganglia (TG) sensory neurons innervating the nasal mucosa were investigated. The effects of asphalt fumes on nasal mucosal innervation were examined by measuring SP and calcitonin-gene-related peptide (CGRP) levels in rat TG neurons projecting to the nasal epithelium. Female Sprague-Dawley rats were exposed to asphalt fumes at 16.0$\pm$8.1 mg/m^3 for 5 consecutive days, 3.5 hr/day. Inflammatory cells were measured in nasal cavity lavage fluid. SP and CGRP immunoreactivity (IR) was measured in the cell bodies of trigeminal ganglion sensory neurons projecting to the nasal cavity. A significant increase in neutrophils and macrophages was observed after asphalt fume exposure, indicating an inflammatory response in the nasal cavity. The percentage of SP-IR neurons increased significantly in the asphalt-exposed rats and the proportion of CGRP-IR neurons was also elevated following asphalt exposure. These results indicate that exposure to asphalt fumes produces inflammation and increases the levels of SP and CGRP in TG neurons projecting to the nasal epithelium. The findings are consistent with asphalt-induced activation of sensory C-fibers in the nasal cavity. Enhanced sensory neuropeptide release from nerve terminals in the nasal cavity may produce neurogenic inflammation associated with nasal irritation following exposure to asphalt fumes.

Key Words: Asphalt; trigeminal ganglia; sensory neurons; substance P; calcitonin-gene-related peptide

INTRODUCTION

Asphalt stone composite is the major paving product in the United States, accounting for over 90% of roadway paving. The road paving industry employed 300,000 workers as of 1990 (Asphalt Institute, 1990). Derived from processing petroleum crude oil, asphalt is a complex mixture of high-molecular-weight organic compounds, including aromatic hydrocarbons and sulfur-nitrogen and oxygen-based heteromeric compounds (Hanley & Miller, 1996). The composition of asphalt varies, depending on the crude oil source and end usage. Application temperatures for paving asphalt range between 135-163°C, producing bitumens, viscous solid or liquid byproducts of petroleum refining to produce fumes. Depending on temperature, these fumes may contain polyaromatic hydrocarbons (PAH), some of which are known human carcinogens (IARC, 1983; IARC, 1985). The immunotoxic potential of PAH has been documented in both animal and human studies (Schnizlein *et al*., 1987; Karakaya *et al.*, 1999). Asphalt fumes may also be responsible for nonmalignant lung diseases such as bronchitis, emphysema, and asthma (Hansen, 1991; Maizlish *et al.*, 1988) through activation of cytochrome P-450 isozyme CYP1A1 (Ma *et al.*, 2002). The current threshold limit value (TLV) set by the American Conference of Governmental Industrial Hygienists (ACGIH) for asphalt fumes is 5 mg/m^3 for the 8-hour time-weighted average (TWA) (ACGIH, 1991). National Institute of Occupational and Health Safety site studies involving asphalt paving workers list nasal, throat and eye irritation as frequently reported symptoms (Hanley & Miller, 1996).

Airway responses to irritant inhalation are mediated in part by sensory and autonomic nerve fibers in the respiratory airways (Barnes, 1986). Irritants, including

cigarette smoke, formalin, histamine and sulfur dioxide, are known to activate sensory nerve fibers in the upper-respiratory-airway mucosa, inducing neurogenic inflammatory responses (Lundberg *et al.*, 1987). The rat nasal mucosa receives sensory innervation from nerve cell bodies of the trigeminal ganglia (TG) (Grunditz *et al.*, 1994). Sensory neurons projecting to the nasal mucosa are branched, small-diameter, capsaicin-sensitive C-fibers that can be activated by noxious chemical, mechanical, or thermal stimuli (Allen, 1924; Anton & Peppel, 1991). Upon activation with chemical stimuli such as capsaicin, the pungent agent in peppers, these fibers release neuropeptides, including substance P (SP) (Jancso *et al.*, 1967). SP, an 11 amino-acid peptide, is localized within cell bodies of the TG and in the peripheral terminals of sensory nerves located near arteries, veins, mucous glands, and epithelium (Lundblad, 1984). Upon release, SP launches a receptor-mediated inflammatory response characterized by mucus secretion, plasma extravasation and vasodilation (Jancso *et al*., 1967; Lundberg & Saria, 1983). Although SP has been implicated as the primary contributor in upper airway sensory responses (Baraniuk *et al.*, 1991), the neuropeptide, CGRP, is co-stored and co-released with (Lee *et al*., 1985; Lundberg *et al.*, 1985). Nerve endings containing CGRP are extensively located along the walls of arterioles and veinules and, upon release, CGRP produces long-lasting vasodilatory effects (Stjarne *et al*., 1989; Zhao & Tao, 1994).

Studies suggest that airway irritants affect neuropeptide levels in sensory neurons. Inhalation of either silica or toluene diisocyanate (TDI) have been demonstrated to transiently increase SP production in TG neurons projecting to the nasal epithelium (Hunter *et al*., 1998; Hunter *et al.*, 2000). Nerve fibers in the airways and the

neuropeptides they release may be involved in regulating the inflammatory responses to inhaled irritants.

The objective of the present study was to determine if inhalation of asphalt fumes activates sensory neurons innervating the nasal cavity. The proportion of TG neurons innervating the nasal epithelium that expresses SP and CGRP following asphalt fume inhalation was investigated. It was hypothesized that nasal irritation and inflammation following asphalt fume inhalation is due in part to enhanced neuropeptide production in TG neurons innervating the nasal cavity.

METHODS

Animals Female Sprague-Dawley rats [Hla: (SD)CVF] weighing 200-250 g and purchased from Hilltop Lab Animals (Scottsdale, PA) were verified by serology to be free of endogenous viral pathogens, parasites, mycoplasms, Helicobacter, and cilia-associated respiratory bacillus. Rats were acclimated in an AAALAC-accredited, specific pathogen-free and environmentally controlled animal facility for two weeks prior to initiation of inhalation exposures. When not in the inhalation chamber rats were kept in filtered, ventilated cages with Alpha-Dri cellulose chips (Shepard Specialties Papers, Kalamazoo, MI) and hardwood Beta-chips (Northeastern Products Corp., Warrensburg, NY) as bedding; provided with HEPA-filtered air, autoclaved Prolab 3500 diet (Purina Mills, St. Louis, MO) and tap water ad lib; and housed under controlled light-cycle (12 hr light/12 hr dark) and temperature (22-24˚C) conditions.

Experimental design Rats were exposed to either asphalt fume or ambient air (n=7 for both groups). In the experimental group, rats were exposed to 16.0 ± 8.1 mg/m^3

(mean±SD) of asphalt fume for five consecutive days (3.5 hr/d). The major sources of variability of fume concentration were when cans of asphalt were changed and day-to-day variation. A lethal dose of sodium pentobarbital (0.2 g/kg body weight, IP) was used to euthanize the rats 18 hr after the last exposure. The right and left nasal cavities were lavaged simultaneously and cytospin slides were prepared for differential cell counts. The TG were then removed and processed for SP and CGRP immunocytochemistry.

Asphalt exposure system An asphalt fume generator (Heritage Research Group, Indianapolis, IN) was purchased and modified to produce an asphalt aerosol and gas suitable for small animal exposure studies. Hot performance grade asphalt was purchased (PG 64-22, Asphalt Materials, Inc., Indianapolis, IN) and heated to 170˚C in a temperature-controlled kettle. The hot asphalt passed through a needle valve, which regulated the flow. As the asphalt left the fume generator, its weight was measured with a digital scale. This value was acquired by the computer, and the asphalt mass flow rate was calculated by dividing the change in weight over the change in time. This flow rate signal was used in a feedback loop to adjust a stepper motor connected to the needle valve to achieve the desired flow rate of 150 g/min onto a 6”-wide 24”-long flat stainless steel plate having a 1.3-degree slope with respect to horizontal. At the entrance to the plate, the asphalt was 160˚C. A 500-watt heater underneath the plate maintained a temperature gradient of 35˚C along the length of the plate to simulate cooling under road paving conditions. The asphalt-covered plate was enclosed within a stainless steel chamber in which conditioned HEPA-filtered air passed through at a rate of 20 L/min. A mixture of air with the aerosol and gases released from the asphalt surface was removed from the chamber and conducted through a ½” stainless steel pipe, heated to 150 ˚C with

three 500-watt heating tapes, into a 0.25 m^3 whole body animal exposure chamber. Light scattering measurements (Personal Data Ram, PDR-1000AN, MIE, Bedford, MA) of the asphalt aerosol within the animal exposure chamber were recorded in addition to gravimetric samples, which were made using a Teflon filter (PTFE, 0.45μ pore size, SKC, Eighty Four, PA) and pump (Gilair5, Gilian, Sensidyne, Clearwater, FL) at a flow rate of 1.0 L/min. The light scattering measurements were used in a computer feedback control system to modulate the diluent air that was mixed with the asphalt fume from the generator to keep the aerosol component of the asphalt fume constant (25 mg/m^3). The gas component of the fume was monitored with a photo ionization detector, (MINIRae2000, RAE Systems, Sunnydale, CA), and gas samples were collected by pulling the filtered fume through a XAD-2 amberlite poly aromatic resin (#226-30-06, SKC, Eight Four, PA) with a constant flow pump (Giliar5, Gilian, Sensidyne, Clearwater, FL) at a rate of 1.0 L/min. The XAD-2 samples were analyzed by mass spectroscopy to determine fume composition, and the results have been described elsewhere (Wang *et al.*, 2001). Temperature and humidity within the exposure chamber were monitored (HMP 233, Vaisala, Wobourn, MA) and recorded throughout each exposure period. The average temperature and relative humidity were 26˚C and 40%, respectively. Control animals were housed in similar chambers and exposed to HEPA-filtered conditioned air maintained at the same temperature and relative humidity as the environment within the asphalt exposure chambers.

Rhodamine-labeled latex microsphere instillation Five days prior to asphalt exposure, the rats were anesthetized with sodium brevital (25 mg/kg body weight, IP). The anterior and posterior regions of the right and left nasal cavities were each instilled with 4

µl of rhodamine-labeled-latex microspheres as previously described (Hunter & Dey, 1998). The microspheres were delivered using a 10 µl Hamilton syringe with plastic tubing covering the tip. The tubing was marked at 0.8 and 1.3 cm lengths to allow correct positioning into the anterior and posterior nasal regions. Even distribution of the instilled material over the entire nasal mucosa was achieved by rotating the rats in a circular pattern around the anterior-posterior axis five times after microsphere instillation. The rhodamine-labeled-latex microspheres were selectively taken up by sensory nerve endings in the nasal epithelium and then transported retrogradely to the corresponding cell bodies in the TG.

Nasal lavage The rats were overdosed with sodium pentobarbital (0.2 g/kg IP) and the lower jaw removed. A syringe with plastic tubing covering the needle was inserted into the posterior nares and sealed by manual pressure. Both sides of the nasal cavity were simultaneously lavaged with sterile phosphate buffered saline (PBS) until 20 ml was collected from the nostrils.

The lavage fluid was centrifuged at 1,500 rpm (352 x g) for 10 min. The supernatant was discarded, and the pellet resuspended in 1.0 ml PBS. Cells were plated on slides at a density of $1.5x10^5$ cells/ml using a Cytospin (Shandon Scientific, Ltd., Cheshire, UK) at 400 rpm (18.06 x g) for 4 min. The slides were processed using the Hema 3 Manual Staining System (Biochemical Sciences Inc., Swedesboro, NJ) for Wright-Giemsa stain. A total of 100 cells were classified as neutrophils, macrophages, eosinophils, basophils, lymphocytes, and epithelial cells using a light microscope (Olympus AX70) with a 40x magnification objective.

Tissue removal and preparation The right TG were removed by cutting distal to the division of the ophthalmic, maxillary and mandibular branches of the trigeminal and at the junction of the trigeminal nerve (V) emerging from the ganglion (Hunter and Dey, 1998). The tissue was immediately fixed in picric-acid formaldehyde fixative consisting of 2% paraformaldehye, 15% saturated picric acid, and 0.15 M phosphate buffer at 4°C (Stefanini *et al.*, 1967). After 3 hr, the tissue was rinsed twice with 0.1 M phosphate-buffered saline containing 0.3% (v/v) Triton X-100 (PBS-Tx, pH=7.8). After the second rinse, the tissues remained in the PBS-Tx overnight at 4˚C. The TG were oriented on corks so the first section would be taken from the ventral surface. The tissue was covered with Tissue Tek OCT compound (Sakura, Torrance, CA), frozen in isopentane cooled by liquid nitrogen and stored in airtight plastic bags at –80˚C.

Continuous-serial-cryostat sections (12 μm thickness) of the entire TG were made as previously described (Hunter & Dey, 1998). Every fifth section was collected on one of three gelatin-coated coverslips. The first coverslip had sections 1, 6, 11…, the second coverslip has sections 2, 7, 12…, and the third coverslip had sections 3, 8, 13…, and so on, until the entire ganglion was sectioned. The first two coverslips were used for SP immunocytochemistry and the third for CGRP immunocytochemistry.

Immunocytochemistry Immunocytochemical procedures for localizing SP and CGRP immunoreactive neurons were identical to those previously described (Dey *et al.*, 1990). Cryostat sections on gelatin-coated coverslips were covered with either rabbit anti-SP (diluted 1:200) or rabbit anti-CGRP (diluted 1:100) (Peninsula, Belmont, CA) primary antiserum. The coverslips were incubated in a humid chamber at 37 °C for 30 min, then rinsed 3 times with PBS-Tx + 1% (w/v) bovine serum albumin (PBS-Tx-BSA), allowing

5 min per rinse. The sections were then covered with a diluted secondary antiserum, fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin IgG (ICN Pharmaceuticals Inc., Costa Mesa, CA) diluted 1:100 in PBS-Tx-BSA and the coverslips were incubated again at 37°C for 30 min. The coverslips again were rinsed 3 times for 5 min increments in PBS-Tx +BSA. Fluoromount (Southern Biotechnology, Birmingham, AL) was used to mount the coverslips onto glass slides. The sections were observed using an Olympus AX70 fluorescence microscope equipped with fluorescein (excitation 495 nm and emission 520 nm) and rhodamine (excitation 540 nm and emission 580 nm) filters.

Analysis of SP and CGRP Immunoreactivity SP immunoreactivity (SP-IR) and CGRP immunoreactivity (CGRP-IR) in the TG cell bodies innervating the nasal epithelium were evaluated by direct observation. Without knowledge of the experimental grouping, neurons containing rhodamine-labeled latex microspheres were identified. Using the rhodamine filter, the presence of microspheres was used as criteria that axons of the identified cell body projected to the nasal epithelium. The same cell body was then visualized with a fluorescein filter and classified as either SP-IR positive or negative. The percentage of SP-IR positive neurons was determined by dividing the positive SP-IR neurons by the total number of microsphere-labeled neurons.

A second method of analysis was also used only to evaluate only SP levels in TG neurons. The intensity of fluorescence of the cell bodies innervating the nasal cavity was determined, converted to a mean gray value (MGV) using digital image analysis and compared against a threshold value to distinguish between positive and negative cell bodies. The system was calibrated using an InSpeck Green (505/515) Microscope Image

Intensity Calibration Kit (Molecular Probes, Eugene, OR). Once the neurons of interest were identified using the rhodamine filter, a black and white image was captured with a SPOT digital camera (Diagnostic Instruments Inc, Sterling Heights, MI), and the perimeter of cell bodies containing microspheres was traced using Optimus, version 6.5, image analysis software (Media Cybernetics, L.P., Silver Spring, MD). Using the same field, an identical image was captured with the fluorescein filter and the outline of the cell bodies superimposed. The MGV was calculated for each neuron of interest using Optimus software. Neurons with an MGV < 50 were considered negative and neurons with an MGV $\geq$ 50 were classified as SP-IR. The cutoff from negative to positive was based on an initial survey of several neurons in the TG directly observed to be either positive or negative and then digitally analyzed. The percentage of SP-IR neurons innervating the nasal epithelium was determined by dividing the positive SP-IR (MGV$\geq$50) microsphere-containing neurons by the total number of microsphere labeled neurons.

Statistical Analysis The means and standard errors were calculated for the percentage of SP-IR and CGRP-IR neurons in the TG and nasal lavage cell differentials. A Student's t test was run with significance set at $p \leq 0.05$.

RESULTS

As determined by direct observation, the frequency of SP-IR positive neurons following asphalt exposure (83.7$\pm$1.4%) was significantly increased from the control group (58.5$\pm$2.9%) (Figure 1). The asphalt group also showed a significant increase in the percentage of CGRP-IR positive neurons over the control group, with values of 65.3$\pm$2.0 and 49.7$\pm$6.8 respectively (Figure 2). The increase in SP-IR following asphalt

exposure was confirmed by a second method of evaluation involving threshold analysis of intensity measurements. The SP-IR of microsphere-containing neurons was determined by converting the immunofluorescent intensity to a MGV (Figure 3). Neurons with a MGV $\geq$ 50 were categorized as SP positive. Using digital image analysis, the percentage of SP positive neurons in the control group (40.5$\pm$8.1%) was found to be significantly lower than that for the asphalt group (61.9$\pm$4.5%; Figures 1).

The nasal lavages showed the presence of inflammatory cells in response to inhalation of asphalt fumes (Figure 4). The percentage of neutrophils (5.3$\pm$1.6) in the nasal lavage fluid of asphalt-exposed animals was increased 4.1-fold over controls (1.3$\pm$0.5). The asphalt-exposed group also had a significantly increased number of macrophages (22.0$\pm$8.1%) in the nasal lavage fluid compared to control animals (2.4$\pm$0.8%). The asphalt exposed and control animals had a large number of epithelial cells in the nasal lavage (61.7$\pm$6.2% and 90.9$\pm$3.1% respectively, data not shown).

DISCUSSION

Past studies have investigated the role of asphalt in cancer, nonmalignant lung diseases, and renal disease (Ma *et al*., 2002; NIOSH, 2001). The present study is the first to evaluate the involvement of neuropeptides in the nasal cavity following asphalt inhalation. Retrograde transport of rhodamine-labeled-latex microspheres instilled into the nasal cavity was used to identify cell bodies of sensory neurons in the TG that projected to the nasal cavity (Hunter & Dey, 1998). Immunocytochemical processing of TG cell bodies containing microspheres was the first step in the evaluation of SP-IR and CGRP-IR.

Our study compared two different methods of neuropeptide measurement, direct observation and threshold analysis. Using both methods, it was determined that in the TG neurons innervating the nasal epithelium SP-IR was significantly increased following asphalt fume exposure. Intensity measurements (40.6±8.1% for control and 61.9±4.5% for asphalt exposed) indicated fewer numbers of positive cells compared to direct measurements (58.5±2.9% and 83.7±1.3%, respectively). The threshold analysis method converts the fluorescent intensity of a TG neuron into an MGV, which eliminates the subjectivity that may occur using the naked eye. Although both methods have limitations, both are sufficient for detecting changes in neuropeptides. Direct observation is an established method for determining SP-IR in TG neurons following irritant inhalation (Hunter *et al*., 2000; Hunter *et al.*, 1998), but the use of digital images and image processing to determine positive and negative neurons reduces possible observer bias.

Previous studies demonstrated a link between neuropeptides, irritants and inflammation. Inhalation of dust or chemical irritants produced rhinitis, nasal and upper airway irritation, and obstructive airway disease (Seixas *et al*., 1992; Vandenplas et al., 1993; Brugsch & Elkin, 1963). In rats exposed to inhaled silica for six months, the proportion of SP-IR neurons innervating the nasal epithelium increased 8.7-fold compared to controls (Hunter *et al.*, 1998). Increased SP-IR in microsphere-containing TG neurons was detectable in rats 48 hr after toluene diisocyanate (TDI) exposure (Hunter *et al.*, 2000). The latter studies also analyzed the level of preprotachykinin (PPT) messenger RNA (mRNA) in TG sensory neurons innervating the nasal epithelium and found an increase in message after both silica and TDI exposure. Since PPT mRNA

codes for SP, the increase in SP content is probably attributed to an increase in transcription of the PPT gene and translation of PPT mRNA.

The increase of SP-IR and CGRP-IR in TG neurons following asphalt exposure demonstrates that synthesis was not enhanced, and suggests an increase in subsequent release of SP and CGRP from nerve fibers in the nasal epithelium. Following synthesis in the TG cell bodies, neuropeptides undergo anterograde axonal transport to corresponding sensory nerve terminals innervating inflamed tissue (Buck *et al.*, 1999). In previous studies, SP levels in epithelial nerve terminals rapidly increased following TDI, suggesting enhanced anterograde-axonal transport of neuropeptides. After peaking 24 hr following TDI exposure, the levels of SP in epithelial nerves steadily decreased and returned to control levels by 96 hr, suggesting that SP was released into the surrounding nasal tissue (Hunter *et al.*, 2000). The neural response to inhaled irritants has been termed neurogenic inflammation and is characterized by increased vascular permeability, plasma extravasation, edema and inflammatory cell chemotaxis, all of which are mediated primarily by SP released from sensory nerve endings (Jancso *et al.*, 1967). In the present study, the asphalt-induced elevation in SP-IR neurons was also associated with an increase in the number of intranasal neutrophils and macrophages. Epithelial damage and shedding, and inflammatory cell influx are common sequelae following irritant inhalation (Harkema, 1990). Studies using capsaicin provided the first evidence that sensory nerves mediated neurogenic inflammation. Application of capsaicin to the nasal mucosa depletes SP from sensory nerve endings but has no effect on parasympathetic or sympathetic nerves (Lundblad *et al.*, 1983). The release of SP from sensory nerve endings in the nasal mucosa is believed to be part of a protective reflex

mechanism, which limits irritants from gaining access to the lower airways (Lundblad, 1984).

Nasal irritation has been reported among asphalt road crew workers (Hanley & Miller, 1996). When extrapolated over 8 hr, the asphalt fume concentration (10.8±1.2 mg/m^3, 3 hr/day) used in an initial study was actually less than current ACGIH TLV; therefore, it is possible that neuropeptide levels may be elevated in asphalt workers with concomitant airway inflammation (Sikora *et al.*, 2001). Inhaled asphalt fume particles may stimulate C-fibers in the nasal epithelium, resulting in neuropeptide production in the corresponding TG neurons and subsequent release from collateral sensory nerve endings in the nasal mucosa. Such a response in asphalt workers requires further investigation.

In conclusion, SP and CGRP levels are increased in upper airway sensory neurons of rats chronically exposed to asphalt fumes. These neuropeptides are involved in neurogenic inflammation, which may explain the influx of neutrophils and macrophages into the nasal cavity and their elevation in nasal lavage fluid. These findings suggest that asphalt fumes can be added to the growing list of chemicals known to stimulate C-fibers in the nasal cavity. In road construction workers, chronic asphalt exposure may activate sensory nerves in the nasal epithelium leading to an increase in neuropeptides and resulting in nasal inflammation and irritation.

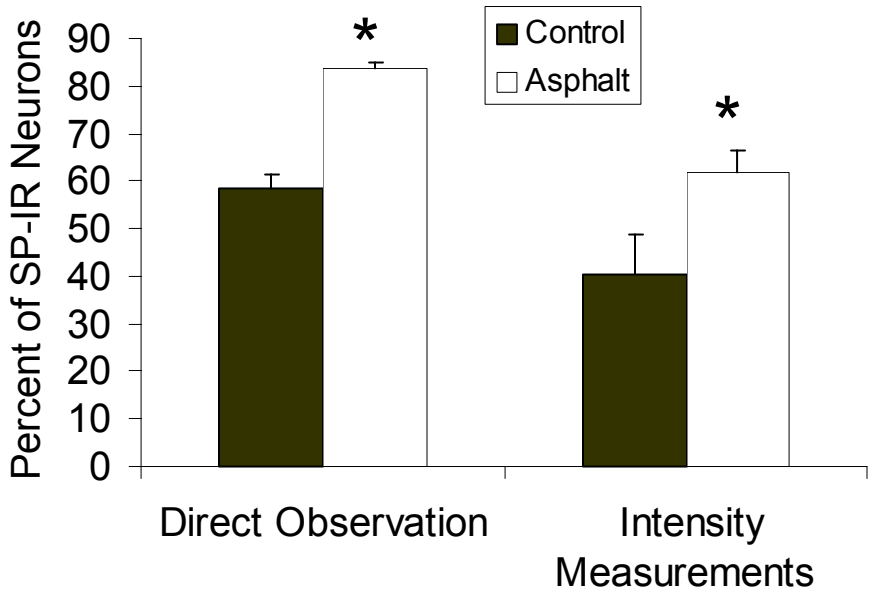

Figure 1 The percentage of SP-IR TG neurons innervating the nasal epithelium detected using both direct observation and intensity measurements by digital analysis following asphalt fume or ambient air (control) exposure. The asterisks denote significant asphalt-induced change relative to controls ($p \leq 0.05$). n=7 for each group.

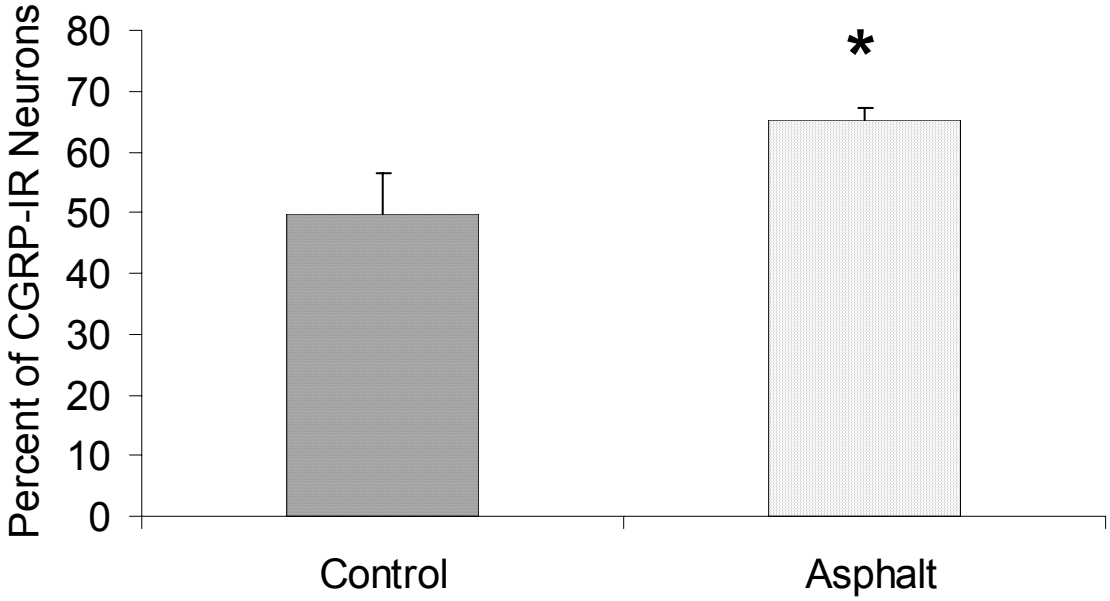

Figure 2 The percentage of CGRP-IR TG neurons supplying the nasal epithelium detected using direct observation after asphalt fume or ambient air (control) exposure. The asterisk denotes significant asphalt-induced change relative to controls ($p \leq 0.05$). n=7 for each group.

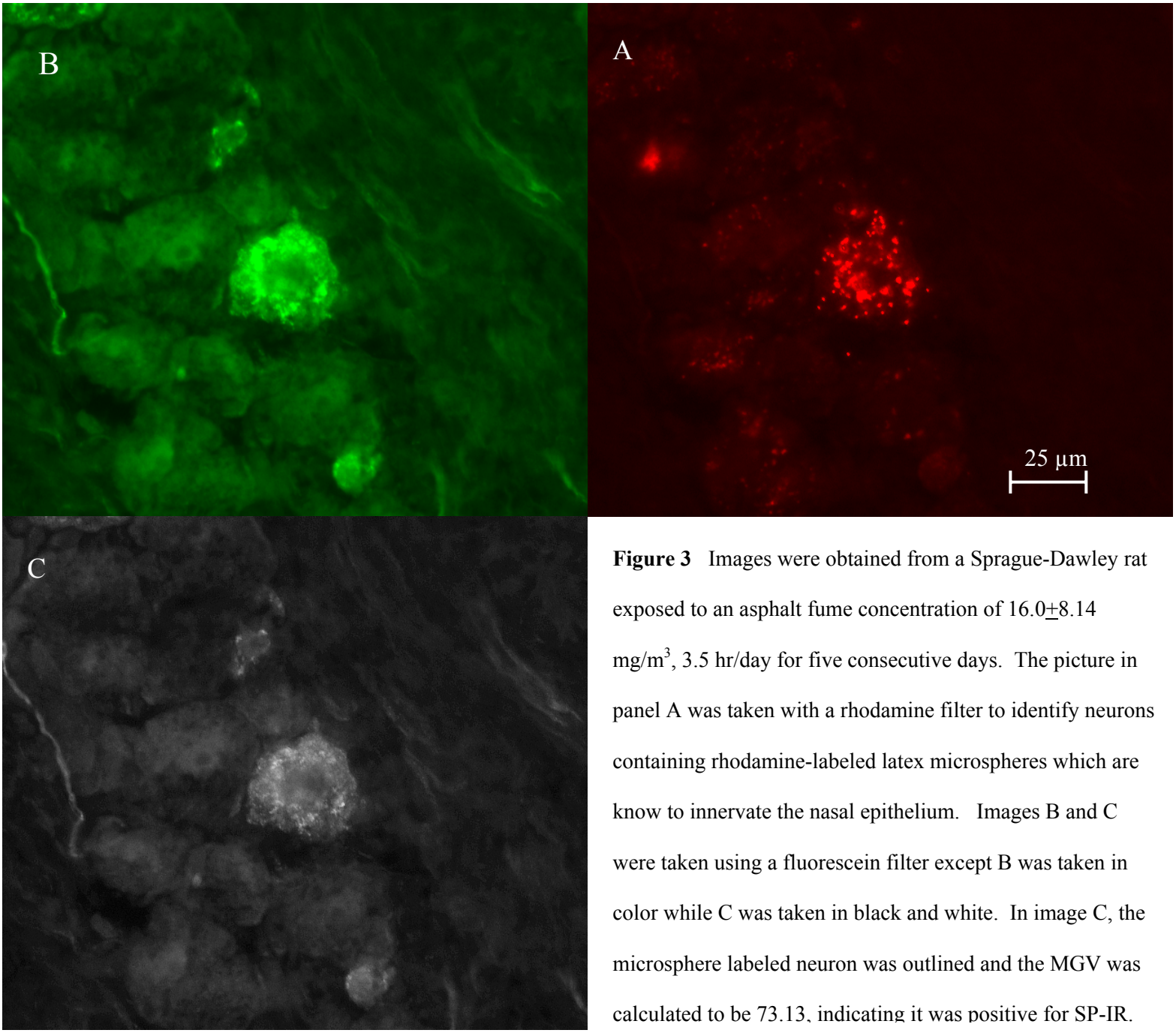

Figure 3 Images were obtained from a Sprague-Dawley rat exposed to an asphalt fume concentration of 16.0±8.14 mg/m^3, 3.5 hr/day for five consecutive days. The picture in panel A was taken with a rhodamine filter to identify neurons containing rhodamine-labeled latex microspheres which are know to innervate the nasal epithelium. Images B and C were taken using a fluorescein filter except B was taken in color while C was taken in black and white. In image C, the microsphere labeled neuron was outlined and the MGV was calculated to be 73.13, indicating it was positive for SP-IR.

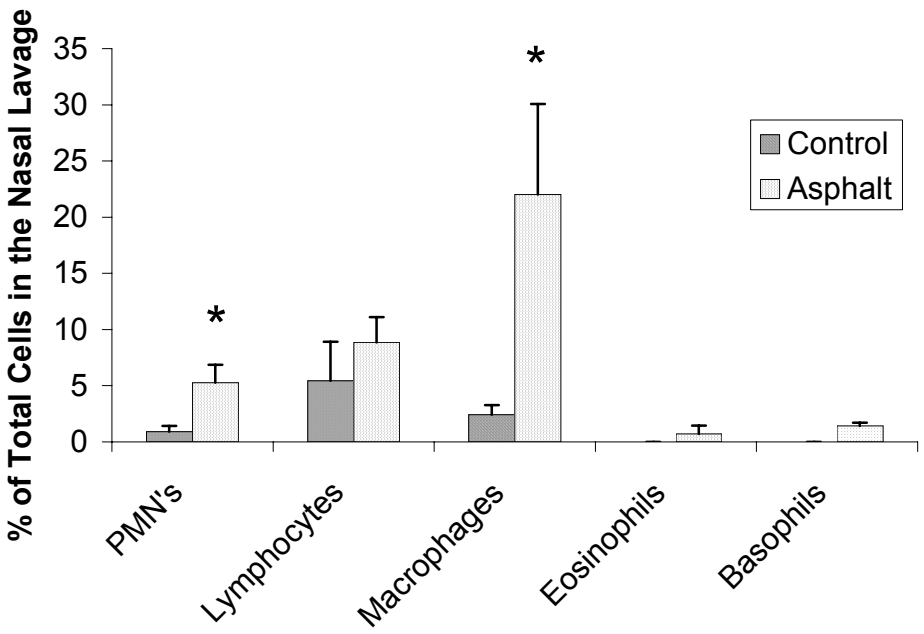

Figure 4 The nasal lavage cell differentials (% of total cells) from asphalt and ambient air (control) exposed rats. The asterisk denotes asignificant difference relative to the controls ($p \leq 0.05$). n=7 for each group.

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STUDY 2

Enhanced Substance P Expression in Sensory Neurons of the Nasal Cavity after Toluene Diisocyanate Exposure Correlates with Increased Levels of NGF

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ABSTRACT

Toluene diisocyanate (TDI) exposure produces rhinitis and nasal irritation and increases the synthesis and release of substance P (SP) from airway sensory nerves. The mechanism leading to enhanced SP production following irritant inhalation remains unclear, but may involve actions of nerve growth factor (NGF). NGF binds trkA receptors located on sensory nerve terminals. Activation of trkA receptors initiates kinase-signaling cascades, which ultimately may increase SP. However, the effects of inhaled irritants on NGF release are not known. In this study, NGF levels in nasal lavages were examined following instillation of 10% TDI into both nasal cavities. NGF was significantly increased 2, 6, 12 and 24 hrs after TDI exposure compared to controls. The increase in NGF preceded the neuronal and mucosal increases in SP. Pretreatment with K252a, a non-selective tyrosine-kinase inhibitor, prevented the increase in SP-immunoreactivity in TG neurons and epithelial nerve fibers and the inflammatory response to TDI exposure. The findings suggest that NGF release into the nasal cavity following TDI precedes SP production in TG cell bodies innervating the nasal epithelium.

INTRODUCTION

Inhaled gases, vapors or particles found in various occupational and environmental settings can act as irritants to the upper respiratory system (Brooks, 1995; Brooks & Bernstein, 1993). Toluene diisocyanate (TDI) is commonly used in the production and manufacture of polymer containing products such as plastics, foams, adhesives and surface coatings. Workers exposed to TDI vapor may develop inflammatory conditions including asthma, rhinitis and nasal irritation (Brugsch & Elkin, 1963; Mapp *et al.*, 1988). At the cellular level, TDI causes the release of neuropeptides, including substance P (SP), from activated sensory nerves in the nasal cavity and bronchi (Gordon *et al*., 1985; Sheppard *et al*., 1988; Thompson *et al.*, 1987). Activation of C-fibers located in the airway mucosa produces neurogenic inflammation characterized by vasodilation, plasma extravasation, mucous secretion and neutrophil chemotaxis resulting from the release of neuropeptides, especially SP and other tachykinins (Jancso *et al*., 1967; Lundberg *et al*., 1991; Lundberg & Saria, 1983). In addition to TDI, a variety of other irritants, including cigarette smoke, acrolein, histamine, sulfur dioxide, and ozone have also been shown to activate sensory nerve fibers and induce neurogenic inflammatory responses in the airways of animal models (Lundberg & Saria, 1983; Morris *et al*., 1999; Turner *et al.*, 1993). Neurogenic inflammation has also been demonstrated in human airways and has been implicated in the pathology of chemical sensitivity syndromes (Meggs, 1993) as well as a number of disease states, including rhinitis and asthma (Barnes, 1992; Joos *et al.*, 1995).

There is substantial evidence that neurogenic inflammation in the airways is mediated by the release of the sensory neuropeptide SP from the peripheral endings of C-

fibers distributed throughout the airway walls (Baluk et al., 1992; Lundberg *et al*., 1982; Lundberg *et al.*, 1983). SP is synthesized in nerve cell bodies through post-translational splicing of preprotachykinin mRNA (Krause *et al.*, 1987). SP-containing sensory nerve endings in the nasal mucosa are found near blood vessels, mucous glands and epithelium (Baraniuk *et al.*, 1991). The nerve cell bodies supplying sensory C-fibers to the nasal cavity are located in the trigeminal ganglia (TG) (Hunter, 1997). Transient increases in SP protein and message have been demonstrated in sensory cell bodies innervating respiratory epithelium of the nose or lung following antigen challenge (Fischer *et al.*, 1996), TDI exposure (Hunter *et al.*), viral infection (Carr *et al.*, 2002) and asphalt fume exposure (Sikora *et al.*, 2003).

Although the contribution of SP to a wide range of airway inflammatory conditions has been recognized, the mechanisms regulating irritant-enhanced SP expression in sensory neurons have not been established. Recent studies suggest that nerve growth factor (NGF), a neurotrophin released from inflamed tissues, may be a key mediator in the up-regulation of SP levels in sensory neurons (Nicholas *et al.*, 1999). NGF regulates neuronal growth and controls neuropeptide levels in mature sensory neurons (Levi-Montalcini *et al.*, 1996). NGF is expressed in non-neuronal cells associated with the process of inflammation, including airway epithelial cell lines (Fox *et al.*, 2001), mast cells (Leon *et al.*, 1994) and lymphocytes (Santambrogio *et al.*, 1994).

Several studies suggest that NGF may play a role in sensory-neural responses during airway inflammation. Superficial nasal mucosal cells obtained from the inferior turbinate bones of healthy human subjects constitutively express mRNA for NGF and NGF protein is present in nasal lavage fluid (Sanico *et al.*, 2000), suggesting that NGF

may be important in maintaining normal levels of sensory innervation. However, airway inflammatory conditions may lead to increased NGF production. Significantly higher levels of NGF have been detected in serum of asthmatics compared to non-asthmatics (Bonini *et al.*, 1996) and patients with allergic rhinitis have significantly higher NGF concentrations in nasal lavage fluid compared to control subjects (Sanico *et al.*, 2000). The involvement of NGF in regulating neuropeptide expression in sensory neurons innervating the airways is also supported by recent studies in animal models. Transgenic mice overexpressing NGF in the airways have higher SP levels in the airway wall and exhibit enhanced sensitivity to capsaicin-induced airway contraction (Hoyle *et al.*, 1998), a response mediated by sensory C-fibers, and also have enhanced inflammatory responses to ozone exposure (Graham *et al.*, 2001). Tracheal instillations of NGF produces increased SP expression in neurons of the nodose and jugular ganglia innervating the guinea pig airways (Hunter *et al.*, 2000) and produces airway hyperresponsiveness through activation of SP-selective, NK-1 receptors in rats (de Vries *et al.*, 1999). NGF levels are increased during viral infections in rats and NGF antibodies attenuate the enhanced NK-1 receptor expression observed during viral infections (Hu *et al.*, 2002). Recent studies demonstrating that transgenic mice deficient in the NGF receptor exhibit reduced inflammatory responses to antigen challenge (Path *et al.*, 2002).

The aim of the present study was to determine if NGF production and release in the nasal cavity increase during irritant exposures and to determine if NGF mediates increased SP expression in airway sensory neurons. We first correlated the time-course for NGF production in the nasal cavity, the arrival of NGF in the cell bodies of sensory neurons and SP production in sensory neurons and nasal inflammatory responses. Then,

we showed that neuronal SP levels and inflammatory responses were attenuated after the NGF transduction pathway was blocked with the tyrosine kinase inhibitor, K252a. The findings support the hypothesis that TDI exposure causes the release of NGF in the nasal cavity and that NGF regulates SP expression in sensory neurons innervating the nasal mucosa.

METHODS AND MATERIALS

Experimental Design Adult male Sprague-Dawley rats (Hla:(SD)CVF) weighing 200-250 g purchased from Hilltop Lab Animals (Scottsdale, PA) were used for all the experiments. For the time-course studies, either 10% TDI or ethyl acetate (vehicle) was instilled into the nasal cavity. Tissues were removed and nasal lavages were done 2, 6, 12, 24, 48 or 96 hrs later (n=6/time point).

For the protein kinase inhibitor studies, either K252a or DMSO (vehicle) was instilled into the nasal cavity 2 hr prior to either TDI or ethyl acetate. Tissues were removed and nasal lavages were performed 24 hr after irritant instillation (n=6/group).

Rhodamine Latex Microsphere Instillation Ten days prior to TDI or K252a/TDI instillations, rats were anaesthetized with an intraperitoneal injection of sodium brevital (50mg/kg body weight; Eli Lilly, Indianapolis, IN). Neurons in the trigeminal ganglion projecting to the nasal cavity were identified using a retrograde neural tracing procedure described previously (Hunter, 1997). Briefly, the anterior and posterior regions of the right and left nasal cavities were each instilled with 4 µl of rhodamine-labeled-latex microspheres using a 10 µl Hamilton syringe with plastic tubing covering the tip. The tubing was marked at 0.8 and 1.3 cm lengths to allow correct positioning into the anterior

and posterior nasal regions. Even distribution over the entire nasal mucosa was achieved by rotating the rats in a circular pattern around the anterior-posterior axis five times after microsphere instillation. The microspheres are endocytosed by sensory nerve endings in the nasal epithelium and then retrogradely transported to the corresponding cell bodies in the TG.

TDI Instillation Ten days following instillation of rhodamine-labeled-latex microspheres the rats were again anaesthetized with an intraperitoneal injection of sodium brevital (50 mg/kg weight dose). Both nasal cavities were instilled with 5 µl of 10% TDI (Aldrich Chemical Co., Milwaukee, WI) or ethyl acetate (vehicle; Sigma Chemical Co., St. Louis, MO) by placing the tip of a 10 µl pipette at the entrance of the nasal cavity.

K252a Instillation Two hours prior to TDI instillation the rats were anaesthetized with an intraperitoneal injection of sodium brevital as described. The anterior and posterior regions of the right and left nasal cavities were each instilled with 8 µl of K252a (100 µg/ml) (Alexis Biochemicals, San Diego, CA) or dimethyl sulphoxide (vehicle, DMSO, Sigma, St. Louis, MO). The K252a and DMSO were delivered using a 10 µl Hamilton syringe with plastic tubing covering the tip. The tubing was marked at 0.8 and 1.3 cm lengths to allow correct positioning into the anterior and posterior nasal regions. NGF action is inhibited by K252a, a carbazole alkaloid, which has been shown to inhibit trkA, trkB and trkC phosphorylation (Ohmichi *et al.*, 1992). K252a binds with high affinity to one site on the cytoplasmic kinase domain of the trkA receptor and inhibits NGF-stimulated phosphorylation of tyrosine residues on trkA receptors (Berg *et al.*, 1992), (Ohmori *et al.*, 1988).

Nasal Lavage. The rats were overdosed with 1.5 ml of 50 mg/ml sodium brevital and the lower jaw was removed. A syringe with plastic tubing covering the needle was inserted into the posterior nares and sealed by finger pressure. Both sides of the nasal cavity were simultaneously lavaged with 15 ml of phosphate buffered saline (PBS). The first 3 ml of lavage fluid were kept separate from the final 12 ml. Both aliquots for the nasal lavage fluid were centrifuged at 1,500 rpm (352 rgf) for 10 min. The supernatant from the initial 3 ml of nasal lavage was aliquoted and frozen at -80°C for subsequent assays. The two resulting cell pellets from each nasal sample were resuspended in a total of 1 ml cold PBS and pooled, plated on glass slides ($1.5x10^5$ cells) using a cytospin (Shandon Scientific, Ltd., Cheshire, UK) at 400 rpm (18.06 rgf) for 4 min and stained with Wright-Giemsa on a Hema-Tek 2000 automated slide stainer (Bayer, Inc., Tarrytown, NY). A total of 100 cells were classified as neutrophils or nucleated cells (primarily epithelial cells) using light microscope (Olympus AX70) with a 40x magnification objective. The percentage of neutrophils was recorded for each slide.

Tissue removal and preparation The right and left TG were removed by cutting distal to the division of the ophthalmic, maxillary and mandibular trigeminal branches and at the junction of the trigeminal nerve with the ganglion. The nasal mucosa was also removed from the anterior and posterior regions of the nasal cavity. All tissue was immediately fixed in picric-acid formaldehyde fixative consisting of 2% paraformaldehyde, 15% saturated picric acid and 0.15 M phosphate buffer at 4°C (Stefanini *et al.*, 1967). After 3 hr, the tissue was rinsed twice in 0.1 M phosphate-buffered saline containing 0.3% (v/v) Triton X-100 (PBS-Tx, pH=7.8). After the second rinse, the tissues remained in PBS-Tx overnight at 4°C. The next day the TG were

oriented on corks so the first section would be taken from the ventral surface. The nasal mucosa was laid flat and then rolled into a cylinder shape and stood upright on the cork. The tissues were covered with Tissue Tek OCT compound (Sakura, Torrance, CA), frozen in isopentane cooled by liquid nitrogen and stored in airtight plastic bags at –80°C.

Continuous serial cryostat sections (12 μm thickness) of the entire TG were made as previously described (Hunter, 1997). The first and second sections were collected on two separate gelatin-coated coverslips. The third, forth and fifth sections were discarded. This was repeated until the entire TG was sectioned. The first coverslip was used for SP immunocytochemistry and the second for NGF immunocytochemistry. The nasal epithelium was sectioned at 12 μm and used to evaluate SP nerve fiber density. A separate coverslip containing 16 sections randomly taken throughout the nasal mucosa was used for SP immunocytochemistry.

Immunocytochemistry Procedures for immunocytochemistry were previously described (Dey *et al.*, 1990). Cryostat sections on gelatin-coated coverslips were covered with either rabbit anti-SP (1:200; Peninsula, Belmont CA) or rabbit anti-NGF (1:100; Chemicon International, Inc., Temecula, CA) primary antiserum diluted in PBS-Tx + 1% bovine serum albumin (PBS-Tx-BSA, pH=7.8). The coverslips were incubated in a humid chamber at 37 °C for 30 min, rinsed 3 times with PBS-Tx-BSA, allowing 5 min per rinse and then covered with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin IgG (ICN Pharmaceuticals, Inc., Costa Mesa, CA) diluted 1:100 in PBS-Tx-BSA and incubated at 37 °C for 30 min. The coverslips were rinsed 3 times for 5 min increments in PBS-Tx-BSA and mounted on glass slides in Fluoromount (Southern Biotechnology, Birmingham, AL). The sections were observed using an Olympus AX70

fluorescence microscope equipped with fluorescein (excitation 495 nm and emission 520 nm, for antibodies) and rhodamine (excitation 540 nm and emission 580 nm for microspheres) filters.

Analysis of Immunoreactivity SP and NGF immunoreactivity was evaluated in TG cell bodies innervating the nasal epithelium. Without knowledge of the experimental grouping, neurons containing rhodamine-labeled-latex microspheres were identified. The presence of microspheres was used as criteria that axons of the identified cell bodies projected to the nasal epithelium. Once the neurons of interest were identified using the rhodamine filter, a black and white image was captured with a SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) and the perimeters of cell bodies containing microspheres were traced using Optimus, version 6.5 image analysis software (Media Cybernetics, L.P., Silver Springs, MD). Using the same field, an identical black and white image was captured with the fluorescein filter. The cell body outline obtained using the rhodamine filter was superimposed on the fluorescein image. The intensity of immunocytochemical labeling for SP or NGF was determined by calculating the mean gray value (MGV) for each neuron using Optimus software. Neurons with an MGV< 50 were considered negative and neurons with an MGV$\geq$50 were classified as immunoreactive for the protein of interest. The cut-off range from positive to negative was based on an initial survey of several neurons in the TG directly observed to be positive or negative with the naked eye and then digitally analyzed. The percentage of SP-IR or NGF-IR neurons innervating the nasal epithelium was determined by dividing the total number of positive microsphere-containing neurons (MGV$\geq$50) by the total number of microsphere-labeled neurons.

SP Nerve Fiber Density (NFD) Following immunocytochemical processing for SP, sections of nasal mucosa were observed on a Zeiss LSM 510 confocal microscope equipped with an argon laser (Zeiss, Germany). Eighteen random images of respiratory epithelium were recorded from each coverslip. Using Optimus, the entire perimeter of epithelium was traced on each image of nasal mucosa. The threshold for each image was optimized so that only SP-IR nerve fibers were visible. The SP NFD was calculated by dividing the SP-IR nerve fiber area by the total area of epithelium outlined. This represents the proportional cross-sectional area occupied by SP-immunoreactive nerve fibers.

NGF ELISA The nasal lavage supernatant samples (initial 3 ml) were frozen at –80 °C. The concentration of NGF (7.8-500 pg/ml) in each sample was assayed using the NGF Emax ImmunoAssay System (Promega, Madison, WI) according to manufacturer's instructions. NGF was detected using an antibody sandwich format in 96 well plates. Each well was initially coated with 100 µl of anti-NGF pAb and incubated overnight followed by a one-hour incubation with blocking buffer (200 µl/well) to prevent nonspecific binding. Either 100µl of lavage supernatant of 100 µl of NGF standard (7.8-500 pg/ml) was added to each well. The plate was incubated for six hours followed by an overnight incubation with anti-NGF mAb (100 µl /well). For color development, an anti-rat IgG-horseradish peroxidase conjugated antibody (100 µl) was added to each well followed by a tetramethlybenzidine solution, which reacts with the peroxidase-labeled conjugates to develop a blue color. The absorbance of each well was measured at 450 nm on a Spectra Max 340pc plate reader (Molecular Devices, Sunnyvale, CA). The concentration of NGF in each lavage sample was extracted from an NGF standard curve.

All samples were run in duplicate or triplicate, and, as a negative control, a PBS sample was run with each assay.

Protein Assays The nasal lavage supernatant samples were also assayed for total protein using the bicinchroninic acid (BCA) protein assay kit (Pierce, Rockford, IL), a modified Lowry assay that measures the concentration of total protein (20-2,000 μg/ml). The assay was performed according to the manufacturers instructions using the microplate procedure. The assay was performed in 96 well plates and 20 μl of unknown or bovine serum albumin standard was added to each well followed by 200 μl of a working reagent. The plate was incubated at 37 ˚C for 30 min before reading the absorbance at 562 nm on a Spectra Max 340pc plate reader (Molecular Devices, Sunnyvale, CA). The concentration of total protein in each lavage sample was estimated from a bovine serum albumin standard curve. All samples were run in duplicate or triplicate.

Statistical Analysis The means and standard errors were calculated for each endpoint measured. For the time-course study, a two-way ANOVA with a Tukey post-hoc test was run with treatment (TDI or EA) and time (2, 6, 12, 24, 48 or 96 hrs) as the variables. For the protein kinase inhibitor studies, a two-way ANOVA with a Tukey post-hoc test was run with pre-treatment (K252a or DMSO) and treatment (TDI or EA) as the variables. Significance was set at $p \leq 0.05$ for each endpoint measured.

RESULTS

I. Time course Studies

NGF in the Nasal Lavage Fluid Following TDI

The concentration of NGF in the nasal lavage fluid was significantly increased over controls at 2, 6, 12 and 24 hr following instillation of 10% TDI into the nasal cavity (Figure 5). NGF increased as early as 2 hr following TDI (104.74±23.86 pg/ml) compared to control animals (42.00±7.16) that received ethyl acetate. The amount of detectible NGF (pg/ml) continued to increase 6 hr (184.38±31.64), 12 hr (194.65±33.62) and 24 hr (229.32±28.54) after TDI exposure compared to controls (64.08±5.71 at 6 hr, 57.07±6.36 at 12 hr and 54.60±11.95 at 24 hr). By 48 hr and 96 hr, there was no significant difference in NGF in the nasal lavages of TDI and control animals. The elevated concentration of NGF in the nasal lavage fluid as early as 2 hr and continued to increase until 24 hr following TDI exposure demonstrates a time-dependent increase during the first 24 hr followed by a return to baseline values. The findings suggest that NGF levels are increased transiently in the nasal cavity following TDI exposure.

SP and NGF Immunoreactivity in TG Neurons Innervating the Nasal Epithelium Following TDI

The percentage of SP-IR in TG neurons innervating the nasal epithelium was significantly increased 24 hr after TDI instillation (55.24+4.63%) compared to controls (10.50±1.71%) (Figures 6 and 7). After a peak at 24 hr, the % of SP-IR neurons in TDI animals was decreased at 48 hr but remained elevated over controls (30.68±2.36 and

7.72±1.28, respectively). By 96 hr, there was no observable difference between the percentage of SP-IR TG neurons in control and TDI exposed animals. These findings suggest that TDI exposure produced increased levels of SP in TG neurons projecting to the nasal cavity.

Changes in NGF-IR in TG neurons innervating the nasal epithelium were also time-dependent after TDI exposure (Figures 8 and 9). The proportion of NGF-IR TG neurons was significantly increased 24 hr after TDI exposure relative to controls (75.58±9.72% and 11.67±0.84% respectively). A significant difference in the percentage of NGF-IR TG neurons, from 7.50±0.46 in control rats to 21.12±5.41 in TDI exposed rats, was still detected at the 48 hr time point. TDI-induced changes in NGF-IR TG cell bodies returned to pre-exposure levels 96 hr after TDI instillation. The occurrence of NGF in the cells bodies of TG neurons probably reflects the uptake and axonal transport of NGF after being released in the nasal cavity.

SP Nerve Fiber Density in the Nasal Epithelium Following TDI

The percent area of SP-IR nerve fibers in the nasal mucosa significantly changed over time following intranasal instillation of TDI (Figure 10). The SP nerve fiber density (NFD) was significantly increased from 0.19±0.02 in control animals to 0.56±0.08 in the TDI group 12 hr following irritant exposure. The percent area of SP-IR nasal mucosal nerve fibers remained elevated at 24 hr in TDI exposed animals compared to controls (0.53±0.02 and 0.20±0.03, respectively). By 48 hr, the SP NFD in TDI-treated animals returned to control levels where it remained at 96 hr. These findings suggest that the

levels of nerve fiber-derived SP in the nasal mucosa is increasing during the period 12 and 24 hr after TDI exposure.

Inflammatory Markers in the Nasal Lavage Fluid Following TDI

The percentage of neutrophils in the nasal lavage fluid was significantly increased above controls at 6, 12, 24 and 48 hr following intranasal TDI instillation (Figure 11). The proportion of neutrophils in the nasal lavage fluid steadily increased from 44.93±2.06 at 6 hr to 68.87±9.00 at 12 hr compared to controls (21.66±2.18 and 12.68±2.94, respectively). The highest neutrophil count, 79.45±2.23%, was observed 24hr following TDI exposure compared to 14.70±4.22% observed in control animals. Thereafter, the percentage of neutrophils began to decrease at 48 hr (55.7±6.14) and returned to control levels by 96 hr (27.57±4.55) compared to control values (13.17±12.87 and 13.75±3.97 respectively). These findings suggest that TDI induces neutrophilic inflammation in the nasal cavity during the first 6 hours and the inflammation lasts at least 2 days.

The concentration of total protein (μg/ml) in the nasal lavage fluid was significantly increased above controls 12 and 24 hr following intranasal TDI instillation (Figure 12). Total protein was significantly increased (236.12±39.00 μg/ml) 12 hr following TDI treatment compared to control levels (38.30±3.71 μg/ml). The largest amount of protein was detected in the nasal lavage fluid 24 hr following TDI compared to controls (393.47±61.00 and 54.11±10.02 μg/ml, respectively). By 48 hr, the concentration of protein (μg/ml) in the nasal cavity of TDI exposed animals (41.49±11.33) had returned to control levels (63.39±22.47) where it remained at 96 hr

(147.94±27.29 and 78.47±34.79, respectively). These results suggests that protein maybe leaking from the nasal vasculature 12 and 24 hr after TDI exposure. Together with the efflux of neutrophils into the nasal cavity during the same period, the findings support the occurrence of inflammation of the nasal cavity during the period between 12 and 24 hr.

II. Protein Kinase Inhibitor Studies

Immunoreactivity in TG Neurons Innervating the Nasal Epithelium Following K252a and TDI

As already observed, a significant increase (52.88±2.63%) in SP-IR TG neurons innervating the nasal epithelium 24 hr after TDI occurred in animals pretreated with DMSO (control for K252a). However, no increase was observed in animals pretreated with K252a (35.92±4.49; Figure 13), suggesting that K252a reduced the TDI-induced elevation of SP level in cell bodies projecting to the nasal cavity. K252a also caused a small increase in the proportion of rhodamine-labeled SP-IR TG neurons (36.24±4.76%) compared to the DMSO/EA group (16.23±2.43%).

A significant increase in the percentage of NGF-IR TG neurons innervating the nasal epithelium was observed in animals pretreated with DMSO prior to TDI (44.31±3.30) but not in animals pretreated with K252a (14.26±1.59; Figure 14). In animals exposed to ethyl acetate, no difference in NGF-IR was detected in TG neurons supplying the nasal epithelium when animals were pretreated with DMSO (13.58±2.33%) or K252a (19.60±3.50%).

SP Nerve Fiber Density in the Nasal Epithelium Following K252a and TDI

The increase in intraepithelial SP-IR nerve fibers observed 24 hr after TDI treatment was not observed in rats pre-treated with the receptor tyrosine-kinase inhibitor (Figure 16), K252a (0.22±0.02) but was observed in rats pre-treated with vehicle (0.52±0.03; Figure 15). In animals exposed to ethyl acetate, no difference in SP-IR NFD was detected when animals were pretreated with DMSO (0.21±0.01) or K252a (0.24±0.03). These findings strongly indicate that inhibiting the receptor tyrosine kinase activity (K252a) prevented the TDI-induced increase in SP nerve fibers innervating the nasal epithelium.

% Neutrophils in the Nasal Lavage Fluid Following K252a and TDI

The increase in the nasal lavage neutrophils observed 24hr after DMSO-TDI treatment (79.40±2.94%) did not occur in rats pre-treated with K252a (20.00±14.36%; Figure 17). There was no significant difference in the proportion of neutrophils in the nasal cavities of animals treated with either DMSO or K252a prior to ethyl acetate (3.25±0.41% and 14.00±9.46%, respectively). The findings show that inhibiting the receptor-tyrosine-kinase activity (K252a) prior to TDI exposure prevented the percentage of neutrophils from increasing in the nasal lavage fluid. The increase in total protein after TDI exposure was not significantly altered by pretreatment with K252a (Figure 18).

DISCUSSION

The production and release of neuropeptides from sensory nerves in the airways is an important part of the inflammatory response to inhaled antigens and to environmental or occupational irritants. The present study was done to investigate the possibility that NGF generated during irritant exposure mediates the SP content of airway neurons. Previous studies have demonstrated up-regulation of SP in airway sensory neurons after instillation of NGF into guinea pig airways (Hunter *et al.*, 2000) and in transgenic mice overexpressing NGF in airway epithelial cells (Hoyle *et al.*, 1998). The present study shows that NGF and SP levels in the nasal cavity and SP levels in sensory neurons innervating the nasal cavity are up-regulated after TDI exposure and that inhibition of NGF substantially reduces the TDI-induced up-regulation of SP and the inflammatory response. These findings support the conclusion that NGF is produced during irritant exposures and influences SP production in sensory neurons of the nasal cavity.

Our studies demonstrate a temporal relationship between NGF production in the nasal cavity, up-regulation of SP in TG neurons and the appearance of inflammatory markers such as increased neutrophil influx and protein levels in nasal lavage fluid. NGF increased in the nasal lavage fluid prior to the observed increases in SP levels in epithelial nerve endings and TG neurons and the inflammatory markers. Previous studies have demonstrated that levels of NGF increased significantly in the nasal lavage fluid of allergic rhinitis patients following allergen challenge (Sanico *et al.*, 2000), and our findings with TDI demonstrate similar increases in NGF. In addition to increased NGF in the nasal cavity, the number of NGF-containing TG neurons innervating the nasal epithelium was increased. Further, the arrival of NGF in the TG cells bodies and the up-

regulation of SP are temporally matched, both being increased at 24 and 48 hours, supporting the possibility that NGF is transported to the cell body to produce an effect.

The apparent latency between the increase in NGF in the nasal cavity, which was apparent at 2 hr, and the increase in SP-containing cell bodies in TG observed at 24 hr, is probably attributable to the processes of receptor binding and transport. Binding of NGF to trkA receptors located on sensory nerve terminals is well established (McMahon, 1996). The subsequent retrograde axonal transport of NGF to TG cell bodies has been demonstrated in dental models of trigeminal innervation where radiolabeled NGF was detected in TG cell bodies 15 hr after injection into the dental pulp (Wheeler *et al.*, 1998). Similarly, sciatic nerve NGF levels were increased by 136% 24 hrs after subcutaneous injection of Freund's adjuvant into the rat foot pad, suggesting that NGF is transported in axons of the nerve (Donnerer *et al.*, 1992). Although our studies suggest that resident cells of the nasal mucosa may produce NGF, other possible sources of NGF have been reported. Most importantly, immunocytochemical studies demonstrated that more than 80% of all TG neurons are immunoreactive for NGF and also contain NGF mRNA (Jacobs & Miller, 1999), suggesting that sensory neurons are capable of NGF synthesis. A number of inflammatory and immune cell types, including mast cells (Leon *et al.*, 1994), lymphocytes (Barouch *et al.*, 2000), eosinophils (Solomon *et al.*, 1998) and macrophages (Braun *et al.*, 1998) are capable of synthesizing and releasing NGF. The lack of significant inflammatory cell influx at 2 hr when NGF levels are already increasing does not support the concept that these cells are the initial source of NGF, although mast cells present in normal airway mucosa could be responsible for NGF

production. A more likely explanation is that epithelial cells produce NGF, an observation supported in studies using airway epithelial cell lines (Fox *et al.*, 2001).

We have provided evidence that NGF may mediate the up-regulation of SP in sensory neurons. Our findings show that treating the nasal cavity with K252a, a non-specific tyrosine-kinase inhibitor previously shown to inhibit or interfere with NGF activity, reduced the TDI-induced increases in SP innervation in the nasal mucosa, SP-positive cell bodies in the TG projecting to the nasal cavity, and inflammation in the nasal cavity. Previous studies showing that K252a inhibits NGF-dependent SP production in cultured sensory neurons (Buck & Winter, 1996) supports an inhibitory effect of K252a on NGF action and the important role of NGF in maintaining SP levels in sensory neurons. Inhibition of NGF action by injections of NGF antibody reduces the SP content of dorsal root ganglion neurons, further emphasizing the important role of NGF in maintaining SP levels in adult sensory neurons (Shadiack *et al.*, 2001).

The attenuation by K252a of the TDI-induced neutrophil influx can be correlated with the reduction in SP innervation of the nasal mucosa. Upon binding to NK-1 receptors localized within epithelium (Baraniuk *et al.*, 1991), SP initiates a receptor-mediated inflammatory response which includes neutrophil chemotaxis (Jancso et al., 1967; Lundberg & Saria, 1983). Thus, the reduced neutrophil influx after K252a is possibly due to decreased amounts of SP released from nerve fibers of the nasal epithelium. However, other effects of K252a have been described in antigen-sensitized and-challenged rats where the neutrophil-chemotactic factor normally produced by leukocytes infiltrating inflamed tissue is decreased in a concentration-dependent manner when K252a is present (Tanabe *et al.*, 1994). In addition to its inhibitory effects of

K252a on tyrosine kinase, K252a also blocks the NGF-mediated release of inflammatory mediators from resident mast cells and leukocytes by inhibiting protein kinase C and calmodulin (Ohmori *et al.*, 1988). Therefore, the effect of K252a on neutrophil influx cannot be unequivocally attributed to the NGF regulation of SP in sensory neurons. An inhibitor that solely targets the trkA receptor would clarify the involvement of SP or K252a. Interestingly, this study demonstrated that the percentage of SP-IR TG neurons innervating the nasal epithelium was increased in the animals that received K252a + EA, suggesting that the K252a produces a mild inflammatory response. However, the K252a treatment clearly inhibited the TDI-induced SP increases in sensory nerve cell bodies and fibers, the resulting influx of neutrophils and the increased protein in nasal lavage.

Limitations regarding the lack of specificity of K252a as a tyrosine-kinase inhibitor must be acknowledged. In addition to inhibiting protein kinase C and calmodulin (Ohmori *et al.*, 1988), K252a inhibits phosphorylation of the neurotrophin receptors trkB and trkC in addition to trkA (Ohmichi *et al.*, 1992). Tyrosine kinase is used as a signaling molecule in cascades activated by other growth factors including platelet-derived growth factor (Chin *et al.*, 1997) and hepatocyte growth factor (Morotti *et al.*, 2002) as well as other neurotrophins including brain derived neurotrophic factor, neurotrophin 3 (Klein *et al.*, 1991) and neurotrophin 4 (Klein *et al.*, 1992). Some data identifies selectivity of K252a for the trkA tyrosine kinases but not for kinases mediated by epidermal growth factor, insulin, and v-src (Ohmichi *et al.*, 1992).

Since NGF binds to the tyrosine kinase-coupled trkA receptor, attenuation of the neural response by K252a further supports a role for NGF as a trophic modulator of neural activation after irritant exposure. However, K252a is non specific and therefore

does not conclusively prove that NGF mediates the observed changes in SP levels in airway sensory neurons. Growth factors, other than NGF, could contribute to or be entirely responsible for the observed changes in sensory nerves after TDI exposure.

Overall, our findings demonstrate a correlation between NGF release in the nasal cavity following TDI exposure and SP up-regulation in TG neurons innervating the nasal epithelium. Based on the time-course of increased NGF in cell bodies of the TG ganglia and the inhibitory effects of the tyrosine kinase inhibitor, K252a, we have presented data supporting the hypothesis that irritant-induced SP expression in sensory neurons may be mediated by NGF acting through a receptor binding mechanism, probably involving the trkA receptors. Although the cellular source of the TDI-induced NGF remains unclear, the early rise in NGF levels in nasal lavage fluid suggests that it is produced in the nasal mucosa. Final assessment of NGF in airway neural changes will depend on the development of suitable inhibitors of NGF action. The findings still have important health implications because human subjects with allergic rhinitis, an inflammatory disease of the upper airways, have significantly higher baseline concentrations of NGF protein in nasal lavage fluids (Sanico *et al.*, 2000). Identifying not only the source of endogenous NGF but also the mechanisms controlling NGF synthesis and release will aid in the understanding and treatment of airway inflammation.

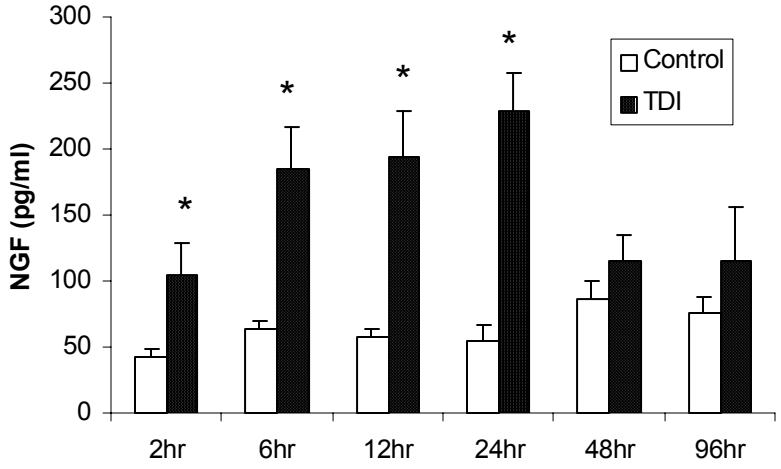


Figure 5 Concentration of NGF (pg/ml) in the nasal lavage fluid of Sprague-Dawley rats at various time points following intranasal instillation of 10% TDI or ethyl acetate (control vehicle). The asterisks denote significant change relative to controls ($p \leq 0.05$). n=6 for each group.

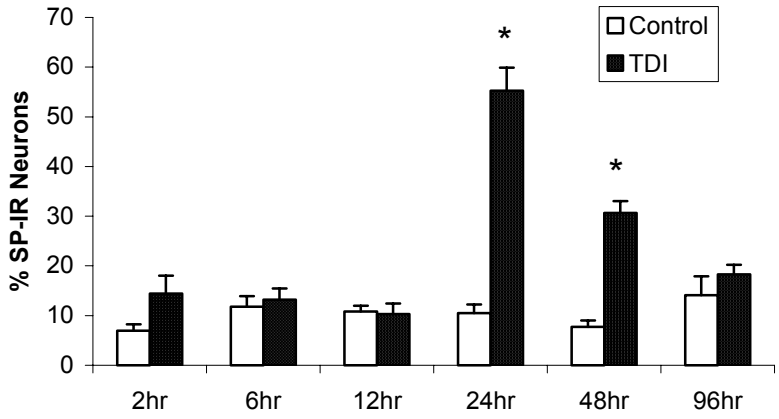


Figure 6 The percentage of SP-IR TG neurons innervating the nasal epithelium of Sprague-Dawley rats at various time points following intranasal instillation of 10% TDI or ethyl acetate (control vehicle). The asterisks denote significant TDI-induced change relative to controls ($p \leq 0.05$). n=6 for each group.

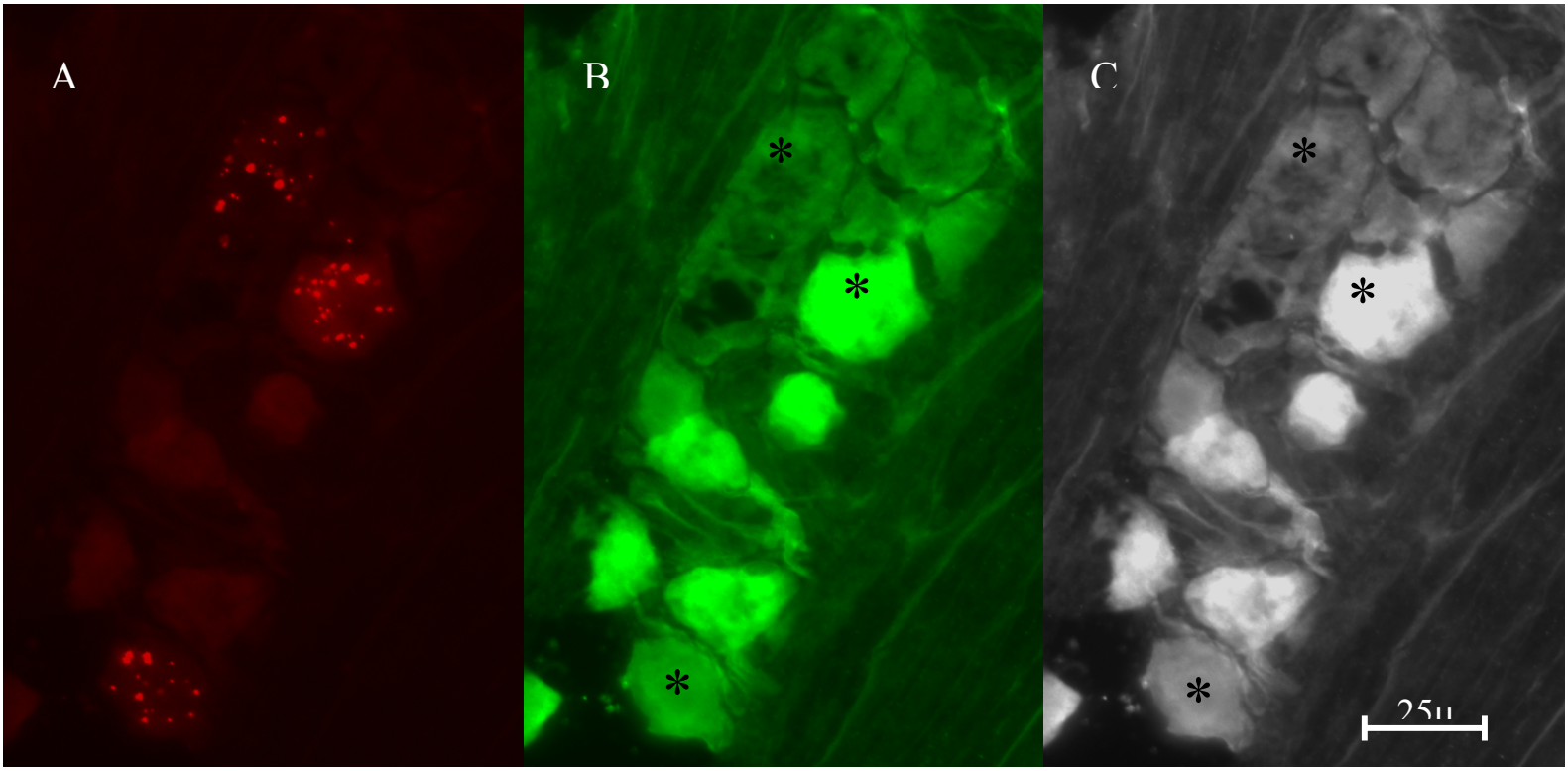


Figure 7 Images of TG processed for SP-IR using immunocytochemistry. Tissue was removed 24 hr following exposure to TDI. Image A was taken with a rhodamine filter to identify neurons containing rhodamine-labeled-latex microspheres, which are known to innervate the nasal epithelium. Images B and C were both taken using a fluorescein filter, except B was taken in color while C was taken in black and white. The asterisks indicate rhodamine-labeled cell bodies identified in A. In image C, the three microsphere-containing neurons were outlined and the MGV was calculated to be 32.14, 100.17, 59.46 (top to bottom). The first neuron was negative for SP-IR (MGV<50) and the other two were SP-IR (MGV>50).

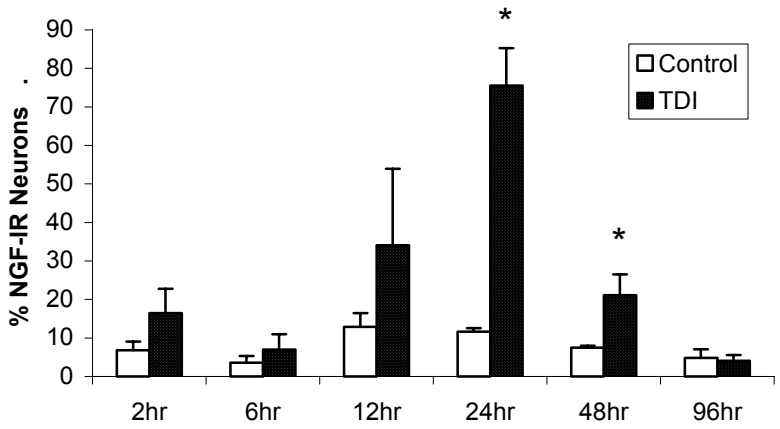


Figure 8 The percentage of NGF-IR TG neurons innervating the nasal epithelium of Sprague-Dawley rats at various time points following intranasal instillation of 10% TDI or ethyl acetate (control vehicle). The asterisks denote significant TDI-induced change relative to controls ($p \leq 0.05$). n=6 for each group.

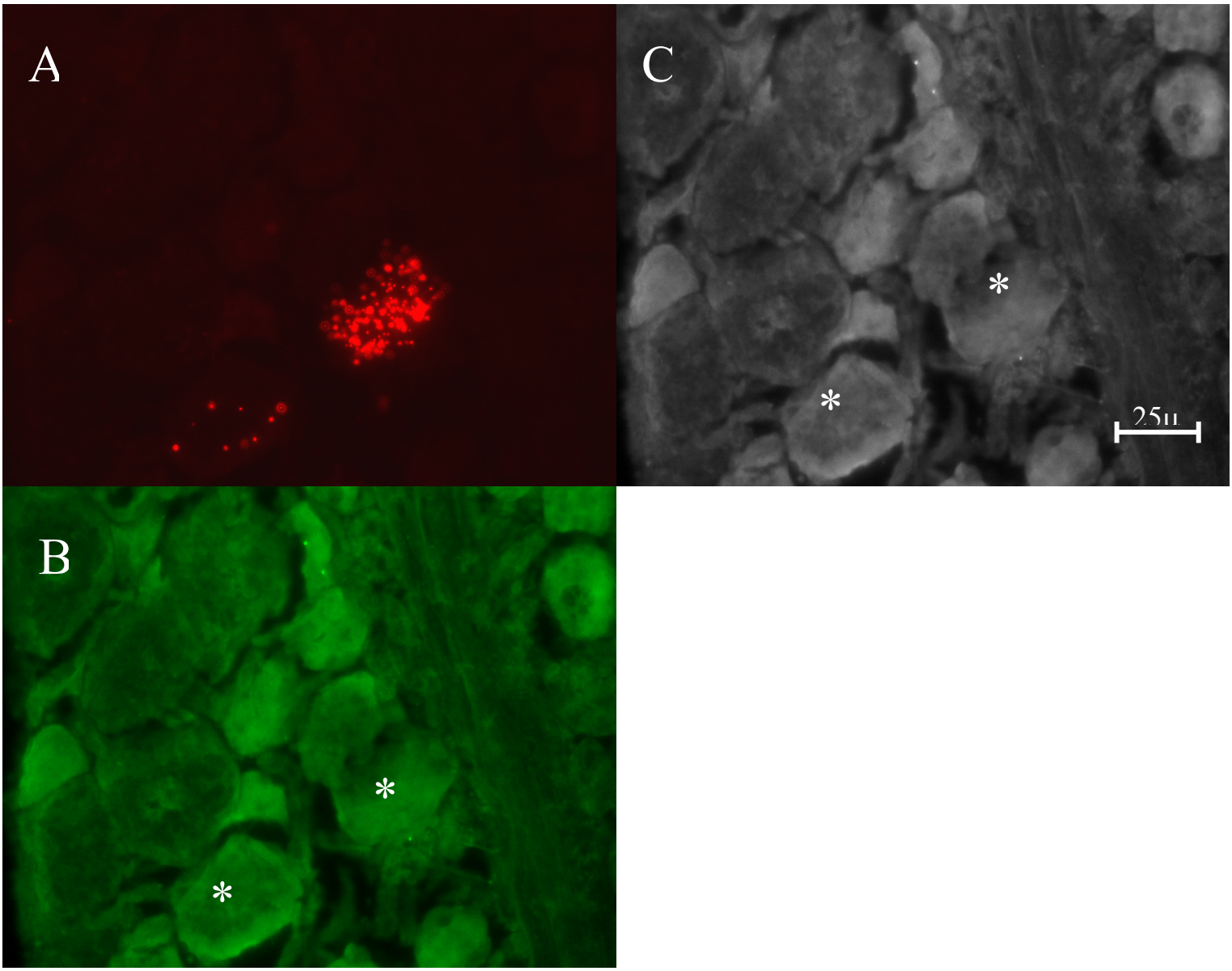


Figure 9 Images of TG sections processed for NGF using immunocytochemistry. Tissue was obtained from Sprague-Dawley rats sacrificed 48 hr after intranasal application of 10% TDI. Image A was taken with a rhodamine filter to identify neurons containing rhodamine-labeled-latex microspheres, which are known to innervate the nasal epithelium. Images B and C were both taken using a fluorescein filter, except B was taken in color while C was taken in black and white. In image C, the two microsphere-containing neurons were outlined and the MGV was calculated to be 65.41 and 61.47 (left to right), indicating both were positive for NGF-IR. Images B and C also contain a number of NGF-IR negative neurons (MGV<50), but none of these neurons supply the nasal epithelium. The asterisks indicate rhodamine-labeled cell bodies identified in A.

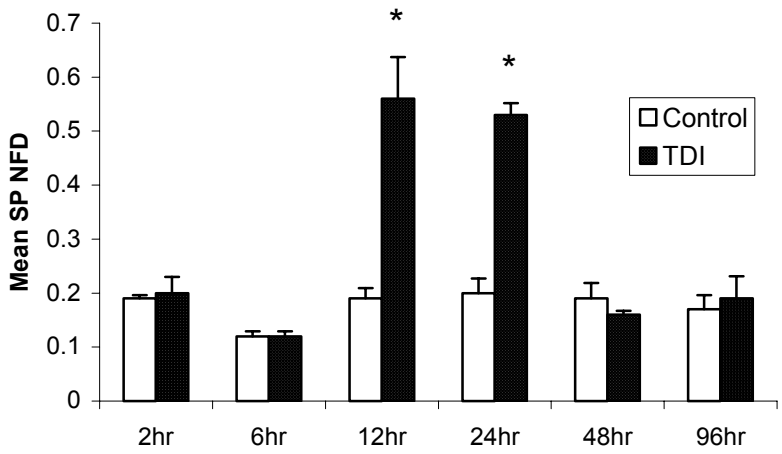


Figure 10 The density of SP-IR nerve fibers in the nasal epithelium of Sprague-Dawley rats at various time points following intranasal instillation of 10% TDI or ethyl acetate (control vehicle). The asterisks denote significant TDI-induced change relative to controls ($p \leq 0.05$). n= 6 for each group.

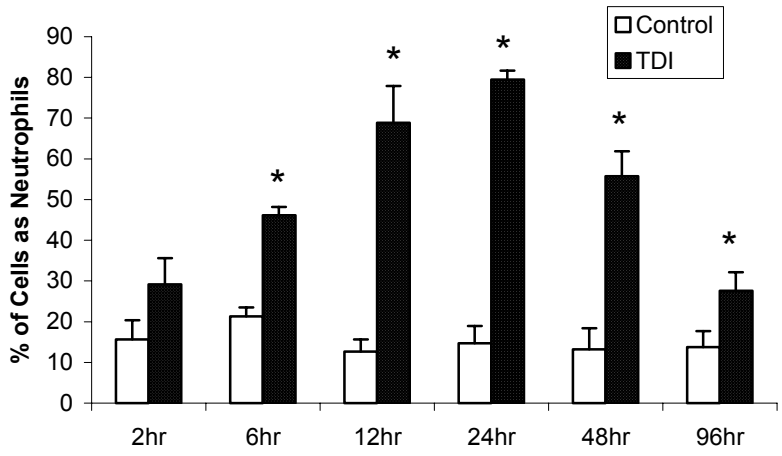


Figure 11 The percentage of nucleated cells that are neutrophils in the nasal lavage fluid of Sprague-Dawley rats at various time points following intranasal instillation of 10% TDI or ethyl acetate (control vehicle). The asterisks denote significant TDI-induced change relative to controls ($p \leq 0.05$). n=6 for each group.

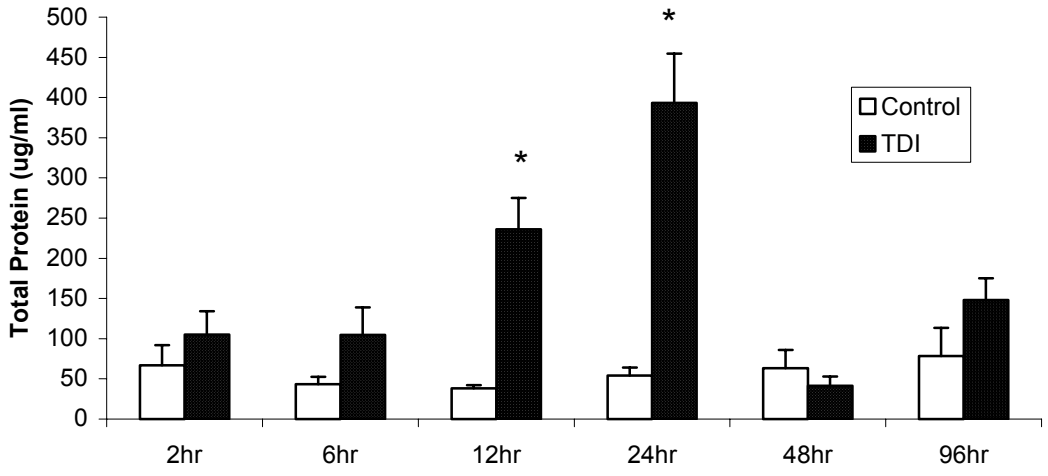


Figure 12 The concentration of total protein (μg/ml) in the nasal lavage fluid of Sprague-Dawley rats at various time points following intranasal instillation of 10% TDI or ethyl acetate (control vehicle). The asterisks denote significant TDI-induced change relative to controls ($p \leq 0.05$). n= 6 for each group.

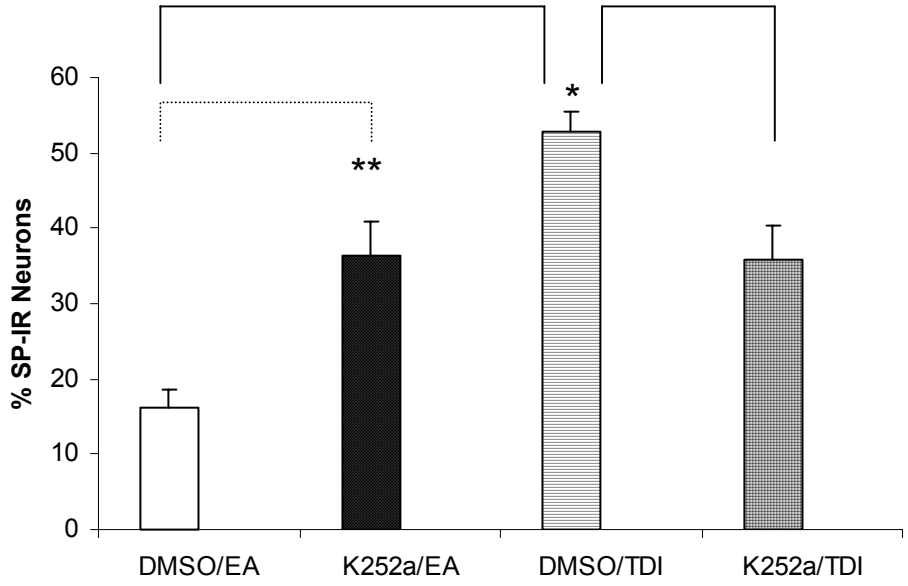


Figure 13 The percentage of SP-IR TG neurons innervating the nasal epithelium of Sprague-Dawley rats pretreated with an intranasal instillation of (100 μg/ml) K252a or DMSO (control vehicle) 2 hr prior to intranasal instillation of 10% TDI or ethyl acetate (control vehicle). The asterisks denote significance ($p \leq 0.05$). n= 5 for DMSO/EA and 6 for all other groups.

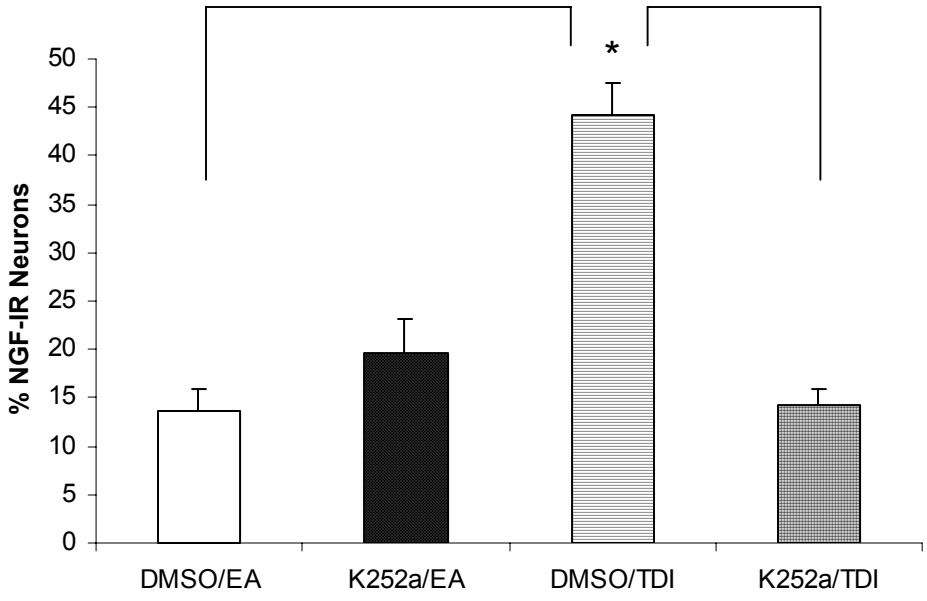


Figure 14 The percentage of NGF-IR TG neurons innervating the nasal epithelium of Sprague-Dawley rats pretreated with an intranasal instillation of (100 μg/ml) K252a or DMSO (control vehicle) 2 hr prior to intranasal instillation of 10% TDI or ethyl acetate (control vehicle) and sacrificed 24 hr later. The asterisks denote significance ($p \leq 0.05$). n=5 for DMSO/EA and K252a/TDI groups and 6 for DMSO/TDI and K252a/EA groups.

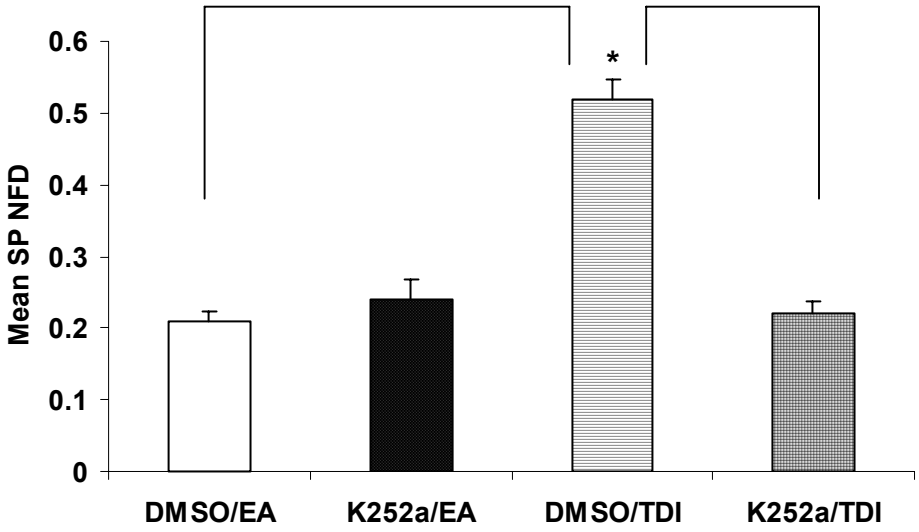


Figure 15 The density of SP-IR nerve fibers in the nasal epithelium of Sprague-Dawley rats pretreated with an intranasal instillation of (100 μg/ml) K252a or DMSO (control vehicle) 2 hr prior to intranasal instillation of 10% TDI or ethyl acetate (control vehicle) and sacrificed 24 hr later. The asterisks denote significance ($p \leq 0.05$). n=5 for K252a/EA group and 6 for the other groups.

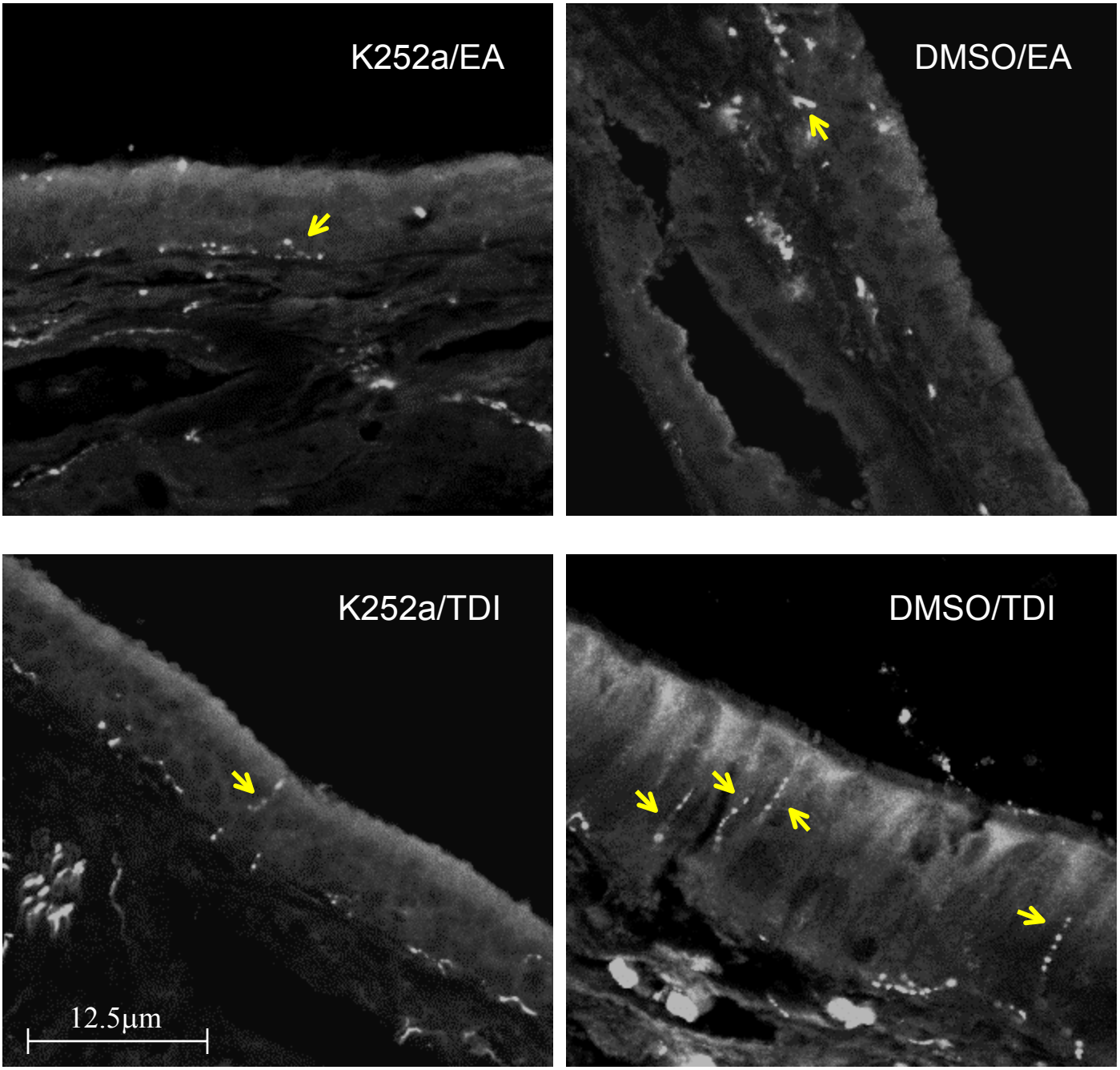


Figure 16 Images of rat nasal mucosa showing SP-IR nerve fibers in the nasal epithelium. The rats were pretreated with either K252a or DMSO prior to either EA or TDI exposure and sacrificed 24 hr later. TDI treatment increases the number of SP-IR nerve fibers projecting between epithelial cells in the nasal mucosa (DMSO/TDI image). Pre-treatment with K252a prevents the increase in SP-IR nerve fibers in the nasal epithelium (K252a/TDI image). Pretreatment with either K252a or DMSO prior to ethyl acetate did not cause an increase in SP-IR sensory nerve fibers in the epithelium. Arrows illustrate location of nerve fibers.

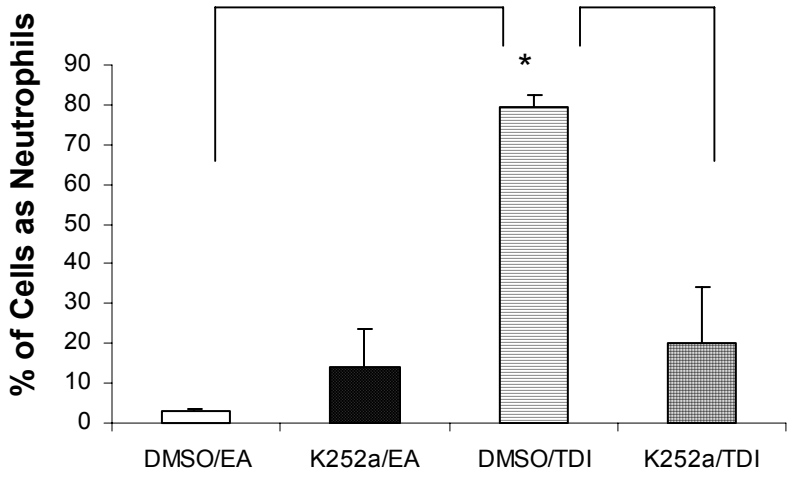


Figure 17 The percentage of neutrophils in the nasal lavage fluid of Sprague-Dawley rats pretreated with an intranasal instillation of (100 μg/ml) K252a or DMSO (control vehicle) 2 hr prior to intranasal instillation of 10% TDI or ethyl acetate (control vehicle) and sacrificed 24 hr later. The asterisks denote significance ($p \leq 0.05$). n=5 for DMSO/TDI and 4 for the other groups.

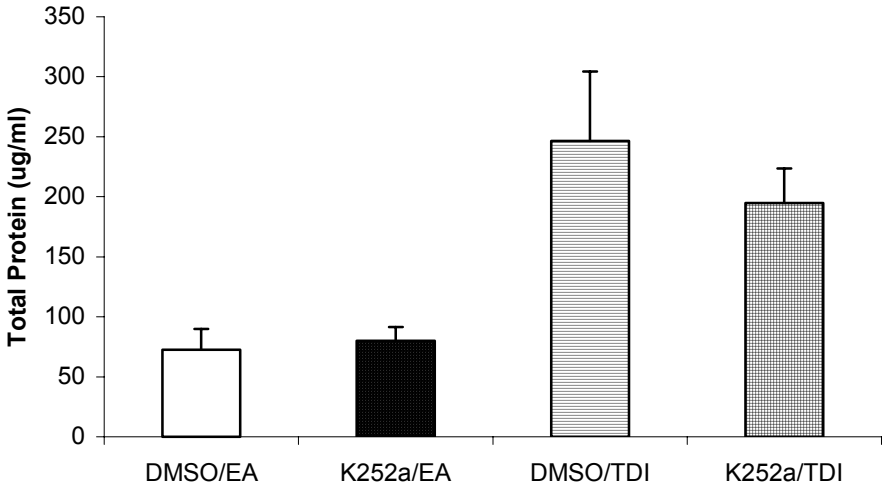


Figure 18 The concentration of total protein (μg/ml) in the nasal lavage fluid of Sprague-Dawley rats pretreated with an intranasal instillation of (100 μg/ml) K252a or DMSO (control vehicle) 2 hr prior to intranasal instillation of 10% TDI or ethyl acetate (control vehicle) and sacrificed 24 hr later. n=6 for K252a/TDI group and 5 for all other groups.

APPENDIX I Experiments conducted in conjunction with Study 2 but not included in the final manuscript

The following graphs are the results of experiments conducted concurrently with studies for Specific Aim #2 and #3. The experimental design and methods used were identical to those listed in the previous manuscript (Study 2). The results contribute to the overall understanding of neurogenic inflammation or the role of NGF in the airways. However, the results were not submitted for publication because the focus of the experiment either did not fit with the manuscript or the results of the experiment did not enhance nor detract from the overall findings.

A. *Glutamate Immunoreactivity in TG Neurons Innervating the Nasal Epithelium Following TDI*

B. *NGF (p75) Receptor Immunoreactivity in TG Neurons Innervating the Nasal Epithelium Following TDI*

C. *Measurement of Albumin in the Nasal Lavage Following TDI Exposure*

D. *NGF and Inflammatory Markers Measured in the Lungs Following Intranasal Instillation of TDI*

E. *NGF Measurements in the Upper Airways Following K252a and TDI*

F. *NGF and Neutrophil Measurements in the Lower Airways Following K252a and TDI*

G. *General Discussion (Study 2 & Appendix I)*

H., I. *References (Study 2 & Appendix I)*

A. GLUTAMATE-IR IN TG NEURONS INNERVATING THE NASAL EPITHELIUM FOLLOWING TDI EXPOSURE

RESULTS

Immunocytochemistry for glutamate was performed with rabbit anti-glutamate (1:50) (Sigma, St. Louis MO) using procedures previously described. Glutamate-IR in TG neurons innervating the nasal epithelium was not significantly different 2 hr (17.11±2.58%), 6 hr (15.74±4.73%) and 12 hr (19.01±3.69%) following TDI compared to time-matched ethyl acetate controls (13.34±3.96% at 2hr, 11.33±2.41% at 6 hr, 21.69±1.50% at 12 hr)(Figure 19). By 24 hr, the proportion of glutamate-IR TG neurons appeared to decrease in the TDI exposed animals, but the change was not significant compared to control values (9.88±1.54 and 23.16±6.95 respectively). The proportion of glutamate-IR TG neurons was 35.92±3.99% 48 hr following TDI, which was significantly higher than the 21.86±3.04% detected in control animals. By 96 hr, neuronal glutamate-IR in TDI treated animals returned to control levels. These findings suggest that TDI exposure produced increased levels of glutamate in TG neurons projecting to the nasal cavity.

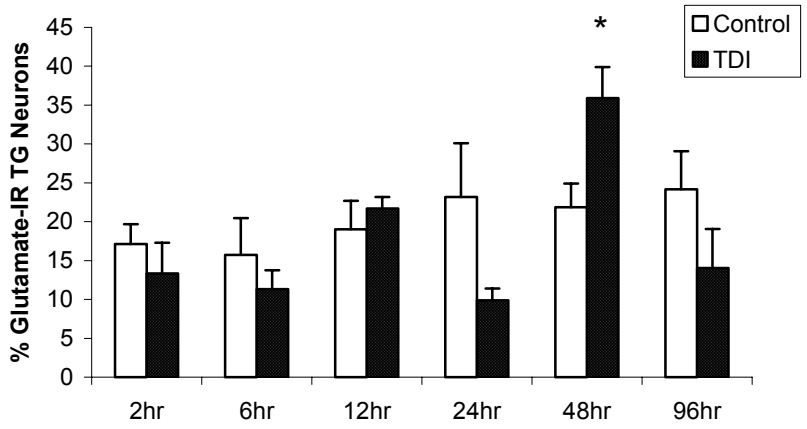


Figure 19 The percentage of glutamate-IR microsphere-labeled TG cell bodies 2, 6, 12, 24, 48 and 96 hr after TDI (n=4) or ethyl acetate (control vehicle, n=4) instillation into the nasal cavity. The asterisks denote significance between the TDI and time matched controls ($p \leq 0.05$).

DISCUSSION

Airway sensory nerve activation results in the generation of action potentials that are conducted orthodromically toward the central nervous system and antidromically down collateral branches resulting in the release of transmitters at both locations (Barnes, 1986). In TG neurons innervating the airways, few details are known about the transmitters involved in signal propagation in the CNS. Histamine or capsaicin inhalation activates sensory nerves in the respiratory tract leading to transmission of airway afferent inputs to the nucleus tractus solitaris resulting in increased cholinergic outflow to the airways. Glutamate released from afferent inputs binds to AMPA receptors in the nucleus tractis solitaris and mediates the reflex airway constriction (Haxhiu *et al.*, 2000).

Previous studies found 24% of the TG neurons to be immunoreactive for glutamate (Kai-Kai & Howe, 1991). In the current study, a significant increase in glutamate-IR was detected in the labeled TG neurons 48 hr after TDI exposure. The return of glutamate-IR to control levels by 96 hr suggest that glutamate was released. These data indicate that TG neurons innervating the airway are glutamate-IR and that exposure to TDI up-regulates glutamate production. However, it is not known if NGF mediates glutamate production in TG neurons. It is also not known if the glutamate produced in TG neurons is transported to central or peripheral projections for release. The role of glutamate in sensory neurons of the upper airway remains unclear.

B. NGF (p75) RECEPTOR-IR IN TG NEURONS INNERVATING THE NASAL EPITHELIUM FOLLOWING TDI EPOSURE

RESULTS

Immunocytochemistry for NGF (p75) receptor was performed with mouse anti-NGF (p75) receptor (1:75; Oncogene Research Products, San Diego, CA) and fluorescein isothiocyanate-labeled goat anti-mouse immunoglobin IgG (1:80; Zymed Laboratories, San Francisco, CA) using methods previously described. TG neurons innervating the nasal epithelium were immunoreactive for the NGF receptor but no change in immunoreactivity was apparent following TDI (Figures 20 and 21). The proportion of NGF receptor-IR microsphere containing TG neurons in control animals at 24 hr (8.20$\pm$2.06%) and 48 hr (11.15$\pm$4.43) was not significantly different from the time-matched TDI animals (13.79$\pm$3.78% at 24 hr and 16.54$\pm$3.79 at 48 hr). There was a consistent pattern of increased p75 expression after TDI, but the difference failed to reach significance with an n=3.

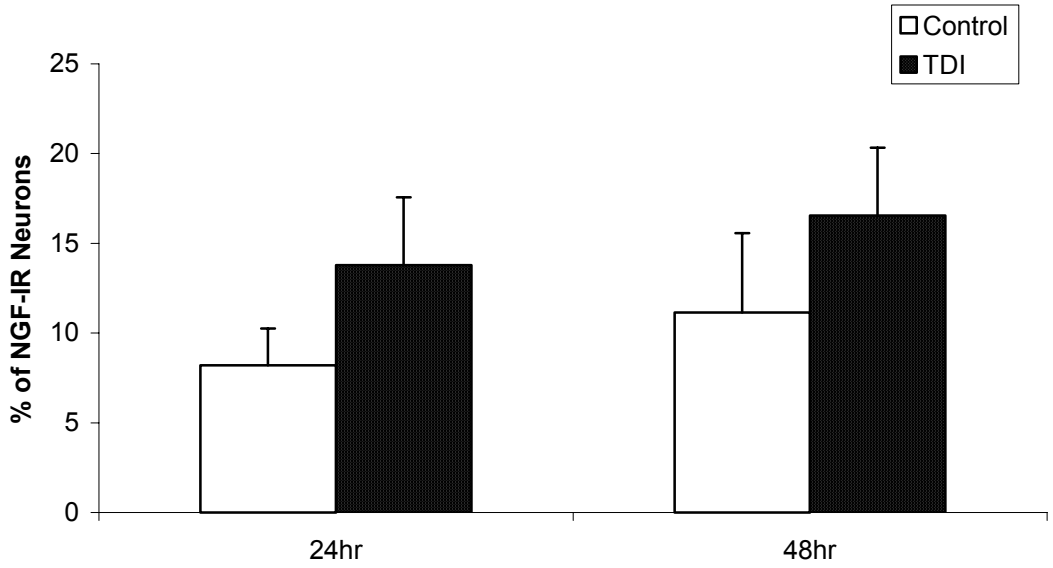


Figure 20 The percentage of NGF (p75) Receptor-IR microsphere-labeled TG cell bodies 24 and 48hr after TDI (n=3) or ethyl acetate (control vehicle, n=3) instillation into the nasal cavity. No significant difference was detected.

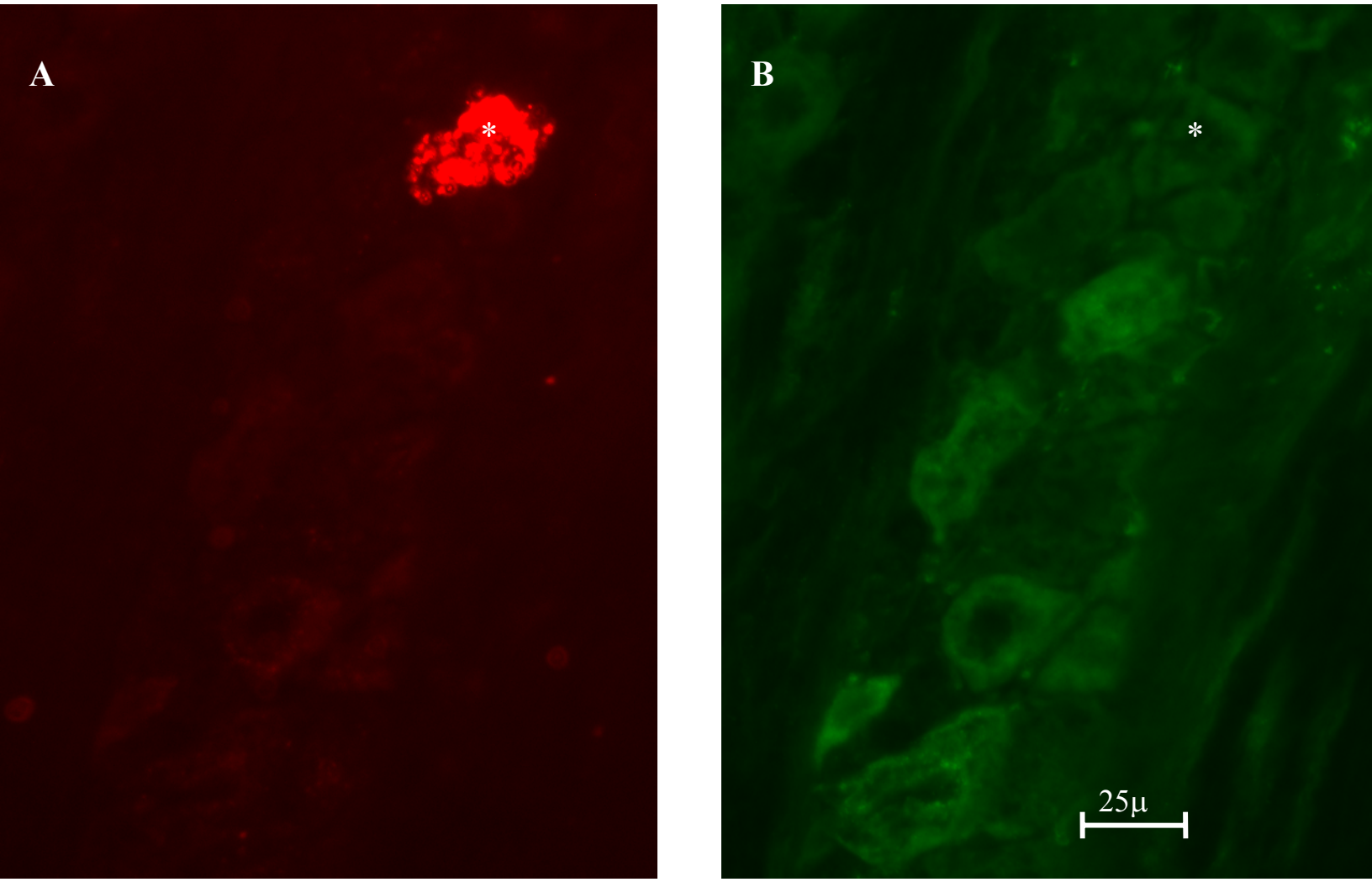


Figure 21 Images of TG processed for NGF p75 receptor-IR using immunocytochemistry. Tissue was removed 24 hr following exposure to TDI. Image A was taken with a rhodamine filter to identify neurons containing rhodamine-labeled latex microspheres, which are known to innervate the nasal epithelium. Image B was taken using a fluorescein filter. A third image, black and white image (not shown) was also taken using a fluorescein filter. Using Optimus software, the microsphere-labeled neuron was determine to be negative for NGFR-IR (MGV<50).

DISCUSSION

Trigeminal ganglion neurons synthesize the high (trkA) and the low (p75) affinity NGF receptors expressed on sensory nerve terminals. The majority of adult rat TG neurons are immunoreactive for the p75 (84.7±9.4) and the trkA receptors (69.2±7.6) (Jacobs & Miller, 1999). Drilling into the rat dental pulp causes small regions of neurogenic inflammation and has been used to investigate the functional relationship between neurotrophins, inflammation and sensory nerves. Following tooth injury, localized increases in NGF occur within 4 hr, retrograde transport of radio-labeled NGF is observed within 15 hr and maximum mRNA expression for the trkA and p75 receptors occurs within 52 hr in TG neurons innervating the injured dental pulp. Based on the timing, NGF at the injury site may regulate NGF receptor expression at the level of the cell body (Wheeler *et al.*, 1998). In the present study, the expression of the p75 receptor in TG neurons innervating the nasal epithelium was increased but not significantly 24 and 48 hr following TDI exposure. There may have been an increase in neuronal p75-IR at 2, 6, 12 or 96 hr but these time points were not analyzed.

C. MEASUREMENT OF ALBUMIN IN THE NASAL LAVAGE FOLLOWING TDI EXPOSURE

PURPOSE /METHODS

The leakage of protein from the nasal vasculature, an indicator of inflammation was measured in the nasal lavage fluid of TDI-and EA-exposed animals using two assays, one measured total protein (Figure 12; Study 2) and the other measured albumin (Figure 22). The concentration of albumin (25-1,000 μg/ml) in the nasal lavage samples was measured using Albumin Reagent (Bromcresol Green) (Sigma Diagnostics, St. Louis, MO). The assay was performed in 96 well plates and 50 μl of sample or bovine serum albumin standard was added to each well followed by 200 μl of Albumin Reagents. After 5 minutes the absorbance was read at 628nm on a Spectra Max 340pc plate reader (Molecular Diagnostics, Sunnyvale CA). The concentration of albumin in each lavage sample was extracted from a bovine serum albumin standard curve. All samples were run in duplicate or triplicate.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) was performed to look at the protein composition of the nasal lavage supernatant. Acrylamide gels containing 7.5% SDS were run with nasal lavage fluid from 12 and 24 hr time points and stained with Coomassie Blue. Each well contained 24 μl of nasal lavage fluid obtained from EA-or TDI-instilled animals, PBS (blank) or protein standard.

RESULTS

The concentration of albumin (μg/ml) in the nasal lavage fluid was significantly increased above controls 12, 24 and 96 hr following intranasal TDI instillation (Figure 22). Albumin began to significantly increase 12 hr following TDI treatment (281.84±28.15 μg/ml) compared to controls (90.79±12.02 μg/ml). The largest increase in albumin (444.70±110.7 μg/ml) was observed 24 hr after TDI compared to control levels, (111.37±12.57 μg/ml). The levels of albumin in the nasal cavities of TDI-exposed animals returned to control levels by 48 hr (150.60±20.02 and 111.37±12.57 μg/ml respectively). Interestingly, 96 hr after TDI albumin levels were again significantly increased relative to controls (273.62±28.69 and 152.55±22.14 μg/ml respectively). These results suggest that protein was leaking from the nasal vasculature 12, 24 and 96 hr following TDI exposure.

The results of the SDS PAGE gel (Figure 23) show a strong single band ran with a slightly weaker band on top was present in wells loaded with nasal lavage fluid from rats exposed to TDI and sacrificed after 12 or 24 hr. The dominant band corresponded with the protein marker band for bovine serum albumin. No significant bands were present in the EA or PBS wells. The SDS-PAGE images indicate that albumin in addition to other proteins is present in the nasal lavage fluid following TDI exposure.

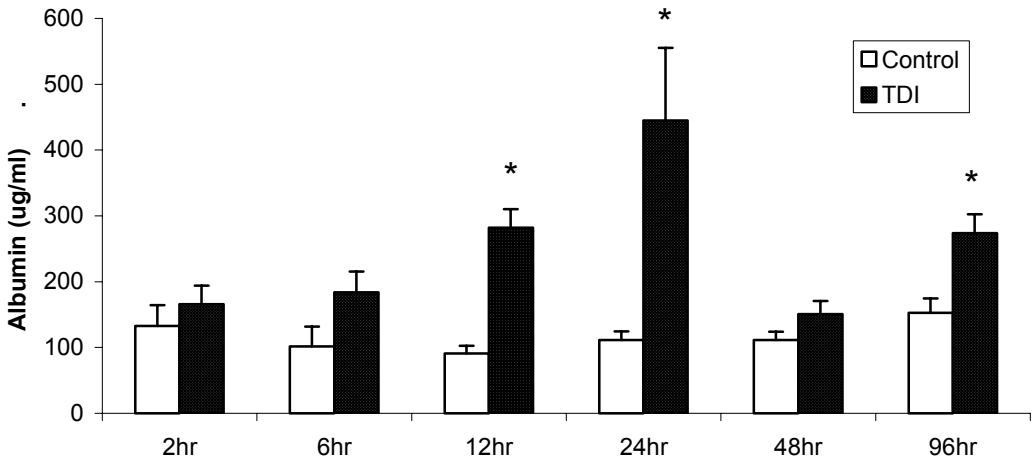
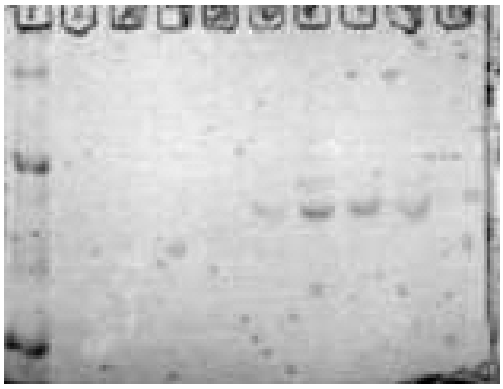


Figure 22 The concentration of albumin (μg/ml) in the nasal lavage fluid 2, 6, 12, 24, 48 and 96hr after TDI (n=6) or ethyl acetate (control vehicle, n=6) instillation into the nasal cavity. The asterisks denote significance between the TDI and time-matched controls ($p \leq 0.05$).

Nasal Lavages Obtained 12hr after TDI or EA

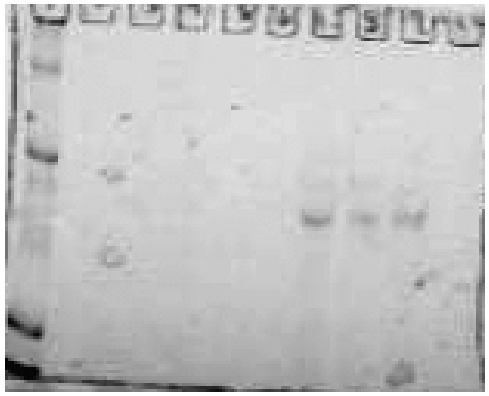
1 2 3 4 5 6 7 8 9 10



Lane 1: Protein Marker (220K-14.3K)
Lanes 2-5: EA n=4
Lanes 6-9: TDI n=3
Lane 10: Blank

Nasal Lavages Obtained 24hr after TDI or EA

1 2 3 4 5 6 7 8 9 10



Lane 1: Protein Marker (220K-14.3K)
Lanes 2-5: EA n=4
Lanes 7-9: TDI n=3
Lane 6&10: Blank

Figure 23 Images of SDS PAGE gels. Acrylamide gels containing 7.5% SDS were loaded with 24 µl of nasal lavage supernatant obtained from Sprague-Dawley rats 12 hr and 24 hr following intranasal instillation of 5 µl of either 10% TDI or EA. Gels were electrophoresed for 2 hr at 125 volts and stained with Coomaissie blue.

DISCUSSION

Results from the total protein assay (Figure 12; Study 2) and the albumin assay indicate that significant plasma extravasation occurs 12 and 24 hr following TDI. Comparison of the protein concentrations measured by each assay raised concern with the reliability of the albumin assay. The concentration of albumin (μg/ml) was either the same or higher than the concentration of total protein (μg/ml) in the nasal lavage samples at each time point when Figure (12 and 22) were compared. This suggested that the amount of albumin in the nasal lavage fluid was equal to or greater than the amount of total protein. Since more than one band was visable on the SDS-PAGE images, this indicates that albumin in addition to other proteins was present in the nasal lavage fluid following TDI exposure. The results from the albumin assay should have therefore been lower than the total protein assay.

D. NGF AND INFLAMMATORY MARKERS MEASURED IN THE LUNGS FOLLOWING INTRANASAL INSTILLATION OF TDI

RESULTS

NGF in the Lung Lavage Fluid Following TDI

The concentration of NGF (pg/ml) in the lung lavage fluid was significantly increased 24 hr following intranasal instillation of 10% TDI (542.03±60.61) compared to controls (117.37±39.33) (Figure 24). By 48 hr, there was no statistical difference between the concentrations of NGF in controls, 245.84±83.27 pg/ml and TDI exposed animals, 369.15±97.71 pg/ml. These findings indicate that NGF levels are increased in the lungs following intranasal TDI exposure.

Percentage of Neutrophils in the Lung Lavage Fluid Following TDI

The percentage of neutrophils in the lung lavage fluid was significantly increased above controls at 6, 12, 24, 48 and 96 hr following intranasal TDI instillation (Figure 25). The proportion of neutrophils in the lung lavage fluid steadily increased from 4.31±0.61% at 6 hr to 11.50±2.02% at 12 hr compared to controls (0.35±0.22% and 0.58±0.58%, respectively). The greatest increase, 28.81±4.90%, was observed 24 hr following TDI compared to the ethyl acetate controls (1.38±0.41%). Significant amounts of neutrophils remained in the lung lavage fluid 48 and 96 hr after TDI (11.42±0.88% and 24.75±3.88%) compared to controls (1.08+0.36% at 48 hr and 0.44+0.29% at 96 hr).

These data indicate that nasal instillation of TDI causes inflammation in the lungs for at least 96 hrs.

Total Protein in the Lung Lavage Fluid Following TDI

The concentration of total protein in the lung lavage fluid was not significantly altered following intranasal treatment with TDI. Total protein 2 hr (173.01$\pm$32.48), 24 hr (89.26$\pm$6.37) and 48 hr (72.63$\pm$2.71) after TDI was not significantly different than the time matched controls (191.95$\pm$51.77 at 2 hr, 58.96$\pm$6.87 at 24 hr and 105.28$\pm$22.93 at 48 hr)(Figure 26). These results indicate that intranasal TDI instillation does not cause significant plasma leakage from the lung vasculature.

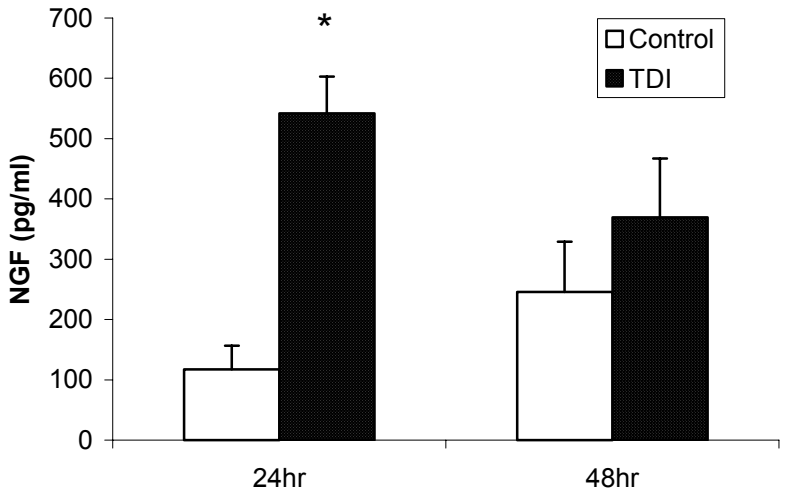


Figure 24 Mean NGF (pg/ml) detected by ELISA in the lung lavage fluid 24 and 48 hours after TDI (n=3) or ethyl acetate (control vehicle, n=3) instillation into the nasal cavity. The asterisk denotes significance between the TDI and time matched controls. ($p \leq 0.05$)

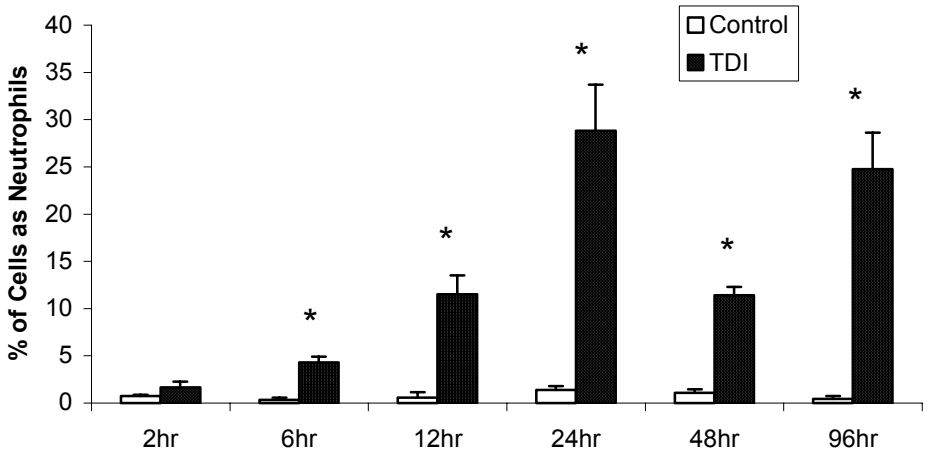


Figure 25 The percentage neutrophils in the lung lavage fluid 2, 6, 12, 24, 48 and 96 hr after TDI or ethyl acetate (control vehicle) instillation into the nasal cavity. The asterisks denote significance between the TDI and time matched controls ($p \leq 0.05$). At 2, 6, 12 and 96 hr, n=3 for TDI and controls. At the 24 and 48 hr time points, n=5 for both groups.

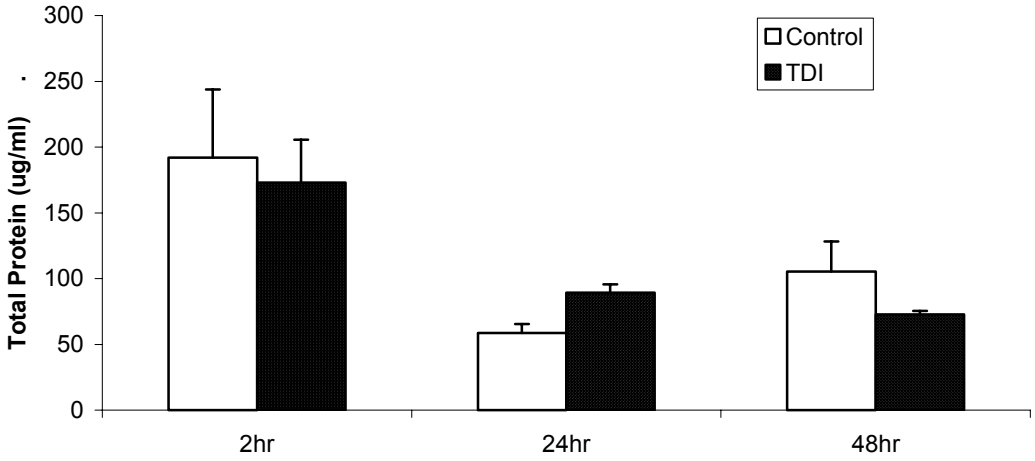


Figure 26 The concentration of total protein (μg/ml) in the lung lavage fluid 2, 24, and 48 hr after TDI (n=4 for 2 hr and n=3 for 24 and 48 hr) or ethyl acetate (control vehicle, n=4 for 2 hr and n=3 for 24 and 48 hr) instillation into the nasal cavity.

DISCUSSION

This was an exploratory study to determine if TDI instillation in the nose could cause inflammation and increased NGF release in the lungs. Either 10% TDI or EA was instilled into rat nasal cavity and the animals were sacrificed after 2, 6, 12, 24, 48 and 96 hr. Although the primary purpose of these studies was to examine changes in NGF, inflammation and neuropeptides in the upper airway, the lungs were also lavaged to see if nasal exposure to an irritant caused inflammation and NGF release in the lungs.

The lung lavage fluid was assayed for NGF at two of the six time points. NGF was significantly increased 24 hr after TDI but returned to control levels by 48 hr. Two markers for inflammation, neutrophil influx and protein extravasation, were also measured. The percentage of neutrophils in the lung lavage fluid was significantly increased, suggesting the presence of inflammation in the lower airways. However, there was no evidence of plasma extravasation, indicated by low total protein concentrations in the lung lavage following TDI. Although clearly capable of causing inflammation and NGF release in the lower airways, the primary site of TDI damage in this model occurs in the upper airways.

These data were omitted from Study 2 because the focus of manuscript was neuropeptide and NGF changes in the upper airway.

E. NGF MEASUREMENTS IN THE UPPER AIRWAYS FOLLOWING K252a AND TDI

RESULTS

In these studies as described in the previous manuscript, two hours prior to TDI instillation the rats were instilled with 8 μl of K252a (100 μg/ml) or DMSO (control vehicle). K252a, a non-specific tyrosine-kinase inhibitor, was used to block the receptor-mediated actions of NGF. As expected, NGF was significantly increased in the nasal lavage fluid 24hr after TDI in rats pre-treated with DMSO (238.11±33.75) but not in rats pre-treated with K252a (67.49±16.68) (Figure 27), suggesting that K252a reduced the TDI-induced release of NGF. Following exposure to ethyl acetate (control vehicle) no difference in NGF (pg/ml) was detected in the nasal lavages of rats pretreated with DMSO (42.71±6.37) or K252a (26.50±15.59). Inhibiting the receptor tyrosine kinase activity (K252a) prevented the release of NGF into the nasal cavity following TDI exposure.

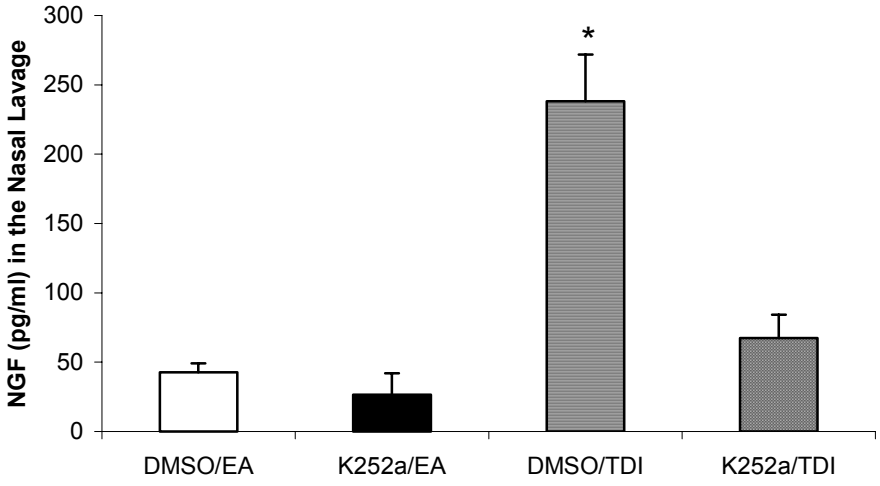


Figure 27 The concentration of NGF (pg/ml) in the nasal lavage fluid of Sprague-Dawley rats pretreated with an intranasal instillation of 5 µl of K252a (100 µg/ml) or DMSO (control vehicle) 2 hr prior to intranasal instillation of 10% TDI or ethyl acetate (control vehicle) and sacrificed 24 hr later. Asterisk denotes significance ($p<0.05$). n=5 for all groups except n=6 for K252a/TDI**.**

DISCUSSION

NGF has been shown to directly bind trkA receptors on sensory nerve terminals *in vivo* (McMahon *et al.*, 1994). K252a binds with high affinity to one site on the cytoplasmic kinase domain of the trkA receptor and inhibits the NGF-stimulated phosphorylation of tyrosine residues, thus inhibiting NGF action (Berg *et al.*, 1992), (Knight, Jr. *et al.*, 1997). Following TDI, K252a was expected to affect trkA receptor activation not the release of NGF. However, NGF did not increase as expected in the K252a/TDI group suggesting that K252a pre-treatment also affected the release of NGF. Epithelial cells (Fox *et al.*, 1997), mast cells (Leon *et al.*, 1994), lymphocytes (Barouch *et al.*, 2000), eosinophils (Solomon *et al.*, 1998), and macrophages (Braun *et al.*, 1998) are capable of synthesizing and releasing NGF in the airways but the mechanism mediating NGF release is unknown. K252a has been shown to block inflammatory mediator release from resident mast cells and leukocytes by inhibiting protein kinase C and calmodulin (Ohmori *et al.*, 1988). In this study, the K252a inhibition of protein kinase C and calmodulin in epithelial cells, mast cells and leukocytes may have interfered with NGF release.

F. NGF AND NEUTROPHIL MEASUREMENTS IN THE LOWER AIRWAYS FOLLOWING K252a AND TDI

RESULTS

NGF in the Lung Lavage Fluid Following K252a and TDI

In these studies as described in the previous manuscript, two hours prior to TDI rats were nasally instilled with 8 μl of K252a (100 μg/ml) or DMSO (control vehicle). K252a, a non-specific tyrosine-kinase inhibitor was used to block the receptor-mediated actions of NGF. NGF failed to increase in the lung lavage fluid 24 hr after TDI in rats pre-treated with either DMSO (239.49±16.44 pg/ml) or K252a (218.61±29.96 pg/ml) (Figure 28). Following exposure to ethyl acetate, the control vehicle, no difference in NGF (pg/ml) was detected in the lung lavages of rats pretreated with DMSO (151.95+25.48) or K252a (130.37±25.90).

Percentage of Neutrophils in the Lung Lavage Fluid Following K252a and TDI

The increase in the lung lavage neutrophils observed 24 hr following TDI treatment (DMSO/TDI 19.6±2.06%) still occurred in rats pre-treated with K252a (17.7±3.25%)(Figure 29). There was no significant difference in the proportion of neutrophils in the lungs of rats treated with either DMSO or K252a prior to ethyl acetate (0.30±0.25% and 0.50±0.22% respectively). Nasal pre-treatment with K252a prior to TDI exposure did not prevent the influx of neutrophils into the lung cavity.

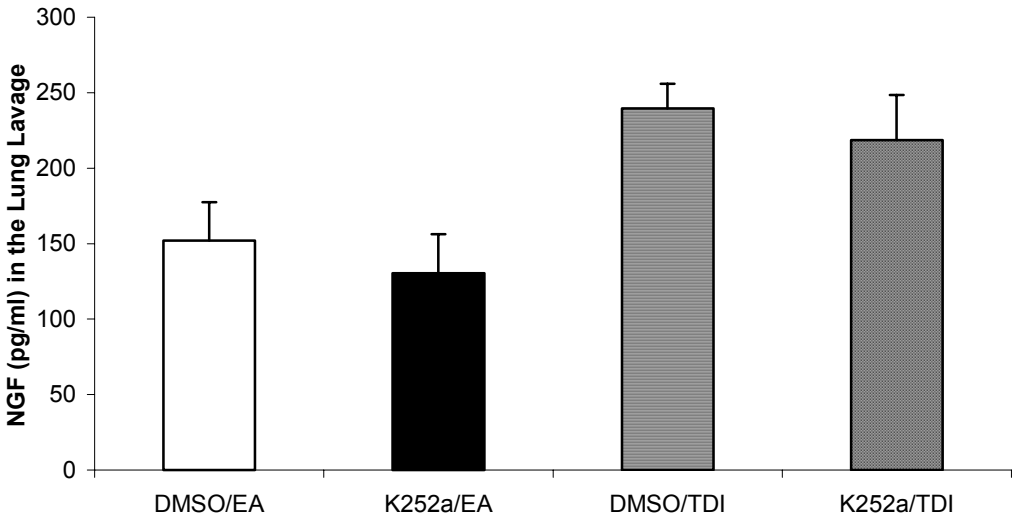


Figure 28 The concentration of NGF (pg/ml) in the lung lavage fluid of Sprague-Dawley rats pretreated with an intranasal instillation of (100 μg/ml) K252a or DMSO (control vehicle) 2 hr prior to intranasal instillation of 10% TDI or ethyl acetate (control vehicle) and sacrificed 24 hr later. Asterisk denotes significance ($p<0.05$). n=4 for all groups except n=5 for DMSO/TDI**.**

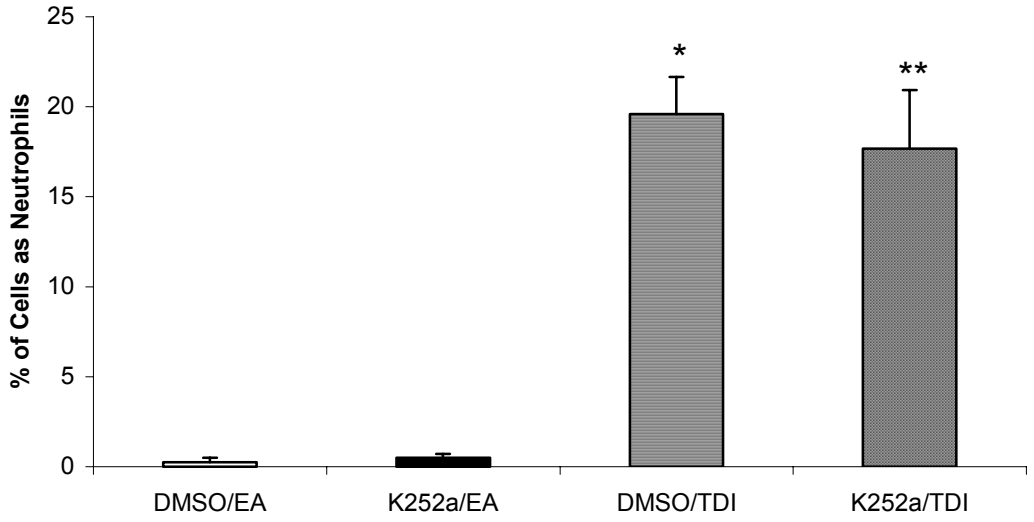


Figure 29 The percentage of neutrophils in the lung lavage fluid of Sprague-Dawley rats pretreated with an intranasal instillation of (100 μg/ml) K252a or DMSO (control vehicle) 2 hr prior to intranasal instillation of 10% TDI or ethyl acetate (control vehicle) and sacrificed 24 hr later. Asterisk denotes significance ($p<0.05$). n=6 for K252a/EA and K252a/TDI, n=4 for DMSO/EA and n=5 for DMSO/TDI**.**

DISCUSSION

The non-specific tyrosine-kinase inhibitor K252a, when intranasally instilled prior to TDI, prevents NGF release and neutrophil influx in the nose. This exploratory study was done to determine if nasal pre-treatment with K252a would also inhibit the NGF release or neutrophil influx commonly observed in the lungs 24 hr after TDI.

NGF binds to trkA receptors on sensory nerve terminals (McMahon *et al.*, 1994) and ultimately up-regulates of SP synthesis in sensory neurons (Lindsay & Harmar, 1989). The SP released from sensory nerve terminals is chemotactic for neutrophils (Jancso *et al.*, 1967); (Lundberg & Saria, 1983). K252a binds with high affinity to one site on the cytoplasmic kinase domain of the trkA receptor and inhibits the NGF-stimulated phosphorylation of tyrosine residues, (Berg *et al.*, 1992); (Knight, Jr. *et al.*, 1997). K252a pre-treatment prevented the release of NGF and influx of neutrophils into the nasal cavity (Figures 27 & 17) but not in the lungs following TDI exposure. This suggests that TDI reaches the lungs but K252a remains in the upper airway following nasal instillation.

Since these studies were in the lung they were outside the focus of the manuscript and omitted.

G. GENERAL DISCUSSION (STUDY 2 + APPENDIX I)

The goal of this study was to characterize the response and regulation of neuropeptides in the upper airway following TDI exposure. TDI is known to activate sensory nerves in the nasal cavity leading to increased synthesis and release of SP (Hunter *et al*., 2000; Kalubi *et al.*, 1992). The events preceding neuropeptide up-regulation are believed to involve NGF. NGF regulates neuropeptide expression at the level of the cell body (Lindsay & Harmar, 1989) through trkA receptor binding at peripheral nerve terminals (McMahon *et al.*, 1994) followed by retrograde transport to corresponding cell bodies (Wheeler *et al.*, 1998). Increases in NGF levels in the airways have been documented in asthma patients (Bonini *et al.*, 1996), rhinitis patients (Sanico *et al.*, 2000) virally-infected rats (Hu *et al.*, 2002) and antigen-challenged mice (Braun *et al.*, 1998).

These studies were the first to show that increases in NGF in the upper airways occured following irritant exposure. Theses studies also demonstrated a temporal relationship between NGF, neuropeptides and inflammatory markers in the nasal cavity. Following exposure to TDI, increases in NGF were detected in the nasal cavity after 2 hr while SP increases were not detected in TG cell bodies innervating the nasal epithelium until 24 hr post-exposure. The binding of NGF to receptors on sensory nerve terminals and retrograde transport to the corresponding cell bodies accounted for the delayed up-regulation of SP. The increase in NGF in TG cell bodies 24 hr following TDI supported the retrograde transport of NGF.

These studies also provided evidence that NGF may mediate the up-regulation of SP in airway sensory neurons. Using K252a, a non-specific tyrosine-kinase inhibitor,

the TDI-induced increases in SP innervation in the nasal mucosa, SP-positive TG cell bodies projecting to the nasal cavity and inflammation in the nasal cavity were reduced. K252a binds with high affinity to one site on the cytoplasmic kinase domain of the trkA receptor and inhibits the NGF-stimulated phosphorylation of tyrosine residues (Berg *et al*., 1992; Knight, Jr. *et al.*, 1997). However, K252a inhibits the phosphorylation of trkB and trkC receptors (Ohmichi *et al.*, 1992). In addition, K252a has also been shown to inhibit protein kinase C and calmodulin, which may have affected neutrophil influx and NGF release following irritant exposure. Therefore, the reduced SP levels and inflammatory markers observed in TDI-exposed animals pretreated with K252a cannot be unequivocally attributed to NGF. These studies do, however, support the concept that NGF release from the nasal mucosa following TDI correlates with SP production in TG cell bodies.

Although the main focus of these studies was NGF and SP in the upper airways, additional parameters were measured in the TG and lower airways. Previous studies reported increased NGF receptor expression in TG neurons innervating injured tooth pulp. These studies suggested that NGF release following tooth injury regulates NGF receptor expression at the level of the TG cell body (Wheeler *et al.*, 1998). Although a pattern of increased NGF receptor expression in TG neurons innervating the nasal epithelium was detected following irritant exposure, the changes were not significant. Viral exposure increased p75 and trkA receptor expression in the lungs (Hu *et al.*, 2002), but this study looked at whole lung, not nerves. Glutamate immunoreactivity was also measured and found to increase in TG neurons 48 hr following TDI exposure. The increase in glutamate, a transmitter involved in signal propagation to the CNS (Bonham

& Chen, 2002), suggests that airway changes following irritant exposure are not mediated solely by neuropeptide up-regulation and release. Glutamate release in the lung promotes lung injury and thus may have important pathological consequences (Said *et al.*, 1996). Since SP and glutamate were both increased 48 hr post-exposure, NGF-mediated-signaling pathways may also regulate glutamate production.

Although previous studies have detected increases in NGF in the lungs following allergen challenge (Braun *et al.*, 1998) and virus inoculation (Hu *et al.*, 2002), changes in NGF in irritant-exposed lungs have not been well documented. In these studies, instillation of TDI into the nasal cavity caused significant and transient inflammation throughout the airways. Neutrophils, but not protein, were elevated in the lung lavage fluid 6 hr post-TDI exposure. Significant levels of NGF were also detected in the inflammed lungs 24 hr following TDI exposure. Since NGF mediates SP synthesis (Lindsay & Harmar, 1989) and SP is chemotactic for neutrophils (Jancso *et al.*, 1967), the synthesis and release of SP may also be increased in the lungs. The mechanism of lung response after nasal instillation of TDI is not clear. The TDI vapor may travel to the lung and cause a direct response. However, another possibility is that a nose-lower airway neural reflex or humoral factors generated during nasal exposure mediate the pulmonary response (Undem *et al.*, 1999). The failure of K252a to abate the pulmonary response may be simply that K252a did not reach the lung. However, it could also be that activation of reflexes in the nasal cavity are not NGF-dependent and, therefore, unresponsive to a tyrosine-kinase inhibitor.

Nasal instillation of TDI is an effective model for studying NGF, neuropeptides and inflammatory markers in the upper and lower airways. Based on these results, NGF release in the nasal cavity following TDI precedes SP production in TG cell bodies.

H. REFERENCES (STUDY 2 & APPENDIX I)

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STUDY 3

The Effect of Nerve Growth Factor Antiserum on Substance P Innervation Following Toluene Diisocyanate Exposure

Not submitted for publication

ABSTRACT

Toluene diisocyanate (TDI) causes rhinitis, nasal irritation and increased synthesis and release of substance P (SP) from airway sensory neurons. The peripheral signaling mechanism responsible for enhanced SP production remains unclear but may involve NGF, which also increases following irritant inhalation. NGF binds to trkA receptors located on sensory nerve terminals. Activation of trkA receptors initiates kinase-signaling cascades, which ultimately may increase SP synthesis. In this study, the role of NGF in neuropeptide up-regulation following TDI exposure was examined at the level of the epithelial nerve fiber using antibodies targeted against NGF. In the first study, rabbit anti-NGF (1:50) was nasally instilled 2 hr before and 2 hr after TDI. In the second study, rats received intraperitoneal injections of sheep anti-NGF 1 hr before and 3 hr after TDI. In both studies, nasal lavages and removal of nasal mucosa were performed 24 hr after TDI instillation and the effect of inactivating NGF was evaluated using SP nerve fiber density (NFD) measurements in the nasal epithelium and neutrophil counts in the nasal lavage fluid. In both studies, pre-treatment with NGF antiserum failed to prevent SP from increasing in epithelial nerve fibers and neutrophil chemotaxis 24 hr following TDI. Interestingly, control animals pre-treated with NGF antiserum also had increased SP NFD in the nasal epithelium. Therefore, these findings are inconclusive and fail to confirm or dispute the role of NGF in SP up-regulation in the airways following TDI exposure.

INTRODUCTION

Toluene diisocyanate (TDI) is a solvent commonly used in the production and manufacture of polymer-containing products such as plastics, foams, adhesives and surface coatings. Workers exposed to TDI vapor can develop a number of inflammatory conditions including asthma, rhinitis and nasal irritation (Brugsch & Elkin, 1963; Mapp *et al.*, 1988) Exposure of rats to TDI leads to the activation of sensory nerves in the nasal epithelium and increased synthesis and release of the neuropeptide substance P (SP) (Gordon *et al.*, 1985; Sheppard *et al.*, 1988; Thompson *et al.*, 1987). The release of neuropeptides from sensory fibers in the nasal cavity leads to vasodilation, plasma extravasation, mucous secretion and neutrophil chemotaxis, which is collectively termed neurogenic inflammation (Jancso *et al.*, 1967; Lundberg & Saria, 1983). Neurogenic inflammation in the airways is mediated by the release of the sensory neuropeptide SP from the sensory nerve endings (Baluk et al., 1992; Lundberg *et al*., 1983; Lundberg *et al.*, 1984) found throughout the nasal mucosa near blood vessels, mucous glands and epithelium (Baraniuk *et al.*, 1991; Lee *et al.*, 1985).

Transient increases in SP protein and message have been demonstrated in sensory cell bodies innervating respiratory epithelium of the nose or lung following antigen challenge (Fischer *et al.*, 1996), viral infection (Carr *et al.*, 2002) and asphalt fume exposure (Sikora *et al.*, 2003). SP synthesis occurs in cell bodies remotely located outside the nasal cavity in the trigeminal ganglia (TG) (Helke *et al.*, 1990).

Following TDI, transient increases in SP have been observed in TG cell bodies innervating the nasal epithelium and in epithelial nerve fibers (Hunter *et al.*, 2000a; Sikora *et al.*, 2003).

The mechanism mediating the up-regulation of SP remains unclear. A signaling cascade, conveying information from the irritant-activated nerve terminals in the nasal mucosa to the trigeminal cell bodies, is most likely responsible for up-regulating the synthesis and release of SP. The neurotrophin, nerve growth factor (NGF), has been shown to bind to receptors on sensory nerve terminals (McMahon, 1996), undergo axonal transport (Sandow *et al.*, 2000) and up-regulate neuropeptide production in neuronal cell bodies (Lindsay & Harmar, 1989).

The involvement of NGF in regulating neuropeptide expression in airway sensory neurons has been demonstrated using a variety of animal models. Transgenic mice overexpressing NGF in the airways have higher SP levels in their airway walls (Hoyle *et al.*, 1998). Tracheal instillation of NGF produces increased SP expression in neurons of the nodose and jugular ganglia innervating the guinea-pig airways (Hunter *et al.*, 2000b). In rats, NGF levels are increased during respiratory syncytial virus infections. Injection of NGF antibodies prior to RSV inoculation reduces neuropeptide receptor expression observed during viral infections (Hu *et al.*, 2002). Recent studies demonstrated that transgenic mice deficient in the NGF receptor exhibit reduced inflammatory responses to antigen challenge (Path *et al.*, 2002). These studies clearly support a role for NGF in airway inflammation and neuropeptide expression.

The aim of the present study was to determine if NGF mediates increased SP expression in airway sensory nerves following irritant exposure. Previously, we have detected increases in NGF in the nasal lavage fluid prior to observed increases in SP levels in epithelial nerve endings, TG neurons and inflammatory markers. In order to assess the contribution of NGF to neuropeptide up-regulation and airway inflammation

following TDI, rats were pre-treated with either rabbit anti-NGF or sheep anti-NGF antibody to inactivate NGF. Changes in SP-IR nerve fibers in the nasal epithelium and neutrophil counts in the nasal lavage were measured.

METHODS AND MATERIALS

Experimental Design Adult male Sprague-Dawley rats (Hla:(SD)CVF) weighing 200-250 g and purchased from Hilltop Lab Animals (Scottsdale, PA) were used for all the experiments. Animals were intranasally treated with either rabbit anti-NGF or PBS before and after TDI or ethyl acetate exposure. The experimental design consists of four groups: anti-NGF/EA, anti-NGF/TDI, PBS/EA and PBS/TDI. Each group consisted of 6 animals. In a separate experiment, rats received an intraperitoneal injection of sheep anti-NGF before and after TDI or ethyl acetate. The experimental design consisted of two groups: anti-NGF/EA and anti-NGF/TDI. Each group consisted of 4 animals. In both experiments, tissues were removed and nasal lavages performed 24 hr after irritant instillation.

Rabbit anti NGF Nasal Instillation Rats were anaesthetized with an intraperitoneal injection of sodium brevital (50 mg/kg). Both nasal cavities were instilled with 8 µl rabbit anti-NGF (1:10; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or PBS (control) using a 10 µl Hamilton syringe with plastic tubing covering the tip. The tubing was marked at 0.8 and 1.3 cm lengths to allow correct positioning into the anterior and posterior nasal regions. After two hours, both nasal cavities were instilled with 5 µl of either 10% TDI or ethyl acetate (control). Rabbit anti-NGF (1:10, 8 µl/nostril) or PBS was re-administered 2 hr following TDI instillation. The anti-NGF doses were adapted from

previous studies which showed that endogenous NGF in the nasal cavity of mice was inactivated following two 25 µl intranasal applications of NGF antibody (1:50 dilution) three hours prior to experimentation (Braun *et al.*, 1998). The concentration and volume of antibody was increased in this study since rats were used.

Sheep anti-NGF Intraperitoneal Injection All rats received an intraperitoneal injection of 500 µl of sheep anti-NGF (1:25) (Chemicon International, Temecula, CA) 1 hr before TDI or EA and 3 hr following TDI or EA. Previous studies successfully blocked NGF in the lung with a single intraparitoneal injection of NGF antibody (1:2000, 4ml/kg) 3 hr prior to inoculation of respiratory syncytial virus (Hu *et al.*, 2002).

TDI Instillation Rats were anaesthetized with an intraperitoneal injection of sodium brevital (50mg/kg). Both nasal cavities were instilled with 5 µl of 10% TDI or ethyl acetate (control vehicle) by placing the tip of a 10 µl pipette at the entrance of the nasal cavity.

Nasal Lavage The rats were overdosed with 1 ml of 50 mg/ml sodium brevital. The nasal cavities were lavaged following removal of the lower jaw and insertion of a syringe into the posterior nares. Both sides of the nasal cavity were simultaneously lavaged with 15 ml of PBS. The nasal lavage fluid was centrifuged at 1,500 rpm for 10 min. Cell suspensions were plated on glass slides ($1.5X10^5$ cells) using a cytospin (Shandon Scientific, Ltd., Cheshire, UK) at 400 rpm for 4 min. The slides were stained with Wright-Giemsa on a Hema-Tek 2000 automated slide stainer (Bayer, Inc., Tarrytown, NY). A total of 100 cells from nasal lavages were classified as neutrophils or nucleated cells using an Olympus AX70 light microscope.

Tissue removal and preparation The nasal mucosa was removed from the anterior and posterior regions of the nasal cavity. All tissue was immediately fixed in picric-acid formaldehyde fixative consisting of 2% paraformaldehyde, 15% saturated picric acid and 0.15 M phosphate buffer at 4°C (Stefanini *et al.*, 1967). After 3 hr, the tissue was rinsed twice in 0.1M phosphate-buffered saline containing 0.3% Triton X-100 (PBS-Tx, pH=7.8). After the second rinse, the tissues remained in PBS-Tx overnight at 4°C. The next day the nasal mucosa were frozen on cork in isopentane cooled by liquid nitrogen and stored in airtight plastic bags at –80°C. The nasal epithelium was sectioned on a cryostat at 12 μm. One coverslip containing 16 sections randomly taken throughout the piece of mucosa were used for SP immunocytochemistry.

Immunocytochemistry Immunocytochemical procedures for localizing SP were identical to those previously described (Dey et al., 1990). Cryostat sections on subbed coverslips were covered with either rabbit anti-SP (1:200) (Peninsula, Belmont CA), diluted in PBS-Tx + 1% bovine serum albumin (PBS-Tx-BSA, pH=7.8). The coverslips were incubated in a humid chamber at 37 °C for 30 min. The coverslips were rinsed 3 times with PBS-Tx-BSA, allowing 5 min per rinse. After covering the sections with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobin IgG (ICN Pharmaceuticals, Inc, Costa Mesa, CA) diluted 1:100 in PBS-Tx-BSA, the coverslips were incubated at 37 °C for 30 min. The coverslips again were rinsed 3 times for 5 min increments in PBS-Tx-BSA. Fluoromount (Southern Biotechnology, Birmingham, AL) was used to mount the coverslips onto glass slides. The sections were observed with an Olympus AX70 fluorescence microscope equipped with fluorescein (excitation 495 nm and emission 520 nm). Immunocytochemistry controls included pre-absorbing the

antibody with the corresponding protein. No fluorescence was detected on the sections proving that the antibody was specific for the indicated protein. A second control included only addition of the secondary antibody to rule out any non-specific reactivity.

SP Nerve Fiber Density Following immunocytochemical processing for SP, the sections of nasal mucosa were observed on a confocal microscope equipped with an argon laser (Zeiss, Germany). Eighteen random images of respiratory epithelium were taken from each coverslip. Using Optimus, version 6.5 (Media Cybernetics, L.P., Silver Spring, MD) the entire perimeter of epithelium was outlined. The threshold for each image was optimized so that only SP-IR nerve fibers were visible. The SP NFD was calculated by dividing the SP-IR nerve fiber area by the total area of epithelium outlined. This represents the proportional cross-sectional area occupied by SP-immunoreactive nerve fibers.

Statistical Analysis The means and standard errors were calculated for each endpoint measured. Two-way ANOVA's were performed for the rabbit anti-NGF studies and one-way ANOVA's were performed for the sheep-anti NGF studies with a Tukey post-hoc test. Significance was set at $p \leq 0.05$ for all studies.

RESULTS

% Neutrophils in the Nasal Lavage Following Nasal Instillation of Rabbit anti-NGF and TDI

Twenty-four hours after TDI, the percentage of neutrophils in the nasal lavage fluid was significantly increased in animals pre-treated with PBS (76.75 ± 4.77) compared to PBS-ethyl acetate control (11.00 ± 4.51) (Figure 30). Neutrophil counts were increased

in rabbit anti-NGF animals exposed to TDI but not ethyl acetate (82.00±3.61% and 9.50±5.42% respectively). Pre-treatment with anti-NGF did not prevent the increase in neutrophils 24 hr after TDI exposure.

SP Nerve Fiber Density (NFD) in the Nasal Epithelium Following Nasal Instillation of Rabbit anti-NGF and TDI

The percent area of SP-IR nerve fibers in the nasal mucosa was significantly increased 24 hr after pretreatment with anti-NGF prior to EA exposure (0.54±0.03) compared to animals receiving PBS prior to EA (0.21±0.03) (Figures 31 and 32). In TDI-exposed animals, increased SP NFD was present in the epithelium of animals pre-treated with PBS and anti-NGF (0.47±0.02 and 0.49±0.03 respectively). The data show that the level of SP in epithelial nerve fibers was increased by either TDI or NGF antibody.

% Neutrophils in the Nasal Lavage Following Intraperitoneal Injection of Sheep anti-NGF and TDI Exposure

Twenty-four hours after TDI, the percentage of neutrophils in the nasal lavage fluid was significantly increased in animals injected with sheep anti-NGF (87.3±4.7) compared to controls (sheep anti-NGF/EA (23.0±0.88; Figure 33). Injections of sheep anti-NGF did not prevent the percentage of neutrophils in the nasal lavage fluid from increasing 24 hr after TDI exposure.

SP NFD in the Nasal Epithelium Following Injections of Sheep anti-NGF and TDI Exposure

Animals injected with sheep anti-NGF had an increased percent area of SP-IR nerve fibers in the nasal mucosa 24 hr after TDI (0.55±0.02) and EA (0.55±0.05) exposure (Figure 34). Pre-treatment with anti-NGF did not prevent the percentage of SP-IR nerve fibers in the nasal epithelium from increasing following TDI exposure.

DISCUSSION

TDI has become a widely accepted agent for studying the neuronal responses to inhaled irritants. A single exposure to TDI causes epithelial injury and acute airway inflammation (Gordon *et al.*, 1985). Repeated application of TDI to the guinea pig nasal cavity increases mRNA levels for SP in TG sensory neurons and stimulates the release of SP from peripheral nerve endings in the nasal mucosa (Kalubi *et al.*, 1992). A 2 hr TDI exposure (60 ppb) also activates sensory neurons in the upper airway indicated by the increased SP mRNA levels in TG neurons supplying the nasal epithelium and increased levels of SP in epithelial nerve fibers 24 hr after exposure (Hunter *et al.*, 2000a). In this study, intranasal instillation of 10% TDI caused the number of SP-IR nerve fibers in the nasal epithelium and the percentage of neutrophils in the nasal lavage fluid to significantly increase.

Prior studies suggested that the increased neuropeptide production observed in airway neurons following irritant exposure may be mediated by NGF. NGF is synthesized and released from a number inflammatory and non-inflammatory cells in the airways. Superficial nasal mucosal cells obtained from the inferior turbinates of healthy

human subjects were found to constitutively express mRNA for NGF. The same subjects also had baseline levels of NGF protein in their nasal lavage fluid (Sanico *et al.*, 2000). Mast cells (Leon *et al.*, 1994), epithelial cells (Fox *et al.,* 2001), lymphocytes (Barouch *et al.*, 2000), eosinophils (Solomon *et al.*, 1998), and macrophages (Braun *et al.*, 1998) are all capable of synthesizing and releasing NGF.

Following release, NGF binds the extracellular domain of trkA, a high-affinity-transmembrane-tyrosine-kinase receptor located on sensory nerve terminals. NGF binding causes autophosphorylation of tyrosine residues located in the C-terminal region of the receptor (McMahon, 1996) leading to the rapid formation of a signaling endosome to retrogradely transport the phosphorylated trkA/NGF complex to the cell body (Greene & Tischler, 1976; Sandow *et al.*, 2000). At the cell body, kinase-signaling pathways are believed to up-regulate the transcription and translation of neuropeptides (Stephens *et al.*, 1994).

The purpose of this study was to block NGF activity in the upper airway using two different approaches: an intranasal delivery of rabbit anti-NGF 2 hr before and 2 hr after TDI exposure and intraperitoneal injections of sheep anti-NGF 1 hr before and 3 hr after TDI exposure. Since NGF is normally detected in the rat nasal lavage fluid and significantly larger amounts are detected as early as 2 hr following TDI, the NGF antiserum was administered before and after TDI. The NGF antiserum was intended to bind NGF, thereby preventing NGF from binding to nerve terminals and up-regulating neuropeptides.

Previous studies have successfully employed NGF antiserum to directly correlate NGF and neuropeptide levels. Daily injections of sheep anti-NGF caused a significant

decrease in the SP and CGRP content of dorsal root ganglion neurons after seven days. The changes were attributed to a decreased availability of endogenous NGF (Shadiack *et al.*, 2001). Injections of anti-NGF 2 and 24 hr after induction of skin inflammation prevented SP from increasing in the sciatic nerve five days later (Donnerer *et al.*, 1992). Nasal application of anti-NGF to allergen-sensitized mice 3 hr prior to ovalbumin challenge significantly prevented the development of neuronally mediated airway hyperreactivity but did not prevent the influx of eosinophils and T cells into the airways (Braun *et al.*, 1998). Intraperitoneal injection of anti-NGF three hours prior to inoculation with respiratory syncytial virus (RSV) reduced NK-1 receptor expression and capsaicin-stimulated plasma extravasation (Hu *et al.*, 2002).

In this study, pretreatment with NGF antiserum failed to confirm or refute NGF's role as a neuronal mediator. Pretreatment with rabbit anti-NGF prior to and after TDI exposure did not prevent the increase in intraepithelial SP NFD. These data alone suggest that NGF does not mediate SP synthesis. However, a significant, unexpected increase in SP NFD was observed in ethyl acetate control rats pre-treated with rabbit anti-NGF. One plausible explanation is that the rabbit anti-NGF instilled into the nasal cavity bound to NGF on sensory nerve terminals where it remained post-mortem and was labeled by the fluorescent goat anti-rabbit antibody used for the immunocytochemical detection of SP. This was tested by resectioning the nasal mucosa obtained from anti-NGF + EA animals and applying only flourescein-labeled goat anti-rabbit antibody to the coverslips. No fluorescence was detected. Therefore, the NGF detected in the nasal epithelium was specific for SP.

The increased SP NFD in control animals may be evidence of an adverse reaction to a foreign protein or the antibodies may have directly activated the nerve fibers. To control for this, a sheep anti-NGF antibody was used for the second studies. Intraperitoneal injections of sheep NGF antiserum produced similar results. Injecting NGF antiserum also failed to prevent SP-IR from increasing in epithelial nerve fibers 24 hr after TDI exposure. Similar to the nasal antibody instillations, control animals injected with antibody displayed increased SP NFD in the epithelium. In previous studies, ethyl-acetate control animals recieving no antibody injections had a SP NFD in the nasal epithelium of 0.20 ± 0.03, which is much lower than the 0.55 ± 0.05 reported in the antibody-injected control animals used in this study. The data in this study are inconclusive despite attempts to control for possible antibody interactions.

Antibody treatment alone, whether intranasally instilled or intraparitoneally injected, increased SP NFD; therefore, any inhibition of NGF following TDI was masked. It was unclear if the large antibodies were even able to access the nasal mucosa. Control animals in these studies should have received intranasal instillation of rabbit serum and intraperitoneal injections of sheep serum prior to TDI or ethyl acetate to control for potential adverse neuronal reactions. Interestingly, neither antibody treatment protocol altered neutrophil levels in the TDI or ethyl acetate exposed animals. In ovalbumin-sensitized and-challenged mice, anti-NGF treatment also failed to prevent the influx of eosinophils into the lungs (Braun *et al.*, 1998).

Prior studies have used NGF antibodies to illustrate the role of NGF in RSV and allergic airway inflammation. However, these studies measured cytokine and smooth

muscle responsiveness (Braun *et al.*, 1998) or NK1 receptor expression and vascular permeability (Hu *et al.*, 2002); neither directly measured changes in nerves.

In conclusion, nasal instillation of TDI is an effective model for studying neuropeptides, inflammation and NGF simultaneously in the upper airways. However, instillation of NGF antiserum and injection of NGF antiserum is not an effective method of manipulating NGF activity in the upper airway. This study was unsuccessful in determining if NGF increases the SP expression in airway sensory nerves following TDI.

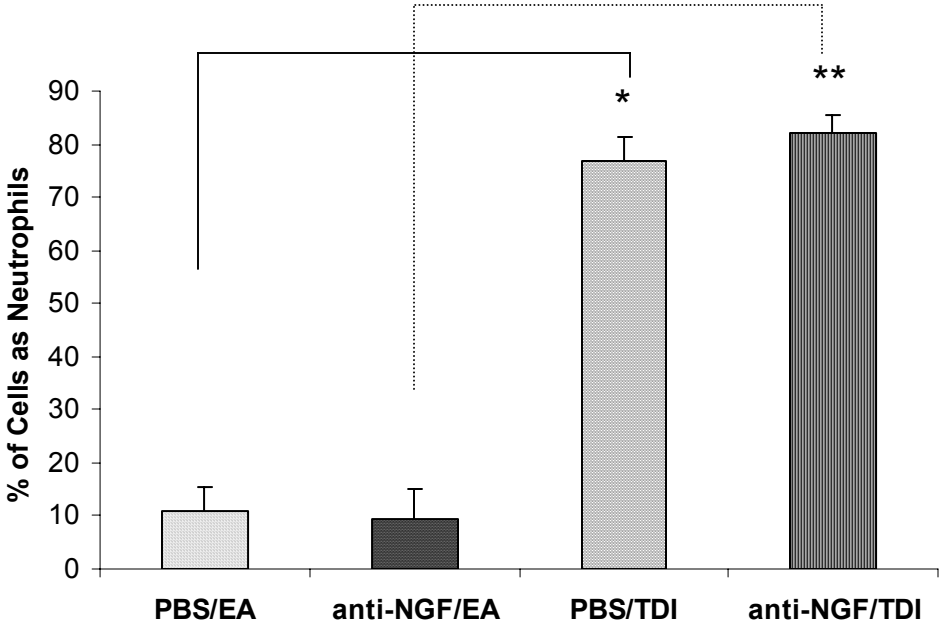

Figure 30 The percentage of neutrophils in the nasal lavage fluid 24 hr after TDI (n=4 PBS/TDI and n=3 anti-NGF TDI) or ethyl acetate (control vehicle, n=3 PBS/EA and n=4 anti-NGF EA) instillation into the nasal cavity. The animals were treated with either PBS or rabbit anti-NGF 2 hr prior and 2 hr following either TDI or ethyl acetate. Asterisk (*) denotes significance over PBS/EA and (**) denotes significance over anti-NGF/EA ($p \leq 0.05$).

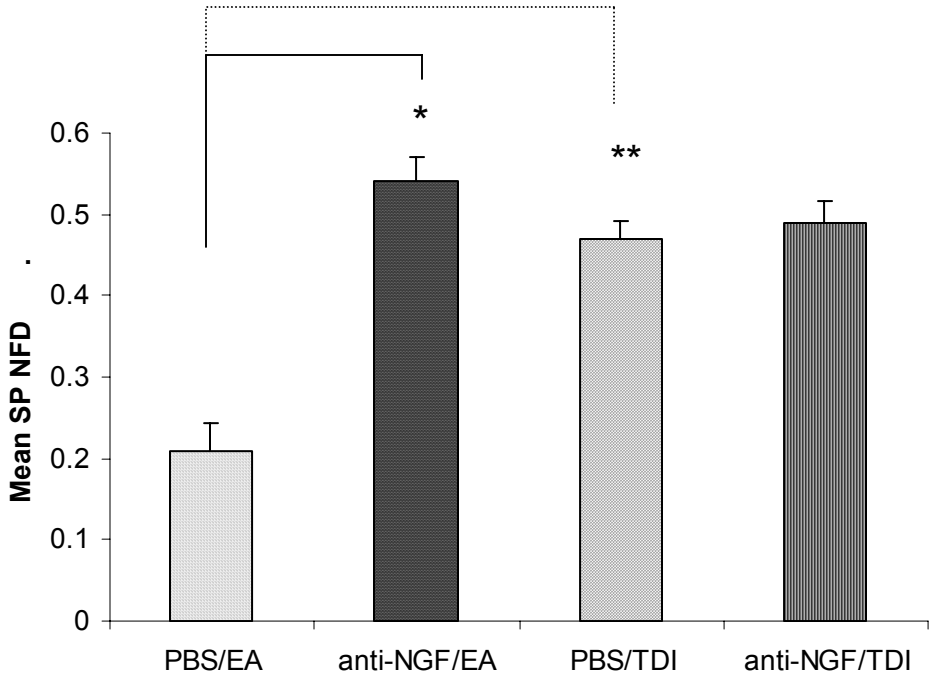

Figure 31 The percentage of SP-IR nerve fibers in the nasal epithelium 24 hr after TDI (n=5 PBS/TDI and n=4 anti-NGF/ TDI) or ethyl acetate (control vehicle, n=4 PBS/EA and n=6 anti-NGF EA) instillation into the nasal cavity. The animals were treated with either PBS or rabbit anti-NGF 2 hr prior and 2 hr following either TDI or ethyl acetate. Asterisk (*) denotes significance increase compared to PBS/TDI and PBS/EA and (**) denotes significant increase compared to anti-NGF/EA and PBS/EA ($p \leq 0.05$).

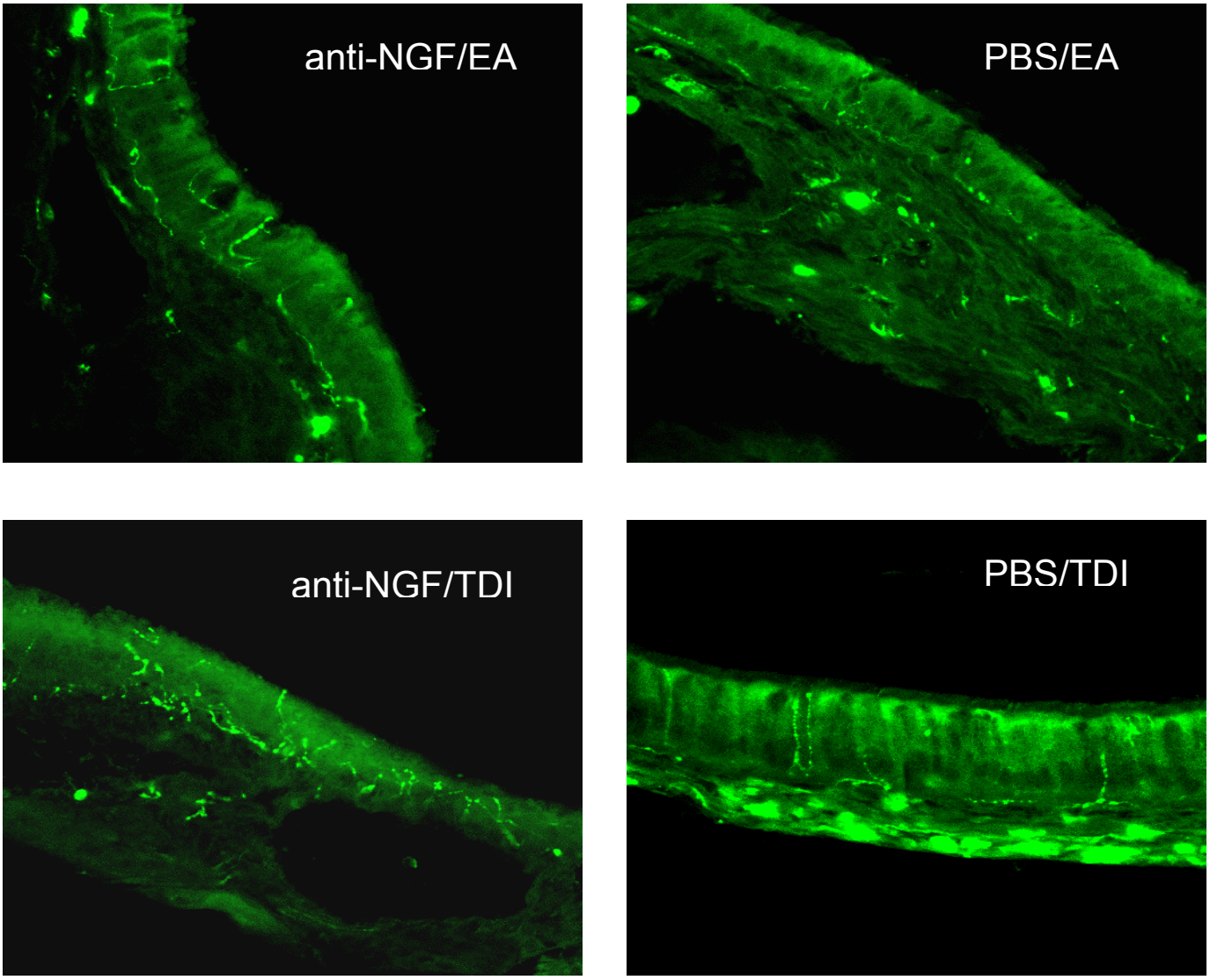

Figure 32 Images of rat nasal mucosa showing SP-IR nerve fibers in the nasal epithelium. Animals were instilled with either PBS or rabbit anti-NGF 2 hr before and 2 hr after TDI or EA treatment. The nasal mucosa was removed 24 hr following TDI or control vehicle. The SP-IR nerves run perpendicular with the basal lamina and project up between epithelial cells. Very few SP-IR nerve fibers were observed projecting between epithelial cells in animals pretreated with PBS prior to EA. However, treatment with TDI caused a significant increase inthe number of SP-IR nerve fibers (PBS/TDI image). Pre-treatment with anti-NGF caused a significant increase in the SP content of nerve fibers in the epithelium of rats exposed to both TDI and EA (anti-NGF/EA and anti-NGF/TDI images).

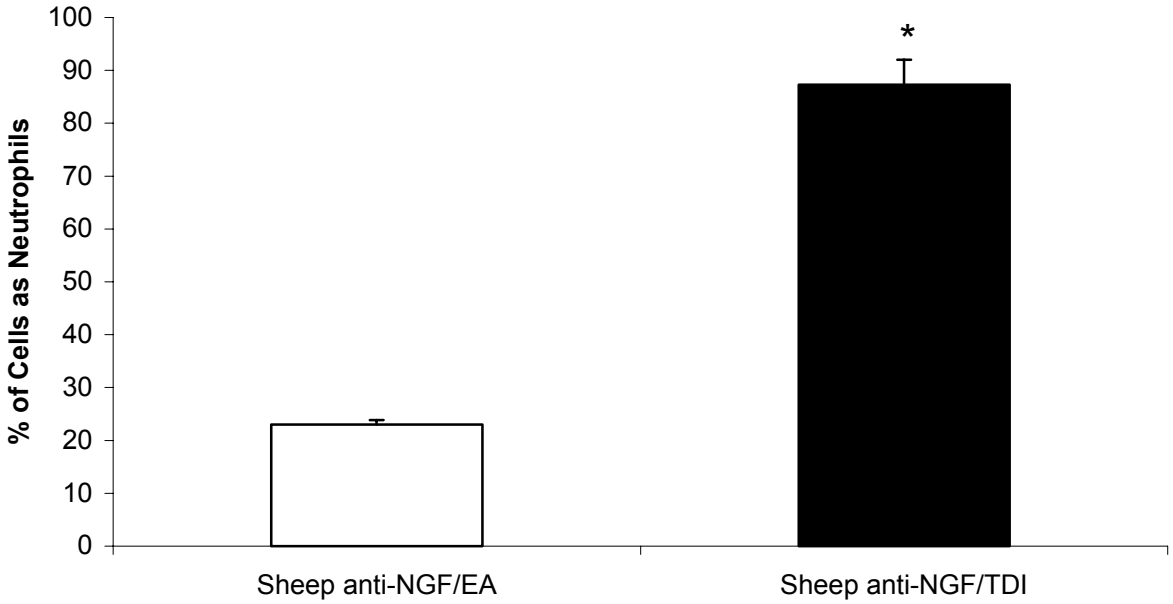

Figure 33 The percentage of neutrophils in the nasal lavage fluid 24 hr after TDI or ethyl acetate instillation into the nasal cavity. The animals were treated with sheep anti-NGF 1 hr prior and 3 hr following either TDI or ethyl acetate. The asterisk denotes significance ($p \leq 0.05$).

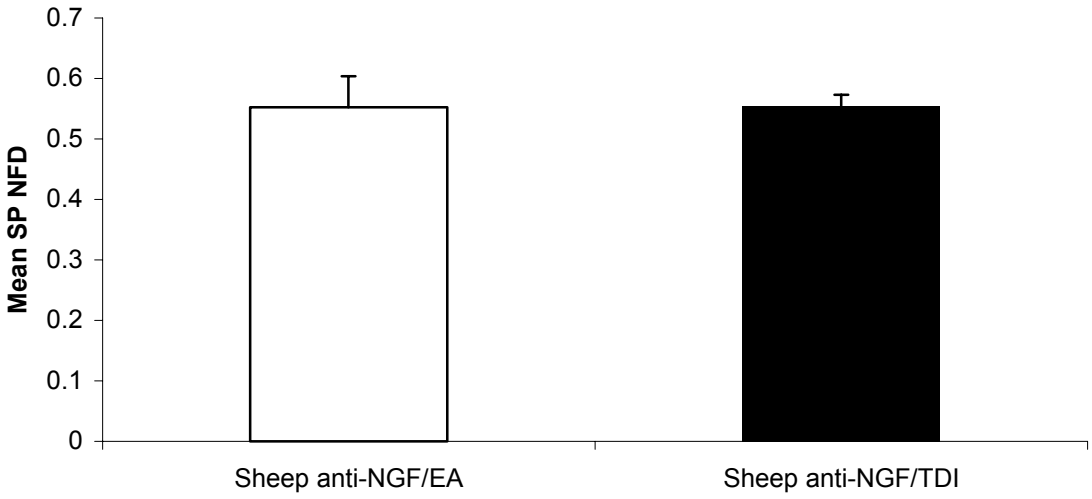

Figure 34 The percentage of SP-IR nerve fibers in the nasal epithelium 24 hr after TDI or ethyl acetate instillation into the nasal cavity. The animals were treated with sheep anti-NGF 1 hr prior and 3 hr following either TDI or ethyl acetate. No significant changes were observed ($p \leq 0.05$).

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STUDY 4

Identifying the Source of Nerve Growth Factor in the Nasal Mucosa Following Depletion of Peripheral Leukocytes and Toluene Diisocyanate Exposure

Not submitted for publication

ABSTRACT

Toluene diisocyanate (TDI) causes rhinitis, nasal irritation and increased synthesis and release of substance P (SP) from airway sensory neurons. The peripheral signaling mechanism responsible for enhanced SP production involves nerve growth factor (NGF), which increases in the nasal cavity following irritant exposure. A number of inflammatory and non-inflammatory cells synthesize and release NGF. In this study, peripheral leukocytes were depleted so that only NGF release from the nasal mucosa following TDI could be measured. The rats received intraperitoneal injections of cyclophosphamide to deplete leukocytes three days before TDI or EA was intranasally instilled into the nasal cavity. Nasal lavages and removal of nasal mucosa were performed 24 hr after TDI instillation. NGF in the lavage fluid, NGF expression in the nasal mucosa, SP nerve fiber density (NFD) in the nasal epithelium and inflammatory markers in the nasal lavage were measured. Pre-treatment with cyclophosphamide destroyed the majority of leukocytes in the nasal cavity. NGF mRNA was detected in the nasal mucosa but was not increased following TDI. NGF significantly increased in the nasal cavity following TDI compared to ethyl acetate controls, but the levels were much lower than in previous studies that did not use cyclophosphamide. Therefore, it could not be concluded if the overall decrease in NGF correlated with leukocyte depletion or if cyclophosphamide directly blocked NGF release. Pre-treatment with cyclophosphamide prevented SP from increasing in epithelial nerve fibers and plasma extravasation 24 hr following TDI. Leukocyte depletion with cyclophosphamide is not an effective model for studying NGF release from the nasal mucosa following TDI.

INTRODUCTION

The neuropeptide, substance P (SP) has potent inflammatory actions and increases in the airways following respiratory viral infection (Carr *et al.*, 2002), allergen challenge (Fischer *et al.*, 1996) and irritant exposure (Hunter *et al.*, 2000). SP is also elevated in the airways of patients with asthma (Nieber *et al.*, 1992) and bronchitis (Tomaki *et al.*, 1995). Upon release, SP causes vasodilation, plasma extravasation, mucous secretion and neutrophil chemotaxis (Jancso *et al.*, 1967; Lundberg & Saria, 1983) all of which contribute to airway inflammation.

SP is released from sensory nerve endings found throughout the nasal mucosa (Baraniuk *et al.*, 1991; Lee *et al.*, 1985) but the transcription of SP is localized in cell bodies located in trigeminal ganglia (TG) (Helke *et al.*, 1990). Transient increases in SP have been observed in TG cell bodies innervating the nasal epithelium and in epithelial nerve fibers following exposure to the airway irritant toluene diisocyanate (TDI) (Hunter *et al.*, 2000).

The neurotrophin, nerve growth factor (NGF) may mediate the irritant-induced up-regulation of SP synthesis and release from TG sensory neurons supplying the nasal epithelium. NGF binds receptors on sensory nerve terminals (McMahon, 1996), undergoes axonal transport (Sandow *et al.*, 2000) and up-regulates neuropeptide production in neuronal cell bodies (Lindsay & Harmar, 1989).

NGF levels are significantly increased in the nasal lavage fluid following exposure to TDI with the largest increase occurring 24 hr after TDI, but the cellular source of NGF in the upper airways remains unclear. Many studies have implicated inflammatory cells, which also increase 24 hr following exposure to TDI and non-

inflammatory airway cells as potential sources of NGF. Lymphocytes (Barouch *et al.*, 2000), eosinophils (Solomon *et al.*, 1998) and macrophages (Braun *et al.*, 1998) are all capable of synthesizing and releasing NGF. NGF mRNA is also present in nasal mucosal scrapings (Sanico *et al.*, 2000) and NGF synthesis and release has been demonstrated in mast cells (Leon *et al.*, 1994) and epithelial cells (Fox *et al.*, 1997). Based on previous studies, potential sources of NGF in the upper airway include the nasal mucosa and inflammatory cells.

The purpose of this study was to determine if nasal mucosal cells synthesize and release NGF following exposure to TDI. Peripheral leukocytes, which migrate into the nasal cavity post-TDI and release NGF, were eliminated with cyclophosphamide.

METHODS AND MATERIALS

Experimental Design Adult male Sprague-Daley rats (Hal:(SD)CIF) weighing 200-250 g and purchased from Hilltop Lab Animals (Scottsdale, PA) were used for all the experiments. Animals were injected with cyclophosphamide (200 μg/g), and three days later either 10% TDI or ethyl acetate (vehicle) was instilled into the nasal cavity. Tissues were removed and nasal lavages performed 24 hr later.

Cyclophosphamide Injections All rats received an intraperitoneal injection of cyclophosphamide (Brand Name: Cytoxan; Bristol Myers Squibb Co., Princeton, NJ; 200 μg/g body weight) to deplete peripheral leukocytes. Cyclophosphamide has been shown to destroy hemopoietic cells at various stages of differentiation in the hemopoietic pathway (Wierda & Pazdernik, 1979). The dose and dose schedule were based on

previous studies, which used cyclophosphamide to deplete peripheral leukocytes in guinea pigs (Castranova *et al.*, 1988).

TDI Instillation Three days after the cyclophosphamide injections, rats were anaesthetized with an intraperitoneal injection of sodium brevital (50 mg/kg weight dose). Both nasal cavities were instilled with 5 µl of 10% TDI (Aldrich Chemical Co., Milwaukee, WI) or ethyl acetate (control vehicle; Sigma Chemical Co., St. Louis, MO) by placing the tip of a 10 µl pipette at the entrance of the nasal cavity.

Nasal Lavage The rats were overdosed i.p. with 1.5 ml of 50 mg/ml sodium brevital and the lower jaw was removed. A syringe with plastic tubing covering the needle was inserted into the posterior nares and sealed by finger pressure. Both sides of the nasal cavity were simultaneously lavaged with 15 ml of phosphate buffered saline (PBS). The first 3 ml of lavage fluid was separated from the final 12 ml. Both aliquots of nasal lavage fluid were centrifuged at 1,500 rpm (352 rgf) for 10 min. The supernatant from the initial 3 ml of nasal lavage was aliquoted and frozen at -80 °C for subsequent assays. The two resulting cell pellets from each nasal sample were resuspended in a total of 1 ml cold PBS, pooled, plated on glass slides at a density of $1.5X10^5$ cells/ml using a cytospin (Shandon Scientific, Ltd., Cheshire, UK) at 400 rpm (18.06 rgf) for 4 min and stained with Wright-Giemsa on a Hema-Tek 2000 automated slide stainer (Bayer, Inc., Tarrytown, NY). A total of 100 cells were classified as neutrophils or nucleated cells (primarily epithelial cells) using light microscope (Olympus AX70) with a 40x magnification objective. The percentage of neutrophils was recorded for each slide.

Tissue removal and preparation The nasal mucosa was removed from the anterior and posterior regions of the nasal cavity. The tissue was either placed directly into RNAlater

(Ambion, Austin, Tx) and stored at -80 °C until RNA isolation or immediately fixed in picric-acid formaldehyde fixative consisting of 2% paraformaldehyde, 15% saturated picric acid and 0.15 M phosphate buffer at 4 °C (Stefanini *et al.*, 1967). After 3 hr, the tissue was rinsed twice in 0.1 M phosphate-buffered saline containing 0.3% (v/v) Triton X-100 (PBS-Tx, pH=7.8). After the second rinse, the tissues remained in PBS-Tx overnight at 4°C. The next day the nasal mucosa was laid flat and then rolled into a cylinder shape and stood upright on the cork. The tissue was covered with Tissue Tek O.C.T compound (Sakura, Torrance, CA), frozen in isopentane cooled by liquid nitrogen and stored in airtight plastic bags at –80°C.

Continuous cryostat sections (12 μm thickness) were taken throughout the nasal mucosa and 16 random sections were collected on gelatin-coated coverslips and used for SP immunocytochemistry (Hunter and Dey, 1997).

Immunocytochemistry Procedures for immunocytochemistry were previously described (Dey *et al.*, 1990). Cryostat sections on gelatin-coated coverslips were covered with rabbit anti-SP (1:200; Peninsula, Belmont CA) primary antiserum diluted in PBS-Tx + 1% bovine serum albumin (PBS-Tx-BSA, pH=7.8). The coverslips were incubated in a humid chamber at 37 °C for 30 min, rinsed 3 times with PBS-Tx-BSA, allowing 5 min per rinse and then covered with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin IgG (ICN Pharmaceuticals, Inc, Costa Mesa, CA) diluted 1:100 in PBS-Tx-BSA and incubated at 37 °C for 30 min. The coverslips were rinsed 3 times for 5 min increments in PBS-Tx-BSA and mounted on glass slides in Fluoromount (Southern Biotechnology, Birmingham, AL).

SP Nerve Fiber Density (NFD) Following immunocytochemical processing for SP, sections of nasal mucosa were observed on a Zeiss LSM 510 confocal microscope equipped with an argon laser (Zeiss, Germany). Eighteen random images of respiratory epithelium were recorded from each coverslip. Using Optimus version 6.5 image analysis software (Media Cybernetics, L.P., Silver Spring, MD), the entire perimeter of epithelium was traced on each image of nasal mucosa. The threshold for each image was optimized so that only SP-IR nerve fibers were visible. The SP NFD was calculated by dividing the SP-IR nerve fiber area by the total area of epithelium outlined. This represents the proportional cross-sectional area occupied by SP-immunoreactive nerve fibers.

RNA Isolation Total RNA was isolated from the nasal mucosa using the RNAqueous-4PCR kit (Ambion, Austin, Tx). DNA-free RNA was obtained according to the manufacturer's instructions. Tissue was removed from –80°C, allowed to thaw on ice and removed from RNAlater solution. The tissue was ground with a mortar and pestle in liquid N_2. The finely ground tissue was transferred to a 1.5 ml microcentrifuge tube containing 500 µl of lysis binding solution. The mixture was vortexed and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Fluka, Buchs, Switzerland) was added to remove lipids. The solution was vortexed for 2 min followed by centrifugation at 12000 RPM for 10 min. The aqueous layer was removed and transferred to a fresh tube containing 500 µl of 64% EtOH. The solution was applied to a silica-based filter, which selectively binds mRNA. The filter was washed 3 times to remove residual DNA, protein and other contaminates. The RNA was eluted in a nuclease-free water/EDTA solution and incubated with DNase I at 37 °C for 30 minutes to remove trace amounts of

DNA. The concentration of RNA was measured on a spectrophotometer (Bausch & Lomb Spectronic 1001; Fisher Scientific, Pittsburgh, PA). Purified RNA was stored at –80 °C in 5 µl aliquots for RT-PCR

RT-PCR One step real-time PCR was used to quantitate relative changes in NGF expression in the nasal mucosa of TDI and EA exposed rats pre-treated with cyclophosphamide. The NGF sense and antisense primers used were CCAAAGGGAGCAGCTTTCTATCCTG and GGCAGTGTCAAGGGAATGCTGAAGT, respectively, which recognize sequences near the 5' end of the cDNA and amplify a 128-bp fragment (Promega Corp., Madison, WI). The RT-PCR conditions were as follows:

Cycle	**Repeats**	**Time**	**Temperature**
1. Reverse Transcription	0	60 min	50 ºC
2. Activation of Taq	0	15 min	95 ºC
3.	40		
Step 1: Denature		15 sec	94 ºC
Step 2: Annealing		30 sec	55 ºC
Step 3: Extension		30 sec	72 ºC (data collection)
4. Melt Curve	80	30 sec (each repeat, temp. decreased by 0.5ºC)	start 95ºC

RT-PCR reactions were set up using the QuantiTect SYBR Green one-step RT-PCR kit (Quiagen, Valencia, CA) according to manufacturers instructions in 96 well plates. Each reaction contained 10 µl of 2x QuantiTect SYBR Green RT-PCR master mix, 100 ng/ml of RNA, 0.2 µl QuantiTect RT mix, 0.5 µM forward (5'-3') primer and 0.5 µM reverse (3'-5') primer and Rnase-free water in a total volume of 20 µL and each

sample was run in triplicate. β-actin was also amplified under the same conditions and used to normalize reactions. Sense and antisense primers to the β-actin gene were TCATGAAGTGTGACGTTGACATCCGT and CTTAGAAGCATTTGCGGTGCACGATG , respectively which recognize sequences near the 5' end of the cDNA and amplify a 285-bp fragment (Promega Corp., Madison, WI).

An icycler (Biorad, Hercules, CA), was used to perform the RT-PCR cycles and fluorescence was quantified as a threshold cycle (Ct) value with Biorad icycler software. NGF expression levels were normalized using β-actin expression levels as an internal control. The differences between the mean Ct values of the gene of interest and the housekeeping gene were denoted (delta-Ct) and the difference between TDI delta-Ct and the EA delta-Ct was labeled delta-delta-Ct. The $\log_2$(delta-delta-Ct) gave the relative quantitation value of NGF expression in the TDI group with the ethyl acetate group expression designated as one. Control wells containing SYBR Green PCR master mix and primers without sample reverse transcriptase emitted no fluorescence after 40 cycles.

NGF ELISA The nasal lavage supernatant samples (initial 3ml) were frozen at –80 °C. The concentration of NGF (7.8-500 pg/ml) in each sample was assayed using the NGF Emax ImmunoAssay System (Promega, Madison, WI) according to the manufacturer's instructions. NGF was detected using an antibody sandwich format in 96 well plates. Each well was initially coated with 100 µl of anti-NGF pAb and incubated overnight followed by a one-hour incubation with blocking buffer (200 µl/well) to prevent nonspecific binding. Either 100 µl of lavage supernatant or 100 µl of NGF standard (7.8-

500 pg/ml) was added to each well. The plate was incubated for six hr followed by an overnight incubation with anti-NGF mAb (100 µl /well). For color development an anti-rat IgG horse radish peroxidase conjugate antibody was added to each well (100 µl) followed by a TMB solution, which reacts with the peroxidase-labeled conjugates to develop a blue color. The absorbance of each well was measured at 450 nm on a Spectra Max 340pc plate reader (Molecular Devices, Sunnyvale, CA). The concentration of NGF in each lavage sample was extracted from an NGF standard curve. All samples were run in duplicate or triplicate, and as a negative control, a PBS sample was run with each assay.

Protein Assays The nasal lavage supernatant samples were also assayed for protein concentration. The first method, the bicinchroninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL) is a modified Lowry assay and measures the concentration of total protein (20-2,000 µg/ml). The assay was performed according to the manufacturer's instructions using the microplate procedure. The assay was performed in 96 well plates and 20 µl of unknown or bovine serum albumin standard was added to each well followed by 200 µl of a working reagent. The plate was incubated at 37 ˚C for 30 min before reading the absorbance at 562 nm on a Spectra Max 340pc plate reader (Molecular Devices, Sunnyvale, CA). The concentration of total protein in each lavage sample was extracted from a bovine serum albumin standard curve. All samples were run in duplicate or triplicate.

Statistical Analysis The means and standard errors were calculated for each endpoint measured and a one-way ANOVA with a Tukey post-hoc test was run. Significance was set at $p \leq 0.05$ for each endpoint measured.

RESULTS

Inflammatory Markers in the Nasal Lavage Fluid Following Cyclophosphamide and TDI

There was no difference in the total number of nucleated cells in the nasal lavage fluid obtained from cyclophosphamide-treated animals exposed to EA (71.6±31.5) or TDI (86.1±27.9)(Figure 35). There was not a significant influx of inflammatory cells into the nasal cavity 24 hr following TDI indicating that cyclophosphamide pre-treatment depleted the majority of peripheral leukocytes. However, of the nucleated cells present there were significantly more neutrophils in the nasal lavage of cyclophosphamide-treated animals exposed to TDI (74.5±9.27%) compared to the EA controls (32.4+7.7%)(Figure 36).

No significant difference in total protein concentration was detected in cyclophosphamide-treated rats exposed to either EA (128.32±17.62) or TDI (108.92±17.26)(Figure 37). These findings suggest that cyclophosphamide pre-treatment prevented the increase in total protein in the nasal lavage 24 hr following exposure to TDI.

SP Nerve Fiber Density (NFD) in the Nasal Epithelium Following Cyclophosphamide and TDI

The percent area of SP-IR nerve fibers in the nasal mucosa was not significantly different when TDI and EA-treated animals injected with cyclophosphamide were compared (Figure 38). The SP nerve fiber density was 0.43±0.17 in control animals and 0.33±0.05 in TDI animals 24 hr following exposure. These findings suggest SP NFD is not increased in the epithelium of rats treated with cyclophosphamide and TDI.

NGF in the Nasal Lavage Fluid and NGF mRNA Expression in the Nasal Mucosa Following Cyclophosphamide and TDI

The concentration of NGF (pg/ml) in the nasal lavage fluid was significantly increased in rats injected with cyclophosphamide and exposed to TDI (Figure 39). No NGF was present in the nasal lavages of EA-treated rats after 24 hr compared to the 12.40±4.18 pg/ml of NGF detected 24 hr following exposure to TDI. The relative expression of NGF mRNA in the nasal mucosa of rats exposed to TDI (0.83) was not significantly different from control animals (Figure 40). These findings suggest that NGF is released into the nasal cavity of rats treated with cyclophosphamide and TDI but expression of NGF mRNA in the nasal mucosa is not increased.

DISCUSSION

TDI is a commonly used solvent in the production and manufacture of polymer containing products such as plastics, foams, adhesives and surface coatings. Workers exposed to TDI vapor can develop a number of inflammatory conditions including

asthma, rhinitis and nasal irritation (Brugsch & Elkin, 1963; Mapp *et al.*, 1988) Exposure of rats to TDI leads to the activation of sensory nerves in the nasal epithelium and increased synthesis and release of the neuropeptide substance P (SP) (Gordon *et al.*, 1985). Twenty-four hours after intranasal instillation of TDI increased levels of SP are detected in epithelial nerve fibers and TG neurons (Hunter *et al.,* 2000) in addition to inflammatory markers and NGF in the nasal lavage fluid (Sikora *et al.*, 2001).

Evidence suggests that the increased neuropeptide production observed in airway neurons following irritant exposure is mediated by NGF. NGF is synthesized and released from a number of resident cells in the airway. Following release, NGF binds the extracellular domain of trkA, a high-affinity-transmembrane-tyrosine-kinase receptor located on sensory nerve terminals (McMahon, 1996) which activates the formation of a signaling endosome to retrogradely transport the trkA/NGF complex to the cell body (Greene & Tischler, 1976);) Sandow *et al.*, 2000) where NGF up-regulates the transcription and translation of neuropeptides (Stephens *et al.*, 1994).

In order to understand better the relationship between NGF, SP and airway inflammation, we attempted in the present study to identify the source of NGF in the nasal cavity. Although a number of inflammatory cells synthesize and release NGF, we were interested solely in the resident cells of the nasal mucosa. Animals were pre-treated with cyclophosphamide to destroy circulating leukocytes, which normally migrate into the nasal mucosa following exposure to TDI. Cyclophosphamide was used in previous studies to examine the effect of peripheral leukocyte depletion on guinea pig pulmonary responses to cotton dust inhalation (Castranova *et al.*, 1988).

In previous studies not involving cyclophosphamide, the total number of nucleated cells in the nasal lavage fluid was significantly higher 24 hr following TDI (382.0±19.8) compared to EA controls (1.5±0.08) (data not shown). In this study, cyclophosphamide pre-treatment prevented the total number of cells in the nasal lavage fluid from increasing following TDI. Cyclophosphamide pre-treatment appeared to destroy most circulating leukocytes but not all indicated by the significant number of neutrophils detected in TDI-exposed animals. However, the neutrophils were not considered a problem since no studies to date have detected NGF mRNA in neutrophils.

The marked leukocyte depletion appeared to affect NGF levels in the nasal lavage fluid 24 hr following TDI exposure. Cyclophosphamide-treated animals exposed to TDI had significantly higher concentrations of NGF in their nasal lavage fluid compared to control animals. However, the levels of NGF detected in this study were much lower than those reported in previous studies. Normally, TDI-treated rats that are not injected with cyclophosphamide have 229.32±28.54 pg/ml of NGF in their nasal lavage fluid 24 hr after exposure (data not shown), which is significantly more than the 12.49±4.18 pg/ml detected in this study. No NGF was detected in the nasal lavage fluid of cyclophosphamide-treated control animals after 24 hr, which is a marked decline from the 54.60±11.95 pg/ml normally detected in non-cyclophosphamide treated ethyl acetate controls (data not shown). This suggests that cyclophosphamide may have affected NGF production and release from resident nasal mucosal cells. The NGF lavage data is supported by the relative quantitation of NGF mRNA expression in the nasal mucosa. No differences in NGF mRNA levels were detected in the nasal mucosa of

cyclophosphamide-treated animals exposed to TDI or EA. This indicates that the nasal mucosa synthesizes NGF but the synthesis is not up-regulated 24 hr after TDI.

Another possible explanation is that inflammatory cells, which migrate into the nasal mucosa following TDI, release the majority of NGF detected in the nasal lavage fluid 24 hr after irritant exposure. Specifically, lymphocytes (Barouch *et al.*, 2000), eosinophils (Solomon *et al.*, 1998), and macrophages (Braun *et al.*, 1998) are all capable of synthesizing and releasing NGF and entering the nasal cavity following irritant exposure. This is unlikely since in previous studies the majority of inflammatory cells in the nasal lavage fluid after TDI were neutrophils.

Normally, 24 hr following TDI there is a significant increase in the SP content of epithelial nerve fibers compared to controls (0.53$\pm$0.02 and 0.20$\pm$0.03 respectively; data not shown). In the present study, no significant increase in SP NFD was present in the epithelium of rats pre-treated with cyclophosphamide and exposed to TDI. NGF, which binds sensory nerve terminals and retrogradely travels to the cell body, is responsible for increasing SP synthesis. The lack of NGF may have prevented the increase in SP airway sensory nerves.

The SP released from sensory nerves contributes to airway inflammation by causing plasma extravasation (Jancso *et al.*, 1967; Lundberg & Saria, 1983) . In previous studies, protein concentration in the nasal lavage fluid was significantly elevated 24 hr following TDI compared to controls (393.47+61.00 μg/ml and 54.11+10.02 μg/ml respectively; data not shown) indicating increased plasma extravasation. However, in this study, protein did not increase in the nasal lavage fluid after TDI, suggesting that SP may not have been released or cyclophosphamide may have affected SP production.

In conclusion, cells in the nasal mucosa synthesize NGF but the amount of NGF released from the nasal mucosa 24 hr following TDI could not be measured in this study. Leukocyte depletion with cyclophosphamide is not an effective model for studying NGF release from the nasal mucosa following TDI because cyclophosphamide also interfered with basal NGF release resident cells in the nasal mucosa. Identifying the source of NGF remains an important question since NGF up-regulates SP expression and SP contributes to airway inflammation.

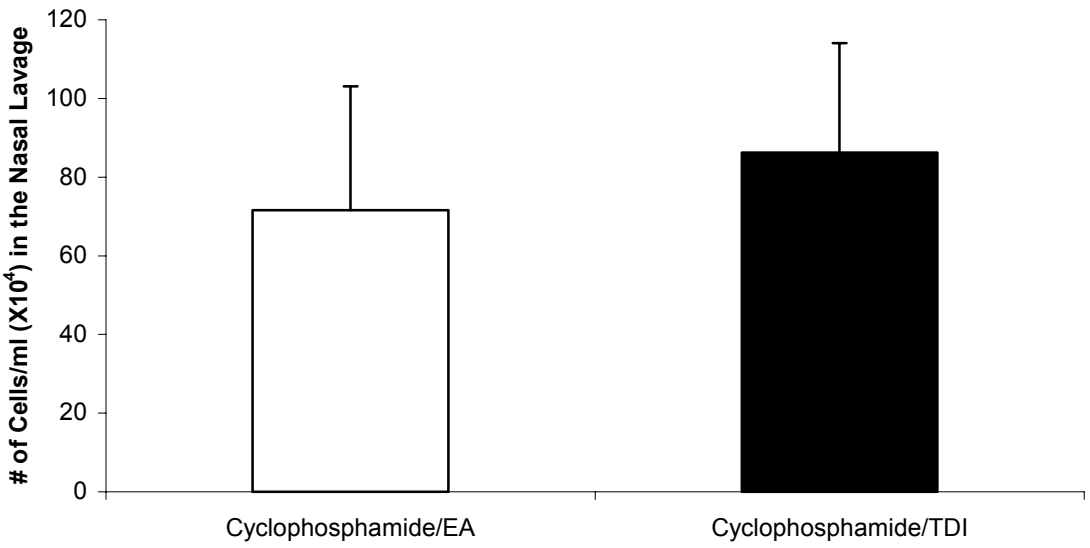

Figure 35 Total number of nucleated cells in the nasal lavage fluid of Sprague-Dawley rats injected with cyclophosphamide (200μg/g) and intranasally instilled with 10% TDI or ethyl acetate (control vehicle) three days later. Rats were sacrificed 24 hr following TDI or ethyl acetate. No significant changes were detected relative to controls. n=5 per group.

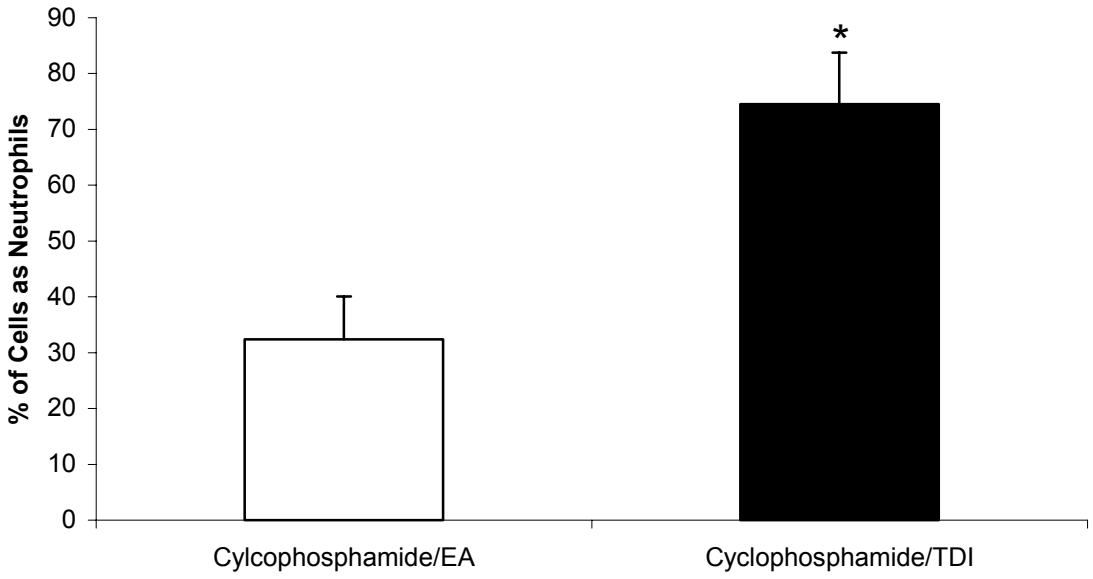

Figure 36 Percentage of neutrophils in the nasal lavage of Sprague-Dawley rats injected with cyclophosphamide (200 μg/g body weight) and instilled intranasally with 10% TDI or ethyl acetate (control vehicle) three days later. Rats were sacrificed 24 hr following TDI or ethyl acetate. The asterisk denotes significant change relative to controls ($p \leq 0.05$). n=5 per group.

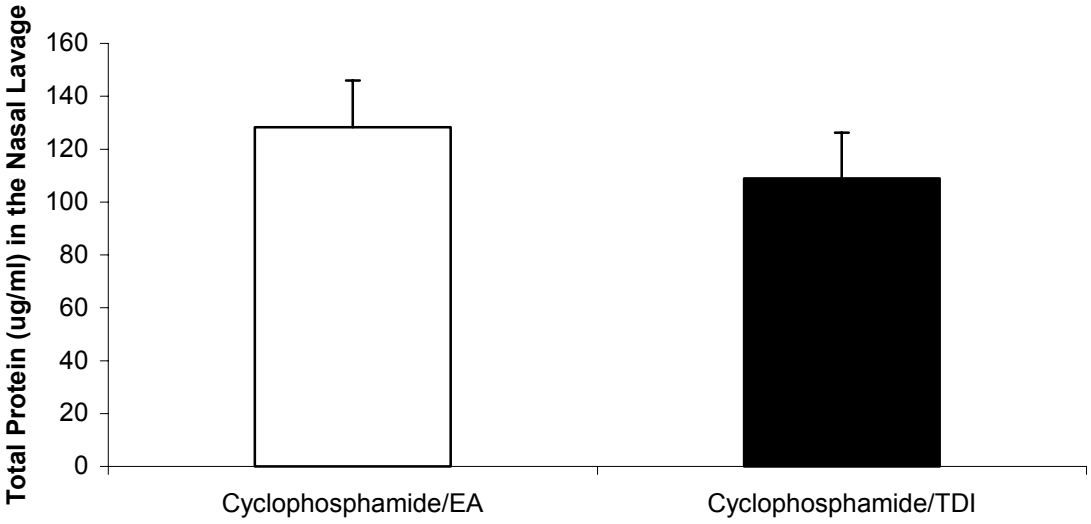

Figure 37 Total protein (μg/ml) in the nasal lavage of Sprague-Dawley rats injected with cyclophosphamide (200 μg/g body weight) and instilled intranasally with 10% TDI or ethyl acetate (control vehicle) three days later. Rats were sacrificed 24 hr following TDI or ethyl acetate. No significant changes were detected. n=5 per group.

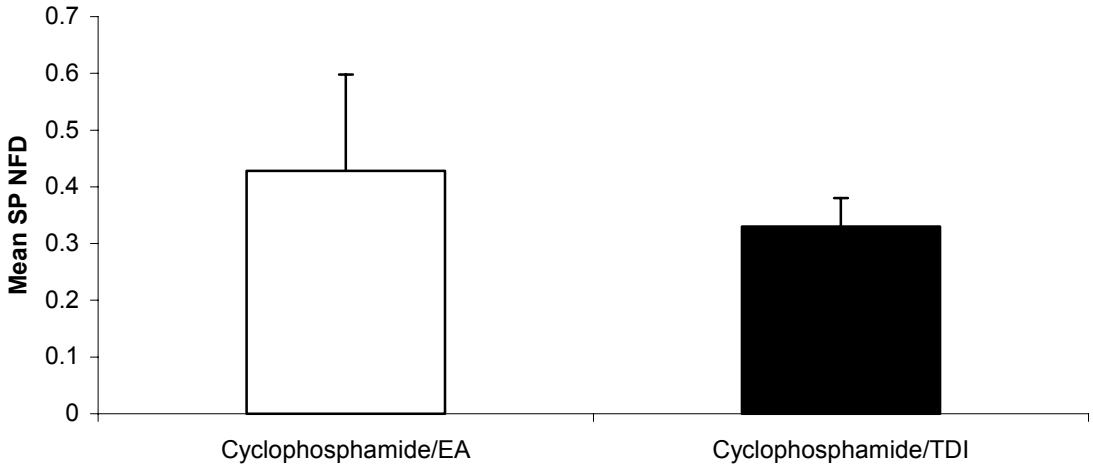

Figure 38 The density of SP-IR nerve fibers in the nasal epithelium of Sprague-Dawley rats injected with cyclophosphamide (200μg/g body weight) and instilled intranasally with 10% TDI or ethyl acetate (control vehicle) three days later. Rats were sacrificed 24hr following TDI or ethyl acetate. No significant changes were detected. n=5 per group.

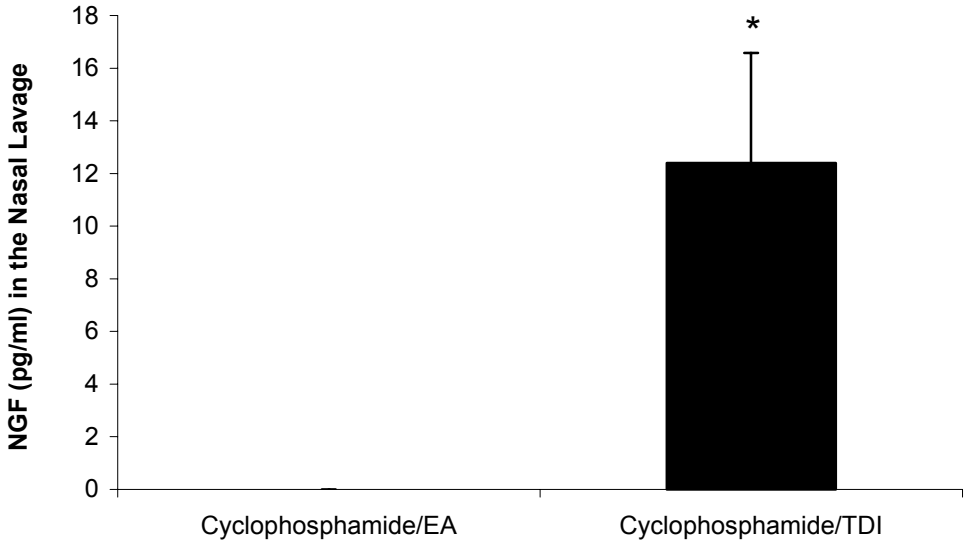

Figure 39 Concentration of NGF (pg/ml) in the nasal lavage of Sprague-Dawley rats injected with cyclophosphamide (200μg/g body weight) and instilled intranasally with 10% TDI or ethyl acetate (control vehicle) three days later. Rats were sacrificed 24hr following TDI or ethyl acetate. The asterick denotes significant change relative to controls ($p \leq 0.05$). n=5 per group.

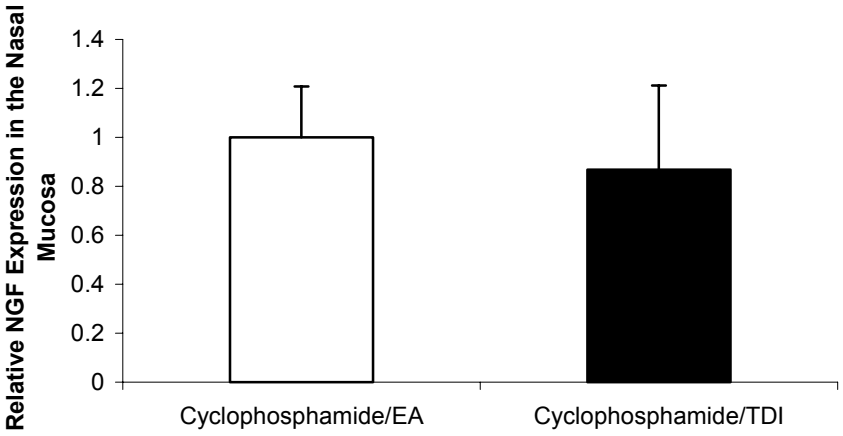

Figure 40 The relative expression of NGF in the nasal mucosa of Sprague-Dawley rats injected with cyclophosphamide (200µg/g body weight) and instilled intranasally with 10% TDI or ethyl acetate (control vehicle) three days later. Rats were sacrificed 24hr following TDI or ethyl acetate. No significant changes were detected. n=5 per group.

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STUDY 5

The Release of Nerve Growth Factor from the Nasal Mucosa following Toluene Diisocyanate

In preparation for submission

Authors: Erin R. Wilfong and Richard D. Dey

ABSTRACT

Toluene diisocyanate (TDI) causes rhinitis, nasal irritation and increased synthesis and release of substance P (SP) from airway sensory nerves. Nerve growth factor (NGF) secretion in the nasal cavity is believed to mediate the irritant-induced up-regulation of SP. NGF levels are significantly increased in the nasal lavage fluid as early as 2 hr following exposure to TDI, but the cellular source of NGF in the nasal mucosa remains unclear. Studies to localize a source of NGF within the nasal mucosa are complicated by inflammatory cell influx into the nasal mucosa following TDI, which obscures immunocytochemical identification of endogenous NGF sources. The purpose of this study was to determine the cellular source of NGF within the nasal mucosa following irritant exposure using a combined *in vivo* and *ex vivo* approach to reduce or eliminate contribution from inflammatory cells. Both nasal cavities of adult Sprague-Dawley rats were instilled with 5 µl of either 10% TDI or control vehicle (ethyl acetate). After 15 minutes, nasal lavages were performed, the nasal mucosa was removed and placed into culture for 3 or 24 hr. NGF was measured using ELISA in the lavage supernatant and the culture media. Fifteen minutes after TDI exposure NGF was significantly increased in the nasal lavage fluid (137.77$\pm$21.5 pg/ml) compared to controls (44.77$\pm$8.6 pg/ml). NGF levels in the culture medium of nasal mucosa from rats exposed to TDI *in vivo* were significantly increased compared to controls following a 3 hr culture (332.65$\pm$62.9 and 134.03$\pm$7.2 pg/ml, respectively). The increase was evident but not significant following 24 hr in culture. NGF immunoreactivity in the nasal epithelium was detected but was not significantly altered by TDI exposure and 3 or 24 hr in culture. These findings suggest that cells in the nasal mucosa release NGF following exposure to TDI.

INTRODUCTION

Initially implicated in neuronal growth and survival during development (Levi-Montalcini, 1987; Levi-Montalcini & Angeletti, 1968), the neurotrophin, nerve growth factor (NGF), also mediates immune and neuronal functions and may contribute to airway inflammation. NGF stimulates the release of inflammatory mediators from basophils (Bischoff & Dahinden, 1992), eosinophils (Hamada *et al.*, 1996) and neutrophils (Kannan *et al.*, 1991) and contributes to monocyte survival (la Sala *et al.*, 2000), lymphocyte growth and differentiation (Mowla *et al.*, 2001) and mast cell accumulation (Aloe & Levi-Montalcini, 1977) and degranulation (Tal & Liberman, 1997) in peripheral tissue. In terms of neuronal interaction, NGF is involved in phenotypic changes in airway sensory neurons (Hunter *et al.*, 2000a), airway hyperresponsiveness (de Vries *et al.*, 1999), increased sensory and sympathetic innervation (Hoyle *et al.*, 1998) and increased neuropeptide expression and release following irritant exposure (Wilfong and Dey, In review).

Increased levels of NGF have been demonstrated in the serum (Bonini *et al.*, 1996) and bronchoalveolar lavage fluid (Olgart *et al.*, 2002) of asthmatics and in the nasal lavage of allergic rhinitis patients (Sanico *et al.*, 2000). Increases in NGF have also been measured in the lungs of rats infected with respiratory syncytial virus (Hu *et al.*, 2002) and in mice with allergic airway inflammation (Braun *et al.*, 1998) .

In previous studies, we have detected significant increases in NGF in the rat nasal cavity following exposure to toluene diisocyanate (TDI), an established occupational irritant (Hunter *et al*., 2000b). Workers exposed to TDI vapor during the production and

manufacture of polymer containing products can develop a number of inflammatory conditions including asthma, rhinitis and nasal irritation (Mapp *et al.*, 1988). In rats exposed to TDI, significant levels of NGF began accumulating in the nasal cavity 2 hr following exposure and levels steadily increased until peaking at 24 hr. Based on previous studies potential sources of NGF include epithelial cells (Fox *et al.*, 1997; Fox *et al.*, 2001) and mast cells (Leon *et al.*, 1994), both of which are present in the nasal mucosa (Lim *et al*., 1995; Bradding *et al.*, 1993) and inflammatory cells, which also significantly increase in number following TDI.

The purpose of this study was to determine if NGF was released from resident cells of the nasal mucosa and to determine if TDI exposure affected NGF release and expression. Previous attempts to identify sources of NGF in the nasal mucosa have been hampered by inflammatory cells, which infiltrate the nasal mucosa and potentially release NGF following TDI. In this study, a combined *in vivo* and *ex vivo* approach was chosen to prevent inflammatory cell influx into the nasal mucosa. To accomplish this, rats were sacrificed 15 min following irritant exposure, prior to leukocyte influx, and the nasal mucosa was removed and maintained in culture for 3 and 24 hr.

METHODS AND MATERIALS

Experimental Design Adult male Sprague-Dawley rats (Hla:(SD)CVF) weighing 200-250 g and purchased from Hilltop Lab Animals (Scottsdale, PA) were used for all the experiments. Animals were intranaslly treated with either TDI or ethyl acetate and sacrificed after 15 min, 3 hr or 24 hr. Following sacrifice at 15 min, the nasal cavities were lavaged and the nasal mucosa was removed and either fixed for

immunocytochemistry or placed into culture for 3 hr or 24 hr. Following sacrifice at 3 or 24 hr, the nasal mucosa was removed for RT-PCR.

TDI Instillation Rats were anaesthetized with an intraperitoneal injection of sodium brevital (50mg/kg). Both nasal cavities were instilled with 5 µl of 10% TDI (Aldrich Chemical Co., Milwaukee, WI) or ethyl acetate (control vehicle; Sigma Chemical Co., St. Louis, MO) by placing the tip of a 10 µl pipette at the entrance of the nasal cavity.

Nasal Lavage The rats were overdosed with 1.5 ml of 50 mg/ml sodium brevital and the lower jaw was removed. A syringe with plastic tubing covering the needle was inserted into the posterior nares and sealed by finger pressure. Both sides of the nasal cavity were simultaneously lavaged with 15 ml of phosphate-buffered saline (PBS). The first 3 ml of lavage fluid was kept separate from the final 12 ml. Both aliquots for the nasal lavage fluid were centrifuged at 1,500 rpm (352 rgf) for 10 min. The supernatant from the initial 3 ml of nasal lavage was aliquoted and frozen at -80°C for subsequent assays. The two resulting cell pellets from each nasal sample were resuspended in a total of 500 µl of cold PBS, pooled and all plated on glass slides using a cytospin (Shandon Scientific, Ltd., Cheshire, UK) at 400 rpm (18.06 rgf) for 4 min and stained with Wright-Giemsa on a Hema-Tek 2000 automated slide stainer (Bayer, Inc., Tarrytown, NY). A total of 100 cells were classified as neutrophils or nucleated cells (primarily epithelial cells) using light microscope (Olympus AX70) with a 40x magnification objective. The number of neutrophils in each lavage sample was determined.

Tissue removal and preparation The nasal mucosa was removed from the anterior and posterior regions of the nasal cavity. All tissue was either immediately fixed in picric-acid formaldehyde fixative (PAF) or the tissue was cultured for 3 or 24 hr and then

placed into fixative. The PAF consisted of 2% paraformaldehyde, 15% saturated picric acid and 0.15 M phosphate buffer at 4°C, (Stefanini *et al.*, 1967). After 3 hr, the tissue was rinsed twice in 0.1 M phosphate-buffered saline containing 0.3% Triton X-100 (PBS-Tx, pH=7.8). After the second rinse, the tissues remained in PBS-Tx overnight at 4°C. The next day the nasal mucosa were frozen on cork in isopentane cooled by liquid nitrogen and stored in airtight plastic bags at –80°C. The nasal epithelium was sectioned on a cryostat at 12 μm. One coverslip containing 16 sections randomly taken throughout the piece of mucosa was used to measure fluorescence intensity of NGF immunoreactivity.

Tissue Culture Following removal from the animal, the nasal mucosa was rinsed in PBS and placed into 60 mm x 15 mm tissue culture treated cell culture dishes (Gibco, Grand Island, NY) containing 3 ml of serum-free tissue culture media comprised of CMRL media (10x), penicillin G and streptomycin (100 μg/ml; Gibco, Grand Island, NY), hydrocortisone hemisuccinate (0.1 μg/ml), bovine recrystalized insulin (1.0 μg/ml), amphotericin B (10 μg/ml), sodium bicarbonate (3.71 mg/ml) and tissue culture grade H_2O (Sigma, St. Louis , MO). The culture dishes were placed into a controlled atmosphere culture chamber and gassed with 45% O_2-5% CO_2-50% N_2. The culture box was placed on a rocker and incubated at 37 ˚C (Dey *et al*., 1999; Wu *et al.*, 2001). After 3 or 24 hr, the culture dishes were removed from the culture chamber. The tissue was fixed and frozen for immunocytochemistry and the 3 ml of culture media was recovered, centrifuged at 1,500 rpm (352 rgf) for 5 min and the supernatant was aliquoted and frozen at -80°C for NGF assay.

Immunocytochemistry Procedures for immunocytochemistry were previously described (Dey *et al.*, 1990). Cryostat sections on gelatin-coated coverslips were covered with rabbit anti-NGF (1:75; Chemicon, Temecula, CA) primary antiserum diluted in PBS-Tx + 1% bovine serum albumin (PBS-Tx-BSA, pH 7.8). The coverslips were incubated in a humid chamber at 37 °C for 30 min, rinsed 3 times with PBS-Tx-BSA, allowing 5 min per rinse and then covered with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin IgG (ICN Pharmaceuticals, Inc., Costa Mesa, CA) diluted 1:100 in PBS-Tx-BSA and incubated at 37 °C for 30 min. The coverslips were rinsed 3 times for 5 min increments in PBS-Tx-BSA and mounted on glass slides in Fluoromount (Southern Biotechnology, Birmingham, AL).

NGF Immunoreactivity (NGF-IR) Measurements The sections were observed using an Olympus AX70 fluorescence microscope equipped with fluorescein (excitation 495 nm and emission 520 nm) following immunocytochemistry. Analysis of NGF-IR was evaluated by converting the intensity of the fluorescence in the nasal epithelium to a mean gray value (MGV) using Optimus, version 6.5, image analysis software (Media Cybernetics, L.P., Silver Spring, MD). The system was calibrated using an InSpeck Green (505/515) Microscope Image Intensity Calibration Kit (Molecular Probes, Eugene, OR). Once the epithelial layer was identified using the fluorescein filter, a black and white image was captured with a SPOT digital camera (Diagnostic Instruments Inc, Sterling Heights, MI), and the perimeter of epithelium was traced. An MGV was calculated for the epithelium using Optimus software. For each animal, at least 16 MGV measurements were made and the average MGV was calculated for the TDI and ethyl acetate groups.

RNA Isolation Total RNA was isolated from the nasal mucosa using the RNAqueous-4PCR kit (Ambion, Austin, Tx). DNA-free RNA was isolated according to themanufacturer's instructions. Tissue was removed from –80 °C, allowed to thaw on ice and removed from RNAlater solution. The tissue was ground with a mortar and pestle in liquid N_2. The finely ground tissue was transferred to a 1.5 ml microcentrifuge tube containing 500 µl of lysis binding solution. The mixture was vortexed and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Fluka, Buchs, Switzerland) was added to remove lipids. The solution was vortexed for 2 min followed by centrifugation (12,000 RPM) for 10 minutes. The aqueous layer was removed and transferred to a fresh tube containing 500 µl of 64% EtOH. The solution was applied to a silica-based filter, which selectively binds mRNA. The filter was washed 3 times to remove residual DNA, protein and other contaminates. The RNA was eluted in a nuclease-free water/EDTA solution. The RNA was then incubated with DNase I at 37 °C for 30 minutes to remove trace amounts of DNA. The concentration of RNA was measured on a spectrophotometer (Bausch & Lomb Spectronic 1001; Fisher Scientific, Pittsburgh, PA). Purified RNA was stored at –80 °C in 5 µl aliquots for RT-PCR

RT-PCR One step real-time PCR was used to quantitate relative changes in NGF expression in the nasal mucosa of TDI and EA exposed rats sacrificed at 3 and 24 hr time points. The NGF sense and antisense primers used were CCAAAGGGAGCAGCTTTCTATCCTG and GGCAGTGTCAAGGGAATGCTGAAGT, respectively, which recognize sequences near the 5' end of the cDNA and amplify a 128-bp fragment (Promega Corp., Madison, WI). The RT-PCR conditions were as follows:

Cycle	Repeats	Time	Temperature
1. Reverse Transcription	0	60 min	50 ºC
2. Activation of Taq	0	15 min	95 ºC
3.	40		
Step 1: Denature		15 sec	94 ºC
Step 2: Annealing		30 sec	55 ºC
Step 3: Extension		30 sec	72 ºC (data collection)
4. Melt Curve	80	30 sec (each repeat, temp. decreased by 0.5ºC)	start 95ºC

RT-PCR reactions were set up using the QuantiTect SYBR Green one-step RT-PCR kit (Quiagen, Valencia, CA) according to manufacturers instructions in 96 well plates. Each reaction contained 10 µl of 2x QuantiTect SYBR Green RT-PCR master mix, 100 ng/ml of RNA, 0.2µl QuantiTect RT mix, 0.5 µM forward (5'-3') primer and 0.5 µM reverse (3'-5') primer and Rnase-free water in a total volume of 20 µl and each sample was run in triplicate. Beta-actin was also amplified under the same conditions and used to normalize reactions. Sense and antisense primers to the beta-actin gene were TCATGAAGTGTGACGTTGACATCCGT and CTTAGAAGCATTTGCGGTGCACGATG , respectively which recognize sequences near the 5' end of the cDNA and amplify a 285-bp fragment (Promega Corp., Madison, WI).

An icycler (Biorad, Hercules, CA), was used to perform the RT-PCR cycles and fluorescence was quantified as a threshold cycle (Ct) value with Biorad icycler software. NGF expression levels were normalized using beta-actin expression levels as an internal control. The differences between the mean Ct values of the gene of interest and the housekeeping gene were denoted (delta-Ct) and the difference between TDI delta-Ct and

the EA delta-Ct was labeled delta-delta-Ct. The $\log_2$(delta-delta-Ct) gave the relative quantitation value of NGF expression in the TDI exposed group with the ethyl acetate group expression designated as one. Control wells containing SYBR Green PCR master mix and primers without sample reverse transcriptase emitted no fluorescence after 40 cycles.

NGF ELISA The nasal lavage supernatant samples (initial 3 ml) and culture media samples (3 ml) were frozen at –80 °C. The concentration of NGF (7.8-500 pg/ml) in each sample was assayed using the NGF Emax ImmunoAssay System (Promega, Madison, WI) according to the manufacturer's instructions. NGF was detected using an antibody sandwich format in 96 well plates. Each well was initially coated with 100 µl of anti-NGF pAb and incubated overnight followed by a one-hour incubation with blocking buffer (200 µl/well) to prevent nonspecific binding. Either 100 µl of lavage supernatant of 100 µl of NGF standard (7.8-500 pg/ml) was added to each well. The plate was incubated for six hours followed by an overnight incubation with anti-NGF mAb (100 µl /well). For color development an anti-rat IgG HRP conjugate antibody was added to each well (100 µl) followed by a TMB solution, which reacts with the peroxidase-labeled conjugates to develop a blue color. The absorbance of each well was measured at 450 nm on a Spectra Max 340pc plate reader (Molecular Devices, Sunnyvale, CA). The concentration of NGF in each lavage sample was extracted from an NGF standard curve. All samples were run in duplicate or triplicate, and as a negative control, a PBS sample was run with each assay.

Protein Assays The nasal lavage supernatant samples were also assayed for protein concentration. The bicinchroninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL) is a modified Lowry assay and measures the concentration of total protein (20-2,000 μg/ml). The assay was performed according to the manufacturer's instructions using the microplate procedure. The assay was performed in 96 well plates and 20 μl of unknown or bovine serum albumin standard was added to each well followed by 200 μl of a working reagent. The plate was incubated at 37 ˚C for 30 min before reading the absorbance at 562 nm on a Spectra Max 340pc plate reader (Molecular Devices, Sunnyvale, CA). The concentration of total protein in each lavage sample was derived from a bovine serum albumin standard curve. All samples were run in duplicate or triplicate.

Statistical Analysis The means and standard errors were calculated for each endpoint measured and a one-way or two-way ANOVA with a Tukey post hoc test was performed. Significance was set at $p \leq 0.05$ for each endpoint measured.

RESULTS

Inflammatory Markers in the Nasal Lavage Fluid 15 Minutes after TDI

The number of neutrophils in the nasal lavage fluid was not significantly increased 15 min after intranasal TDI exposure (850±405) compared to controls (950±350; Figure 41). The concentration of total protein was significantly increased in the nasal lavage fluid from 102.41±24.17 μg/ml in the ethyl acetate controls to 205.72±26.97 μg/ml in the TDI-exposed animals (Figure 42). These data indicate that

nasal instillation of TDI causes plasma extravasation but not neutrophil accumulation after 15 min.

NGF in the Nasal Lavage Fluid and NGF Immunoreactivity (NGF-IR) in the Nasal Epithelium 15 Minutes after TDI

The concentration of NGF increased significantly in the nasal lavage fluid 15 minutes following intranasal TDI exposure compared to controls (137.72+21.52 and 44.77+8.64 pg/ml, respectively; Figure 43). There was not a significant difference in NGF-IR in the nasal epithelium 15 minutes following TDI exposure (56.35±4.39) compared to ethyl acetate controls (48.66±2.77; Figure 44). These data suggest that NGF is released into the nasal cavity immediately following TDI but not from the nasal epithelium.

NGF in the Culture Media and NGF-IR in the Nasal Epithelium 3 and 24 hr after Culture

The structural integrity of the nasal mucosa is maintained after 24 hr in culture and is illustrated in Figure 45. The concentration of NGF (pg/ml) was significantly increased after 3 hr in the culture media of nasal mucosa exposed to TDI *in vivo* compared to controls (332.65±62.91 and 134.02±7.17 respectively; Figure 46). There was no significant difference in NGF concentration in the culture media of TDI (449.84±32.71 pg/ml) and ethyl acetate (412.39±22.43 pg/ml) exposed nasal mucosa after 24 hr. NGF-IR in the nasal epithelium did not differ between control and TDI

exposed tissue cultured for 3 hr (50.85±1.90 and 48.18±3.15 respectively) or 24 hr (68.69±0.29 and 57.39±1.89 respectively; Figures 47 and 48).

Relative Expression of NGF mRNA in the Nasal Mucosa 3 and 24hr following TDI

The relative expression of NGF mRNA in the nasal mucosa of rats exposed to TDI and sacrificed after 3 and 24 hr was not significantly different from control animals (Figure 49). The expression of NGF mRNA in the nasal mucosa was almost identical to control animals 3 hr following TDI (1.01). Twenty-four hours following TDI the expression of NGF mRNA (0.61) appeared to decrease relative to controls but the change was not significant.

DISCUSSION

Previous *in vitro* studies have identified human pulmonary fibroblasts (Olgart & Frossard, 2001), human airway smooth muscle cells (Freund *et al.*, 2002) and human lung epithelial A549 cells derived from alveolar type II cells (Pons *et al*., 2001; Fox *et al.*, 2001) as sources of NGF. The present study provides evidence that the rat nasal mucosa is also capable of synthesizing and secreting NGF and that secretion is increased following irritant exposure.

TDI is a highly reactive industrial chemical and the leading cause of occupational asthma (Karol & Jin, 1991; Mapp *et al.*, 1988). In exposed rats, TDI activates sensory nerves in the nasal epithelium leading to increased synthesis and release of the neuropeptide SP (Hunter *et al.*, 2000b). In addition, inflammatory markers and NGF are also elevated in the nasal lavage fluid following TDI (Sikora *et al.*, 2001). Evidence

strongly suggests that NGF binds to trkA receptors located on sensory nerve terminals (McMahon, 1996) and the trkA/NGF complex is retrogradely transported to the cell body (Greene & Tischler 1976; Sandow *et al.*, 2000) where NGF mediated-kinase-signaling pathways are believed to up-regulate the transcription and translation of neuropeptides (Stephens *et al.*, 1994).

In previous studies (Study 2), NGF, inflammatory markers and neuronal changes were first measured 2 hr following TDI. Only NGF was elevated at that time point. In the present study, significant increases in NGF and total protein were detected in the nasal lavage fluid 15 minutes following TDI exposure. Potential sources of NGF include resident cells of the nasal mucosa and migratory inflammatory cells. Since neutrophils, which represent inflammatory cell influx were not elevated 15 minutes following TDI, the source of NGF immediately following TDI is likely to reside in the nasal mucosa. Epithelial cells, which line the entire nasal mucosa, are considered a primary source of NGF since previous studies found that TDI binds to the ciliary tubulin of human pulmonary epithelial cells (Lange *et al.*, 1999) and epithelial cells release NGF (Fox *et al.*, 1997; Fox *et al.*, 2001). Changes in the NGF immunoreactivity (NGF-IR) were measured in the nasal epithelium. Although the nasal epithelium is immunoreactive for NGF, no decrease in NGF-IR was detected 15 minutes following TDI.

To confirm that the nasal mucosa was a source of NGF, a combined *in vivo* and *ex vivo* approach was used. The nasal mucosa was removed and placed into culture 15 minutes following TDI exposure. Lymphocytes (Barouch *et al.*, 2000), eosinophils (Solomon *et al.*, 1998) and macrophages (Braun *et al.*, 1998) synthesize and release NGF but significant levels of inflammatory cells were not detected in the nasal lavage fluid 15

minutes after TDI; therefore, the NGF detected in the culture media was released from resident cells of the nasal mucosa. After 3hr in culture, the TDI-exposed nasal mucosa released significantly more NGF than the control tissue. Possible cellular sources of NGF include epithelial cells (Fox *et al.*, 1997; Fox *et al.*, 2001) and mast cells (Leon *et al.*, 1994), which are present in the nasal mucosa and have previously been shown to release NGF.

After 24 hr in culture, the amount of NGF released from the TDI-exposed nasal mucosa continued to increase but a comparable amount of NGF was also released from the control tissue. The largest increase in NGF in the nasal lavage fluid in previous studies occurred 24 hr after TDI exposure. In this study, the majority of NGF appears to be released after 3 hr; therefore inflammatory cells migrating into the nasal mucosa may contribute to the large amounts of NGF observed at 24 hr *in vivo*. The culture conditions may have also played a role. Previous studies have successfully maintained rat nasal muocsa in culture for up to 14 days (Fanucchi *et al.*, 1999). In this study, rat nasal mucosa was cultured for just 24 hr, but serum-free media was used due to the measurement of growth factors. Although the nasal epithelium and submucosa remained intact and the cilia continued to beat, the culture conditions may have stressed the tissue contributing to the release of NGF from control tissue after 24 hr.

NGF-IR in the epithelial layer of the nasal mucosa was also measured following a 3 and 24 hr culture. NGF was detected in the epithelial layer after 3 and 24 hr; however, no NGF-IR differences, which would indicate NGF release or increased NGF synthesis were detected between irritant exposed and control tissue. Rapid turnover of NGF

within the cells and sensitivity limitations with the immunocytochemistry technique may also explain why no NGF-IR differences were observed in the epithelial layer.

Relative NGF mRNA expression was also measured in the nasal mucosa obtained from animals sacrificed 3 and 24 hr following TDI or ethyl acetate exposure. The nasal mucosa expresses NGF mRNA but the expression remains unchanged 3 and 24 hr following TDI exposure. Similar to these results, Sanico *et al*., (2000) reported increased NGF concentrations in the nasal lavages of rhinitis patients but not increased NGF mRNA levels in superficial nasal scrapings.

In conclusion, these studies indicate that the nasal mucosa is capable of synthesizing and releasing NGF and that NGF release is increased following irritant exposure. Based on these results, the epithelium does not appear to be the primary source of NGF. Further studies are needed to identify the specific NGF-producing cells in the nasal mucosa and the mechanisms controlling NGF release following irritant exposure. This is important since NGF released from the nasal mucosa may up-regulate SP expression and SP contributes to airway inflammation.

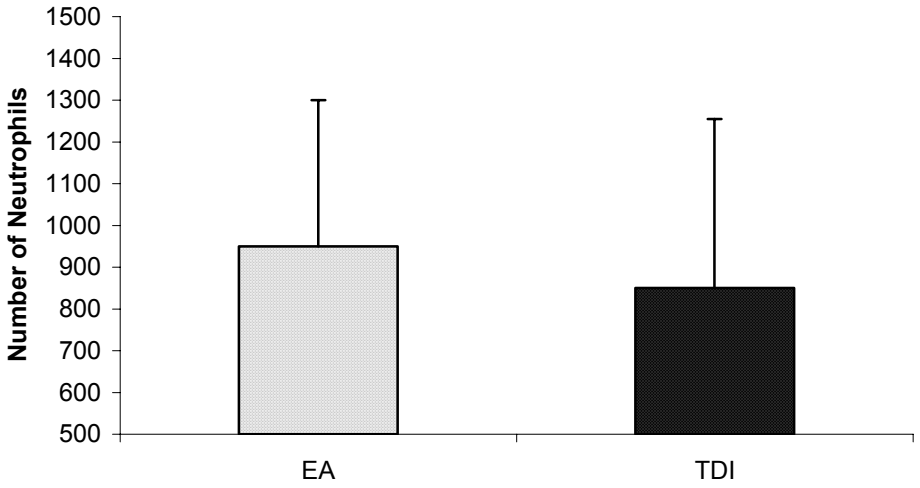

Figure 41 The number of neutrophils in the nasal lavage fluid of Sprague-Dawley rats 15 minutes following intranasal instillation of 10% TDI (n=4) or ethyl acetate (control vehicle; n=4). No significant changes were detected ($p<0.05$).

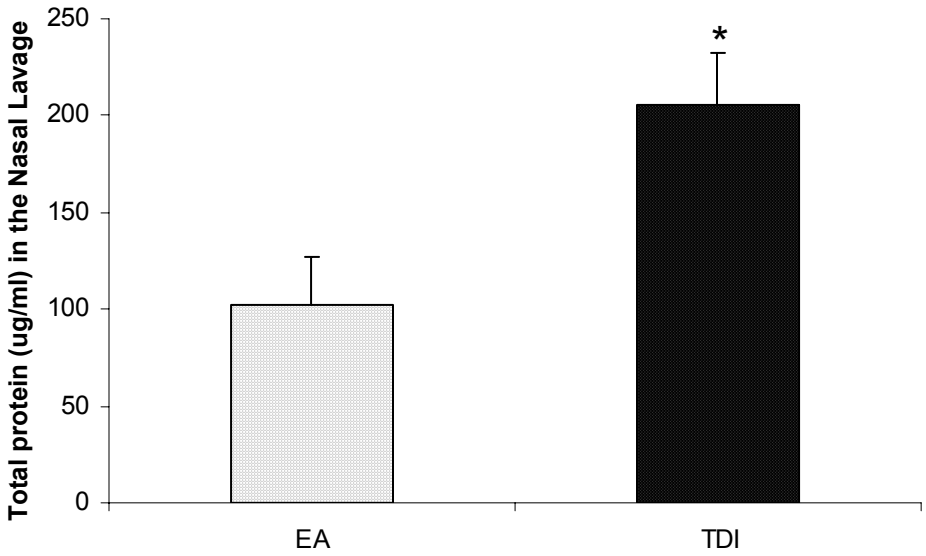

Figure 42 The concentration of total protein (μg/ml) in the nasal lavage fluid of Sprague-Dawley rats 15 minutes following intranasal instillation of 10% TDI (n=6) or ethyl acetate (control vehicle; n=6). The asterisk denotes significant TDI-induced change relative to controls ($p \leq 0.05$).

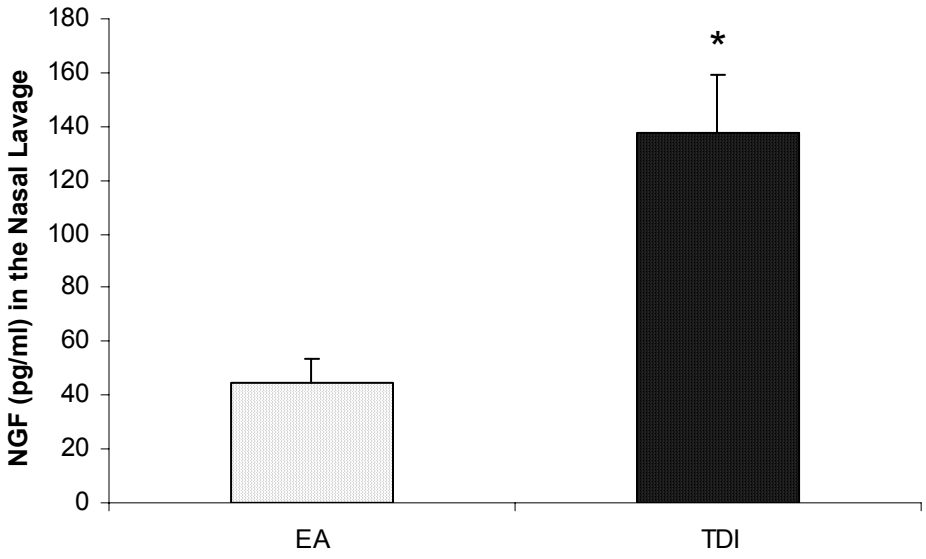

Figure 43 The concentration of NGF (pg/ml) in the nasal lavage fluid of Sprague-Dawley rats 15 minutes following intranasal instillation of 10% TDI (n=6) or ethyl acetate (control vehicle; n=6). The asterisk denotes significant TDI-induced change relative to controls ($p \leq 0.05$).

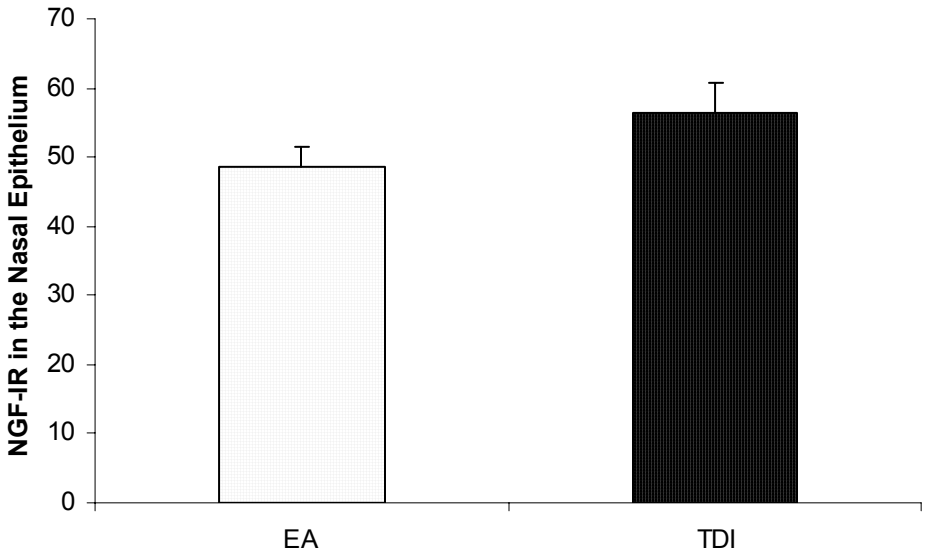

Figure 44 Immunoreactivity for NGF in the nasal epithelium of Sprague-Dawley rats intranasally instilled with either 10% TDI or ethyl acetate (control vehicle) and sacrificed after 15 minutes (n=6). No significant changes were detected ($p \leq 0.05$).

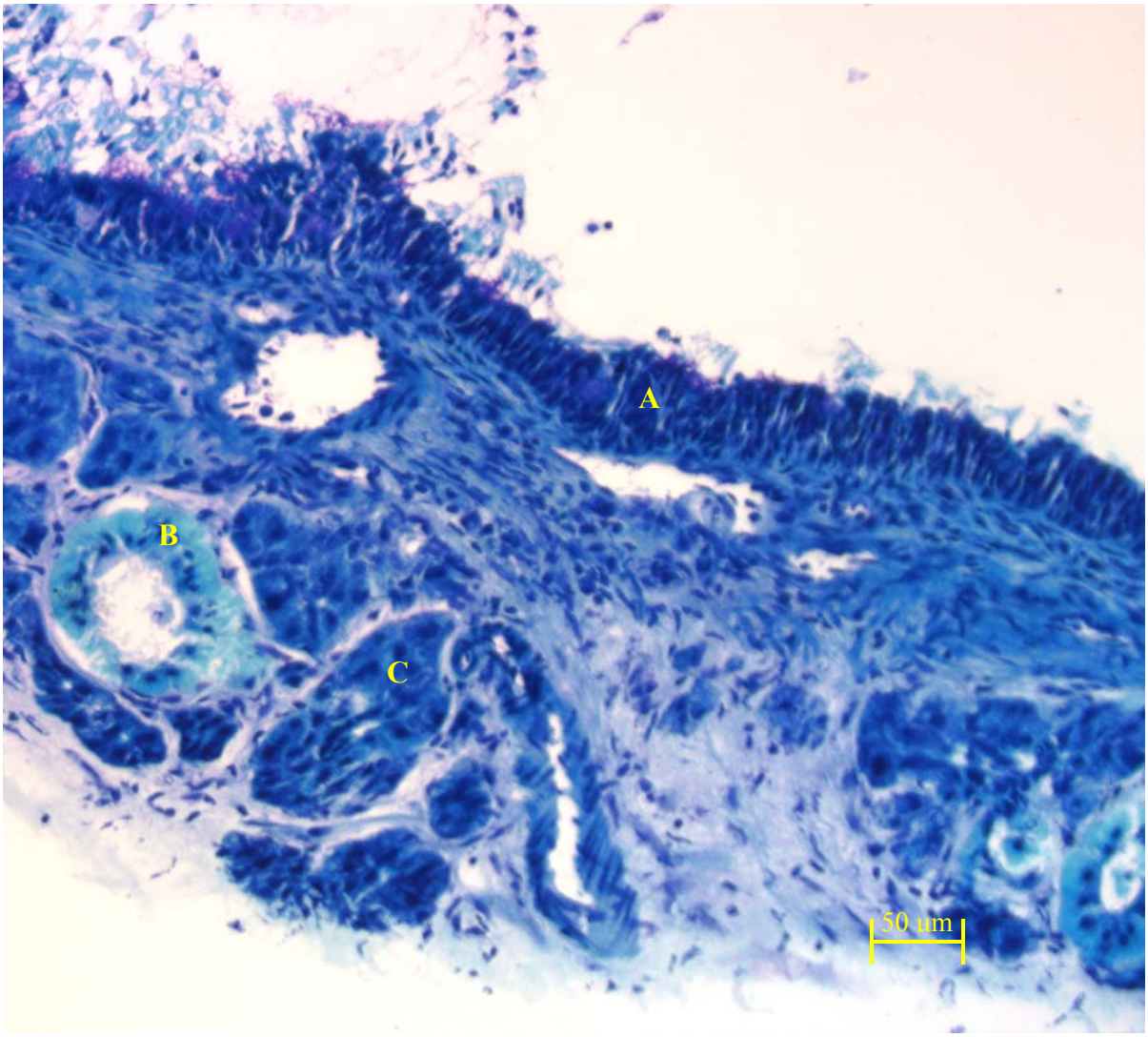

Figure 45 Image of rat nasal mucosa stained with toluidine blue illustrating the general structure after culture. Animals were instilled with TDI and the nasal mucosa was removed after 15 minutes and placed into culture for 24 hr. After culture, the tissue was fixed, frozen, sectioned and stained with toluidine blue. These images show that the epithelium (A), blood vessels (B) and mucous glands (C) remain intact after 24 hr in culture.

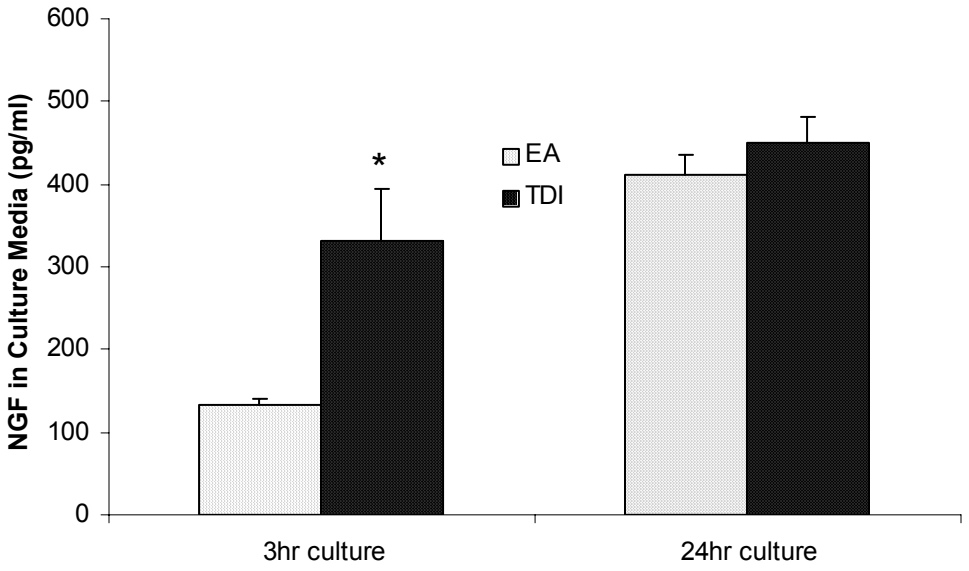

Figure 46 The concentration of NGF (pg/ml) released into the culture media from nasal mucosa cultured for either 3hr or 24hr. The nasal mucosa was removed from Sprague-Dawley rats 15 minutes following intranasal instillation of 10% TDI or ethyl acetate (control vehicle)(n=6). Asterick denotes significant TDI-induced change relative to controls ($p \leq 0.05$).

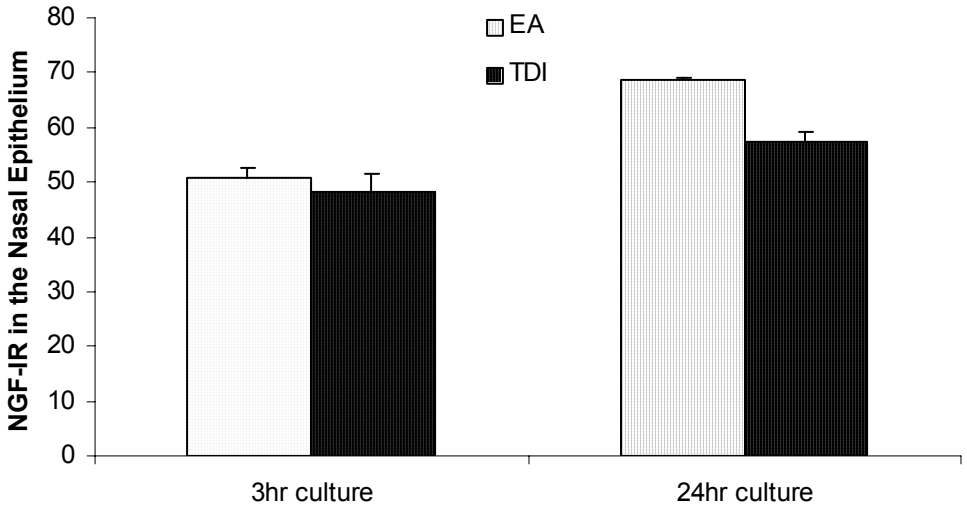

Figure 47 The immunoreactivity of NGF in the epithelial layer of nasal mucosa cultured for either 3 hr or 24 hr. The nasal mucosa was removed from Sprague-Dawley rats 15 minutes following intranasal instillation of 10% TDI or ethyl acetate (control vehicle)(n=4). No significant changes were detected ($p \leq 0.05$).

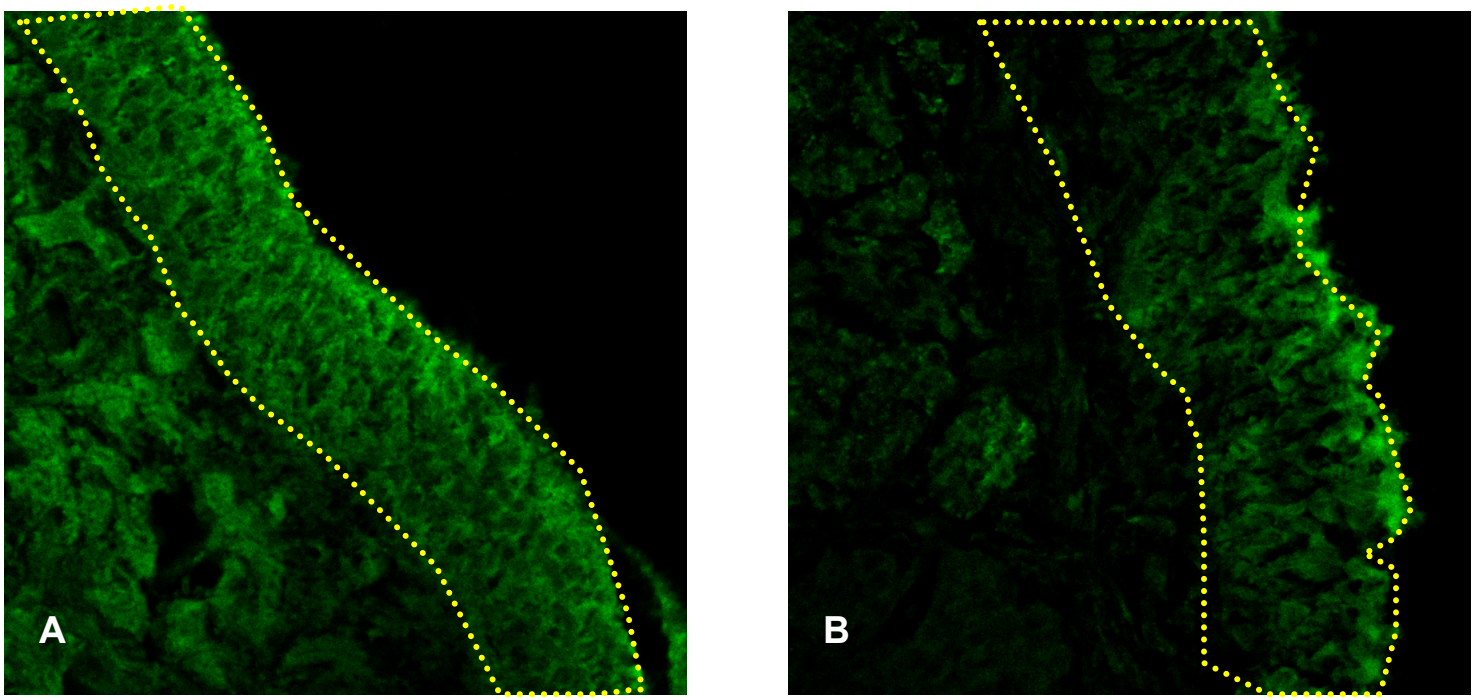

Figure 48 Images of rat nasal mucosa showing NGF-IR in nasal epithelium (yellow outline). Animals were instilled with either EA (image A) or TDI (image B) and the nasal mucosa was removed after 15 minutes. The nasal mucosa was placed into culture for 24 hr and then processed for immunocytochemistry. Although less NGF-IR is present 24 hr after culture in the epithelium obtained from the rats exposed to TDI (B) compared to EA (A), the difference was not significant.

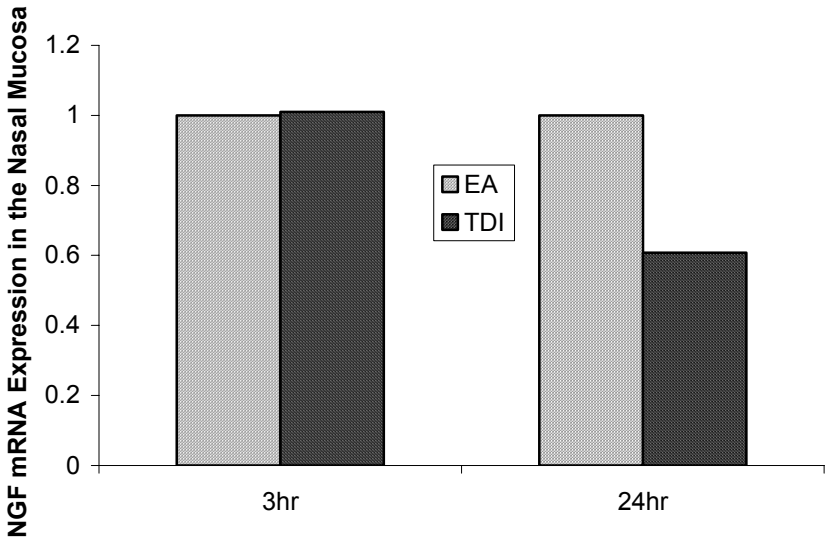

Figure 49 The relative expression of NGF mRNA in the nasal mucosa 3 and 24 hr following intranasal instillation of 10% TDI or ethyl acetate (control vehicle; n=3). No significant changes were detected.

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GENERAL DISCUSSION

Inhaled gases, vapors or particles found in various occupational and environmental settings can act as irritants to the upper respiratory system (Brooks, 1995). The responses to irritant inhalation are mediated in part by sensory and autonomic nerve fibers in the airways (Barnes, 1986). Irritants known to activate sensory nerves in the nasal mucosa include cigarette smoke, acrolein, histamine, sulfur dioxide and ozone (Lundberg & Saria, 1983; Morris *et al*., 1999b; Turner *et al.*, 1993). Activation of sensory nerves produces neurogenic inflammation characterized by vasodilation, plasma extravasation, mucous secretion and neutrophil chemotaxis (Lundberg & Saria, 1983; Lundberg *et al.*, 1991).

Neurogenic inflammation is mediated by the release of neuropeptides, especially substance P (SP), from sensory nerve endings throughout the airway walls (Baluk *et al*., 1992; Lundberg *et al.*, 1983). SP is synthesized in cell bodies housed in the trigeminal ganglia (TG) (Hunter & Dey, 1998) and released from sensory nerve endings found near blood vessels, mucous glands and epithelium (Baraniuk *et al*., 1991; Lundberg *et al.*, 1984b). Increases in SP protein and message have been demonstrated in sensory cell bodies innervating respiratory epithelium of the nose following exposure to TDI (Hunter *et al.*, 2000a) and silica (Hunter *et al.*, 1998), which are both occupational irritants. The activation of sensory nerves, up-regulation of SP and subsequent inflammatory response may all contribute to the symptoms of rhinitis and nasal irritation commonly reported following exposure to either irritant (Brugsch & Elkin, 1963; Chan-Yeung *et al.*, 1978).

Nasal irritation to asphalt fumes is also a frequently reported symptom among asphalt paving workers (Hanley & Miller, 1996). Therefore, the initial study examined

the effect of asphalt fume inhalation on the synthesis and release of neuropeptides from TG neurons innervating the nasal epithelium. Rats exposed to asphalt fumes at 16.0±8.1 mg/m3 for 3.5 hours a day for 5 consecutive days had increased SP immunoreactivity (SP-IR) and CGRP-IR in TG neurons innervating the nasal epithelium. Increases in SP-IR are attributed to increased SP synthesis (Hunter *et al.*, 2000a), and following synthesis, neuropeptides undergo anterograde transport to corresponding sensory nerve terminals (Buck *et al.*, 1999). Neuropeptides are involved in chemotactic responses during neurogenic inflammation; thus, SP may contribute to the influx of neutrophils and macrophages into the asphalt-exposed nasal cavities. Asphalt fumes can be added to a growing list of chemicals known to stimulate sensory nerves in the nasal cavity. The enhanced neuropeptide production may produce neurogenic inflammation and manifest as symptoms of nasal irritation in road construction workers exposed to asphalt fumes.

Since occupational exposure to airway irritants has health implications that may be related to increased neuropeptide production, the remaining studies investigated the mechanisms regulating SP production. Nerve growth factor (NGF), a neurotrophin released from inflamed tissues, may be a key mediator in the up-regulation of SP levels in sensory neurons (Nicholas *et al.*, 1999). NGF is expressed in non-neuronal cells associated with the process of inflammation including airway epithelial cell lines (Fox *et al.*, 2001), mast cells (Leon *et al.*, 1994) and lymphocytes (Santambrogio *et al.*, 1994). Upon release, NGF binds trkA receptors on sensory nerve terminals (McMahon *et al.*, 1994), undergoes axonal transport (Sandow *et al.*, 2000) and up-regulates neuropeptide production in neuronal cell bodies (Lindsay & Harmar, 1989).

Several studies suggest that NGF may play a role in sensory-neuronal responses during airway inflammation. Increased levels of NGF have been demonstrated in the bronchoalveolar lavage fluid of asthmatics (Olgart *et al.*, 2002) and in the nasal lavage fluid of allergic rhinitis patients (Sanico *et al.*, 2000). Increases in NGF have also been measured in the lungs of rats infected with respiratory syncytial virus (Hu *et al.*, 2002), and in mice with allergic airway inflammation (Braun *et al.*, 1998). Tracheal instillations of NGF produces increased SP expression in neurons of the nodose and jugular ganglia innervating the guinea-pig airway (Hunter *et al.*, 2000b) and airway hyperresponsiveness through activation of SP-selective NK-1 receptors in rats (de Vries *et al.*, 1999).

Since previous studies suggested that NGF may play a role in sensory-neuronal responses during airway inflammation, the next two studies focused on determining if NGF levels increase in the nasal cavity following irritant exposures and if NGF mediates increased SP mRNA expression in airway sensory neurons. The occupational irritant TDI was used for these studies because increases in SP synthesis and release after one exposure are well documented (Hunter *et al.*, 2000a). In this study, rats were intranasally instilled with either 10% TDI or ethyl acetate (control vehicle) and sacrificed at one of six time points ranging from 2 to 96 hr post-exposure. The results demonstrated a temporal relationship between NGF production in the nasal cavity, up-regulation of SP in TG neurons and the appearance of inflammatory markers in the nasal lavage fluid. This was the first study of which we are aware to demonstrate that NGF levels significantly increased in the nasal cavity following irritant exposure. Increases in NGF in the nasal lavage fluid occurred hours prior to observed increases in SP levels in epithelial nerve endings and TG neurons and inflammatory markers. NGF reportedly

binds to trkA receptors located on sensory nerve terminals (McMahon *et al.*, 1994) and undergoes retrograde transport to corresponding cell bodies (Wheeler *et al.*, 1998). The apparent latency between the increase in NGF in the nasal cavity at 2 hr, and the increase in SP-IR cell bodies at 24 hr, is attributable, at least in part, to the processes of receptor binding and transport (Figure 50). Although these studies did not specifically study the retrograde transport of NGF, the increase in NGF immunoreactivity in TG cell bodies 24 hr after TDI also supports the process of receptor binding and transport. The increase in SP-IR in TG neurons innervating the nasal epithelium 24 and 48 hr following TDI exposure have been demonstrated previously and is attributed to increased SP synthesis (Hunter *et al.*, 2000a). In addition to neuropeptides, increases were detected in glutamate and NGF receptor immunoreactivity. Glutamate release in the lung promotes lung injury and thus may have important pathological consequences (Said *et al.*, 1996). Therefore, neuropeptide up-regulation may not be the only consequence of airway sensory nerve activation. This also suggests that NGF may regulate the production of various proteins in TG cell bodies.

The purpose of the next study was to determine if NGF mediates the increased SP expression in airway sensory neurons following irritant exposure. Previous studies successfully employed NGF antiserum to directly correlate NGF and neuropeptide levels. Daily injections of sheep anti-NGF caused a significant decrease in the SP and CGRP content of dorsal root ganglion neurons after seven days. The changes were attributed to a decreased availability of endogenous NGF (Shadiack *et al.*, 2001). In order to assess the contribution of NGF to neuropeptide up-regulation and airway inflammation following TDI, rats were pre-treated intranasally with rabbit anti-NGF antibody or

intraparitoneally with sheep anti-NGF antibody to inactivate NGF (Figure 51). However, pretreatment with either NGF antiserum failed to confirm or refute NGF's role as a modulator of SP regulation. Both antibodies failed to prevent the increase in intraepithelial SP nerve fiber density and influx of neutrophils following TDI exposure. However, significant unexpected increases in SP NFD were observed in the ethyl acetate control rats pre-treated with either rabbit anti-NGF or sheep anti-NGF antibodies, which suggests that the presence of the antibodies caused an adverse reaction, at least in neurons. Previous airway studies successfully blocked NGF using either intranasally instilled NGF antiserum to prevent neuronally mediated airway hyperreactivity in allergen-sensitized mice (Braun *et al.*, 1998) or intraparitoneally instilled NGF antiserum to reduce NK-1 receptor expression and capsaicin stimulated plasma extravasation in rats infected with respiratory syncytial virus (Hu *et al.*, 2002). While both studies demonstrated a role for NGF in the neuronal responses during airway inflammation, neither study monitored changes at the level of the sensory nerves. Possible technical differences between these studies and the current study include differences in antibody treatment schedules and the source of the antibody. Unfortunately, NGF antiserum did not prove to be an effective method of manipulating NGF activity in the upper airway following irritant exposure in the present study.

A second method of blocking NGF activity included the use of K252a, a non-specific tyrosine-kinase inhibitor (Figure 51). Pre-treatment with K252a reduced the TDI-induced increases in SP innervation in the nasal mucosa, SP-positive TG cell bodies projecting to the nasal epithelium and inflammation in the nasal cavity. However, there are a number of limitations regarding the lack of specificity of K252a. In addition to

inhibiting the phosphorylation of trkA receptors, K252a also inhibits trkB and trkC receptors (Ohmichi *et al.*, 1992) and protein kinase C and calmodulin (Ohmori *et al.*, 1988). The observed changes in sensory nerves after TDI exposure suggest, but do not confirm, involvement of tyrosine kinase-coupled receptors. Since selective antagonists for the trkA receptor are not currently available, and NGF antiserum elicited an inflammatory response, K252a provided the only data supporting the concept that irritant-induced SP expression in sensory neurons is mediated by NGF.

In order to understand better the relationship between NGF, SP and airway inflammation, the remaining studies investigated the cellular source of the NGF in the upper airway. NGF has previously been detected in various inflammatory and non-inflammatory cells. The early rise in NGF levels prior to inflammatory cell influx suggests that the nasal mucosa was a possible source of NGF following TDI exposure. Previous studies detected mRNA for NGF in nasal mucosal scrapings (Sanico *et al.*, 2000) and NGF synthesis and release has been demonstrated in mast cells (Leon *et al.*, 1994) and alveolar epithelial cell lines (Fox *et al.*, 2001). Studies to indentify the source of NGF within the nasal mucosa have been complicated because inflammatory cells, which also release NGF, migrate into the nasal mucosa following TDI and obscure immunocytochemical identification of endogenous NGF sources. Two different experimental approaches were used to overcome this problem. In the first approach, rats received an intraparitoneal injection of cyclophosphamide three days before TDI or ethyl acetate exposure. The cyclophosphamide substantially depleted circulating leukocytes and allowed NGF release to be measured solely from the nasal mucosa after irritant exposure. Cyclophosphamide was used in previous studies to examine the effect of

peripheral leukocyte depletion on guinea pig pulmonary responses to cotton dust inhalation (Castranova *et al.*, 1988). In the current studies, cyclophosphamide pre-treatment affected NGF release in the nasal cavity 24 hr following TDI exposure. Significantly more NGF was present in the nasal lavage of cyclophosphamide-treated animals exposed to TDI compared to ethyl acetate controls but overall the levels, in both control and TDI treated animals were much lower than those reported in previous studies. The absence of NGF-releasing inflammatory cells, which normally migrate into the nasal mucosa post-irritant exposure may account for the depressed levels of NGF in TDI-exposed animals but not controls. A more likely explanation is that cyclophosphamide impaired the release of NGF from cells in the nasal mucosa. NGF mRNA was detected in the nasal mucosa but no difference in expression was detected in cyclophosphamide treated animals exposed to TDI or EA. Although these results demonstrate that cells in the nasal mucosa synthesize and release NGF, leukocyte depletion with cyclophosphamide is not an effective model for studying NGF release from the nasal mucosa following TDI.

The second approach used a combination of *in vivo* and *ex vivo* techniques to measure NGF release from the nasal mucosa following TDI exposure. In this study, the nasal mucosa was removed and placed into culture 15 minutes following TDI exposure. Significant numbers of inflammatory cells were not detected in the nasal lavage fluid 15 minutes after TDI exposure; therefore, the NGF detected in the culture media was presumed to be released from resident cells of the nasal mucosa. After 3 hr in culture, the TDI-exposed nasal mucosa released significantly more NGF than control tissue. NGF immunoreactivity (NGF-IR) in the epithelial layer of the TDI-exposed and cultured

nasal mucosa was unchanged compared to controls suggesting that epithelial cells were not the primary source of NGF. Relative NGF mRNA expression was measured in nasal mucosa removed from a rat 3 hr after TDI exposure. No increase in expression was detected. This observation is consistent with previous studies in rhinitis patients, which reported increased NGF concentrations in the nasal lavage but not in NGF mRNA expression in nasal scrapings (Sanico *et al.*, 2000). These studies clearly indicated that the nasal mucosa is capable of synthesizing and releasing NGF and that NGF release is increased following irritant exposure. However, the cellular source of NGF was not determined, although it did not appear to originate from the epithelium.

In conclusion, these studies demonstrated that neuropeptide production is increased in TG cell bodies innervating the nasal epithelium following asphalt exposure. Asphalt is one of many occupational irritants that have been shown to activate airway sensory nerves. These studies also provided insight on neuropeptide regulation following irritant exposure. Based on the temporal relationship between NGF production in the nasal cavity and up-regulation of SP in TG neurons and the inhibitory effects of the tyrosine kinase inhibitor, K252a, we have presented data supporting the concept that irritant-induced SP expression in sensory neurons may be mediated by NGF. Although the nasal mucosa releases NGF following TDI, the specific cell types involved are unknown. Identifying not only the specific cellular source of NGF but also the mechanisms controlling NGF synthesis will aid in the understanding of airway inflammation.

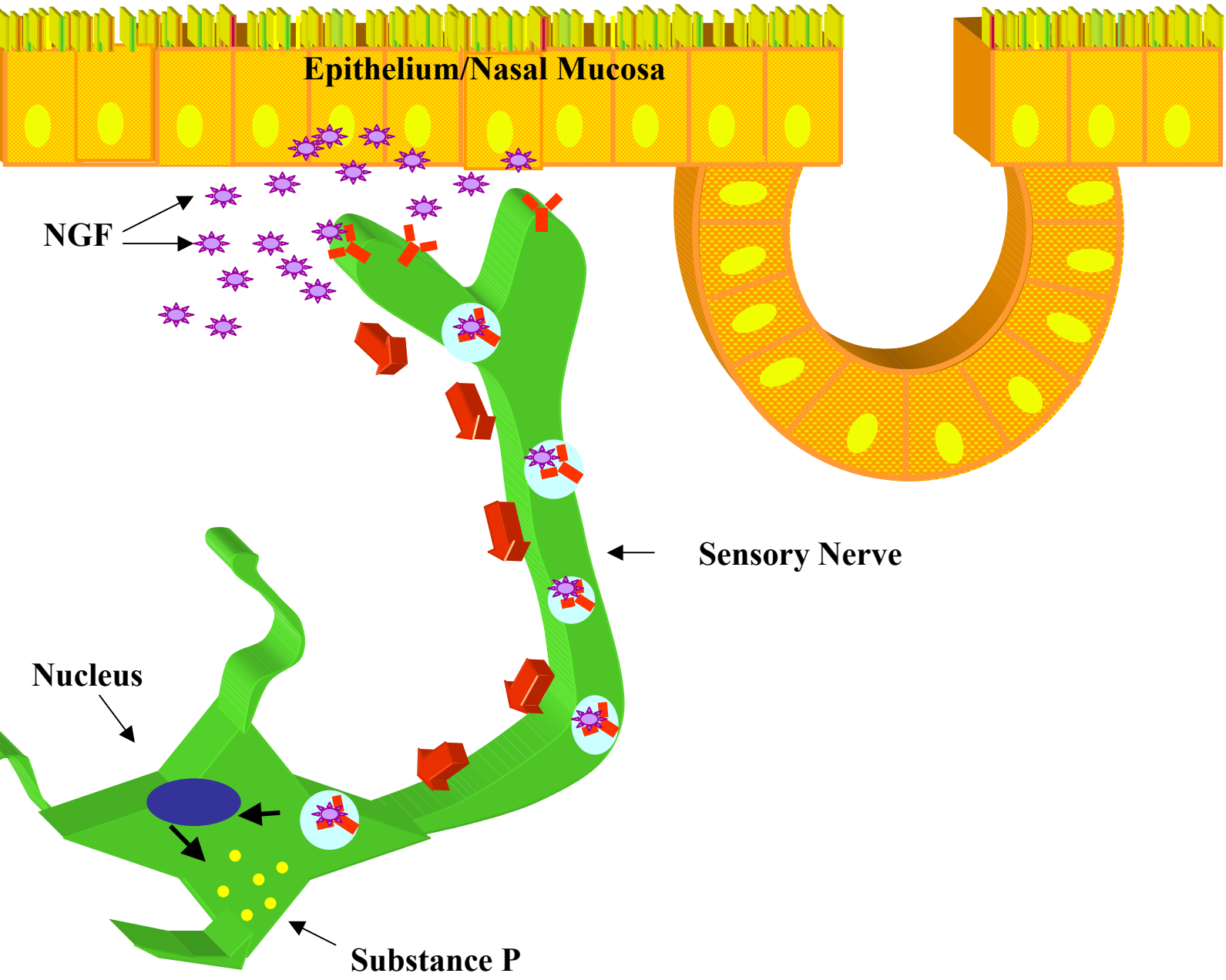


Figure 50 NGF released in the submucosa following TDI binds to trkA receptors on sensory nerve terminals. The NGF-trkA complex is endocytosed into a vesicle at the nerve terminal. The ligand-receptor complex is retrogradely transported to the cell body. At the cell body, the activated receptor signals the transcription and translation of SP.

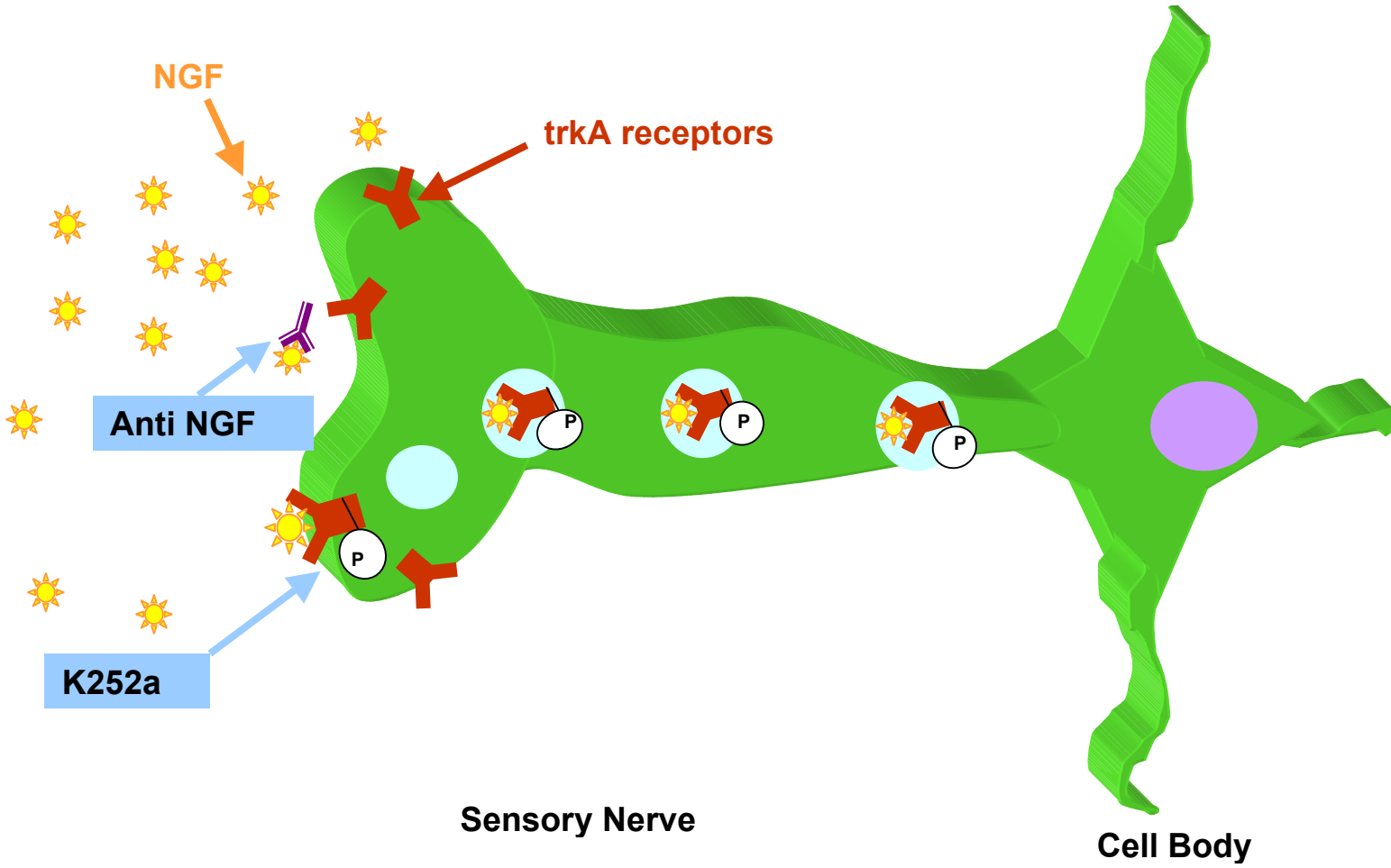


Figure 51 Binding of NGF to the extracellular domain of the trkA receptor located on the sensory nerve terminal autophosphorylates the tyrosine residues located in the C-terminal region of the receptor. The ligand-receptor complex is rapidly endocytosed into an organelle, which is transported to the cell body where kinase-signaling pathways are believed to up-regulate the transcription and translation of SP. NGF antiserum binds NGF and prevents NGF from binding trkA receptors while K252a blocks autophosphorylation of the trkA receptor.

APPENDIX II

The following section contains step-by step instructions for various methods used in the experiments mentioned in previous sections.

Tissue Culture Media Preparation

(for 500ml of media)

1. 50 ml of CMRL (10x) Medium (Gibco cat# 11540-010).
2. 25 ml Fetal Bovine Serum, 5% of total volume of media (Gibco, cat# 16140-063)

*[** do not add if you are assaying for growth factors, increase water by 25ml]*

3. 1ml of 1.85g/10ml of sodium bicarbonate in tissue grade H2O (Sigma, cat# S-5761).
4. 100 µl of 0.05g/ml Amphotericin B in DMSO (Sigma, cat# 16F40507)
5. 5 ml of 10 mg/ml Penicillin G (1000 units/ml) + Streptomycin (1000 units/ml) in tissue culture grade H2O (Gibco cat# 15145-014).
6. 10 µl of 0.05 g/ml Bovine recrystalized insulin in tissue culture grade H2O (Sigma cat# I1882).
7. 10 µl of 0.05g/10ml hydrocortisone hemisuccinate in EtOH (Sigma cat# 27F-8835).
8. 418.88 ml of tissue culture grade H2O (Sigma cat# W-3500)

Total Protein Assay

BCA Protein Assay (cat#23223, Pierce)

1. Use 96 well microtiter plates

2. Run each sample in duplicate or triplicate

3. To make up standards start with a stock solution of 1mg/ml BSA (use PBS no Tx) and follow table below:

Standard Final Conc.	**Volume of 1mg/ml Stock**	**Volume of PBS**
0 µg/ml	0 µl	400 µl
25 µg/ml	10 µl	390 µl
50 µg/ml	20 µl	380 µl
125 µg/ml	50 µl	350 µl
250 µg/ml	100 µl	300 µl
500 µg/ml	200 µl	200 µl
750 µg/ml	300 µl	100 µl
1,000 µg/ml	400 µl	0 µl

4. Gently vortex unknown samples and standards and add 25 µl to each well.

5. Make up working reagent by adding 50 parts of reagent A to 1 part reagent B (50:1). You need 20ml for each 96 well plate. Add 200 µl of working reagent to each well.

6. Mix on plate shaker for 5 minutes

7. Incubate for 30 minutes at 37 ˚C.

8. Read absorbance at 562 nm.

NGF ELISA NGF Emax Immunoassay System (cat# G7630, Promega)

1. Use Nunc MaxiSorp (Cat# 011-439454) 96 well plates
2. Add 10 µl of anti-NGF pAb to 10 ml of carbonate coating buffer (0.0210 g sodium bicarbonate, 0.0265g sodium carbonate, 10ml ddH2O, pH 9.7). Add 100 µl/well and cover and incubate at 4 ˚C overnight.
3. Wash plate once with TBST wash buffer (20mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.05% (v/v) Tween 20).
4. Add 200 µl of blocking buffer to each well (34.4 ml ddH20 and 8.6 ml 5x Block buffer). Incubate at room temperature for 1 hr.
5. Remove samples from -80 C and thaw on ice
6. Prepare dilutions for standard curve (7.8-500 pg/ml). Dilute NGF standard (1 µg/ml stock) 1:2,000 with 1x block buffer. Kit directions suggest performing serial dilutions in wells but it is difficult to mix solution in the wells without scratching bottom surface. Make dilutions in tubes and then transfer to wells.
7. Wash plate once and add either 100 µl of sample or standard to each well. Do not touch bottom of well with pipette tip. Run each sample and standard in duplicate or triplicate. Cover plate and incubate at room temperature for 6 hrs on a plate shaker.
8. Wash plate 5 times. Add 100 µl of anti-NGF mAb to each well (2.5 µl of antibody and 10 ml block buffer 1x). Cover and refrigerate overnight.
9. Wash plate 5 times. Add 100 µl of anti-rabbit IgG, HRP conjugate to each well. Incubate with shaking for 2.4hrs.
10. Remove TMB solution from 4 C and warm to room temperature and warm up plate reader.
11. Wash plate 5 times. Add 100 µl of TMB solution to each well. Incubate with shaking for 10 minutes.
12. Stop reaction with 100 µl/well 1N hydrochloric acid. Read absorbance at 450 nm within 30 minutes

RNA Isolation (RNaqueous 4 PCR, Ambion)

*(*also works for lungs but you must use a piece between 40-50mg, anything larger solution volumes must be adjusted and filter may become clogged)*

1. Remove nasal mucosa from both nasal cavities of rat or left and right trigeminal ganglia.
2. Place into sterile microfuge tube with 1ml of RNA later (Ambion)
3. Store at –80 ˚C for up to six months
4. Clean Mortar/Pestle and wipe down with Rnase Zap (Ambion Cat# 9780-84) and place at –20 ˚C at least 30 minutes before starting
5. Warm heat block to 80 ˚C. Pipette 50 ul of elution solution into a clean RNase free 1.5ml tube and heat
6. Remove DNAse and DNAse buffer from -20 ˚C and thaw on ice.
7. Thaw tissue sample on ice. Do not take tissue from -80 and warm directly to room temperature!
8. Have liquid nitrogen on hand
9. Remove mortar/pestle from freezer and fill with liquid N_2
10. Remove tissue from RNA later and wipe excess liquid on paper towel.
11. Put tissue in mortar, add more liquid N_2 if needed and grind tissue into fine powder
12. Use cell scrapper to transfer powder from mortar to sterile microfuge tube. Do not let tissue thaw, re-freeze mortar with liquid N_2 if needed.
13. Add 500 ul of lysis binding solution to tube (kept in fridge)
14. Mix well/Vortex until solution is homogenous and no tissue pieces are present
15. Add an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1)(kept in fridge) and vortex for about 2 min (results in a milky white mixture)
16. Spin 12000 RPM for 10 min
17. Remove tube from centrifuge but continue holding tube at an angle. Pipette off top aqueous layer and place in a fresh sterile tube. Do not take more than 450 ul and re-spin if pipette tip touches bottom layer.
18. Bottom layer is organic waste and should be placed in organic waste bottle in hood.

19. Add equal volume of 64% Ethanol (500ul)
20. Invert tube several times to mix (do not vortex; solution should be clear)
21. Add half of solution to filter column and spin for 1min at 10,000 RCF or until all solution passes through filter
22. Discard flow through and repeat with remaining solution
23. Discard flow through
24. Add 700 ul of Wash Solution #1 to filter and spin for 1 min at 10,000 RCF
25. Discard flow through
26. Add 500 ul of Was Solution #2/3 to filter and spin for 1 min at 10,000 RCF
27. Discard flow through
28. Repeat steps 26-27
29. Cut cap off tube
30. Spin 30 sec at 10,000 RCF to dry filter (Carry-over ethanol will contaminate PCR)
31. Place filter in a clean, sterile microfuge tube. Add 40ul of warmed elution solution to center of filter
32. Spin 30 sec at 10,000 RCF
33. Add remaining 10 ul and repeat step 32
34. Discard filter and keep microfuge tube
35. Add 1ul of DNAse and 5ul of Dnase buffer. Mix gently and incubate 45min at 37C
36. Add 5ul of Inactivation reagent (fridge) mix well first.
37. Mix gently and incubate at RT for 2 min
38. Spin 1 min 10,000 RCF and draw off supernatant (RNA)
39. Aliquot RNA. 5ul/tube and store at –80C
40. To determine concentration of RNA add 5ul to 495ul of TE buffer and read on spec at 260 and 280

Concentration RNA (ug/ml)= Absorbance at 260x 100 (dilution factor) x 40 (constant)
Estimation of RNA purity = ratio of 260/280 absorbances. For RT-PCR, ratio should be between 1.8 and 2.1.

RT-PCR

1. Turn on icycler before plating reaction
2. Use 96 well icycler iq PCR plates (Cat# 2239441, BioRad)
3. Keep all reagents and plate on ice.
4. Add in order the following reagents to each well (total reaction volume 20 ul). Components a and e are taken from Quantitect SYBR Green RT-PCR kit (Cat # 204243, Quiagen)

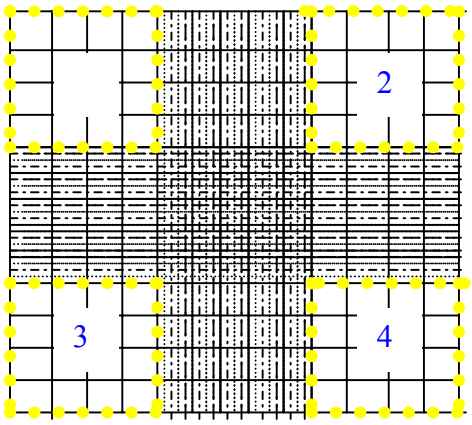
	Component	Volume	Final Concentration
a.	2x SYBR Green Mastermix	10 µl	1x
b.	Primer 5'	2 µl	0.5 µM
c.	Primer 3'	2 µl	0.5 µM
d.	Rnase free water	20 - (14.2+f)	
e.	Quantitect RT Mix	0.2 µl	
f.	Template RNA	100ng/well	5 ng/µl

5. RNA should be kept at -80 C and RT should be kept at -20 C until needed.
6. Thaw remaining reagents on ice mix well and quick spin before using (do not vortex)
7. After all reagents are added cover plate with optical tape (Cat # 223944, BioRad)
8. Run reaction using the following conditions

	Cycle	Repeats	Time	Temperature
1	Reverse Transcriptase		60 min	50 C
2	Activation of Taq		15 min	95 C
3		40		
	1) Denature		15 sec	94 C
	2) Annealing		30 sec	6-8 C below Tm of primers
	3) Extension		30 sec	72 C
4	Melt Curve	80	30 sec	start 95C decr temp by 0.5C
5	Hold		infinite	4 C

Nasal/Lung Lavages

1. For nasal cavity, lavage 15ml of cold PBS through nasal cavity (keep first 3ml separate if doing protein or NGF assay).
2. For lungs, lavage 40ml of cold PBS into lungs (keep first 12ml separate if doing protein or NGF assay)
3. Centrifuge samples at 1,500 rpm for 10 minutes.
4. Pour off supernatant and add 1ml of cold PBS to resuspend cell pellet (mix well but gently, do not vortex)
5. Determine total number of cells in the lavage sample using a hemocytometer w/ coverslip.
6. Load 10ul of lavage sample onto hemocytometer and count the total number of cells (not RBC) in each of the outer 4 quadrants (see areas outlined in yellow below; each quadrant is comprised of 16 squares). Calculate average number of cells per quadrant.



7. Each quadrant is 1mm x 1mm and when a coverslip is placed on top the height is 0.1mm. Therefore the volume of each quadrant is equal to $0.1mm^3$.
8. (Note: 1 mm^3=1 µl)
9. #of cells/mm^3= # of cells counted/mm^2 x 10
10. (Note: 1,000 mm^3 = 1 cm^3 and 1 cm^3 = 1 ml)

11. Total number of cells in the lavage fluid per ml = Average cells per quadrant x 10^4
12. Plate cells on slides at a density of $1.5x10^5$ cells/ml using a cytospin.
13. Volume (ml) of cells needed for cytospin = total number of cells/$1.5x10^5$ cells per ml
14. (Note a minimum volume required for cytospin is 100 µl and maximum is 500 µl; qs to 100 µl with PBS or spin down and resuspend in 500 µl if needed)
15. Stain cytospin slides with Wright-Giemsa manually or use slide stainer.

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