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Ozone Induces Nerve Growth Factor Release from Rat Tracheal Epithelial Cells during an Early Postnatal Critical Period

Lynnsey A. Carrell-Jacks

**Thesis submitted to the School of Medicine
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in partial fulfillment of the requirements
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**Master of Science
In
Biomedical Sciences**

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2011**

ABSTRACT

Ozone Induces Nerve Growth Factor Release from Rat Tracheal Epithelial Cells during an Early Postnatal Critical Period

Lynnsey A. Carrell-Jacks

Ozone, one of the major air pollutants in urban areas, produces epithelial cell injury and inflammation in the airways upon exposure. Previous studies have shown that ozone exposure leads to increased substance P (SP) expression in nerves innervating the smooth muscle in the extrapulmonary airway. Nerve growth factor (NGF), a neurotrophin, is known to increase SP expression. To determine the effect of ozone exposure on the release of NGF, a cell culture technique was developed for studying a homogenous rat tracheal epithelial cell population for analysis of NGF mRNA and protein expression. Furthermore, to determine if this effect is age-dependent, a critical period exposure paradigm was used. Rat pups exposed to ozone at postnatal day (PD) 6 showed an increase in the level of NGF mRNA while pups exposed after PD 6 showed no change in NGF expression. When rat pups exposed to ozone on PD 6 were reexposed at a later date, an increase in NGF mRNA was observed. These data suggest that exposure to ozone has an effect on NGF expression in early postnatal life and that this exposure might play a role in ozone sensitivity later in life

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

TABLE OF FIGURES

I. INTRODUCTION

Children are especially susceptible to airway pollutants. Early life exposure to inhaled environmental pollutants may play an important role in the pathogenesis of childhood asthma and other respiratory disorders. It has been shown that early postnatal exposure to passive cigarette smoke, allergens, and environmental pollution increases the incidence of respiratory illness and asthma later in life (Miller et al., 2009). Children are a susceptible population due to heightened sensitivity during early life and may be at risk of encountering high ozone levels while playing outside. West Virginia is ranked fourth overall for childhood asthma prevalence in the United States, with over 11% of the under-18 population suffering from asthma (Asthma Among West Virginia Children, 2006). Pediatric asthma is a national problem and a crisis in our local community.

Previous studies have shown that neurotrophin levels in the airway increase significantly following exposure to airway irritants and that this increase is followed by an upregulation of SP expression in nerves innervating the airway (Kessler & Black, 1980; Otten et al., 1980; Schwartz et al., 1982; Wilfong & Dey, 2004). The role of neurotrophins released from tracheal epithelial cells that might contribute to the symptoms associated with asthma is not well understood. The neuropeptide SP is considered one of the major initiators of neurogenic inflammation and airway hyperresponsiveness in asthma (Lembeck & Holzer, 1979; Koto et al., 1995). Although tracheal epithelial cells are known to release the neurotrophins nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) and are especially sensitive to inhaled irritants (Hahn et al., 2006; Dey, et al., 1999), more work needs to be done to elucidate the possible role of airway epithelial cells in the pathogenesis of asthma. It is important to understand the role of released neurotrophins from tracheal epithelial cells in response to early postnatal ozone exposure

because of the potential regulatory impact that neurotrophins have on airway sensory nerves,

which regulate inflammatory responses associated with the pathogenesis of childhood asthma.

II. BACKGROUND

A. Ozone and Pediatric Asthma

Ozone (O_3) exposures have a substantial negative impact on pulmonary health. Exposure to O_3 has been shown to decrease pulmonary function (Kim et al., 2010) and increase airway inflammation (Krishna et al., 1998) in healthy subjects. The effects are more severe in sensitive subjects, such as asthmatics or smokers (Frampton et al., 1997a; Frampton et al., 1997b). The effects of O_3 exposure are especially significant in children. Pediatric asthma diagnoses, attacks, hospitalizations, and deaths have increased significantly in the last two decades (Downs et al., 2001; Akinbami and Schoendorf, 2002; Babin et al., 2004). Pediatric respiratory disorders and infant mortality have been correlated to increased levels of inhaled environmental pollutants, including O_3 (Loomis et al., 1999; Triche et al., 2006). Exposure of infants to O_3 during development initiates drastic changes in the epithelial-mesenchymal trophic unit leading to compromised airway growth and development that persists or worsens, even without continued O_3 exposure (Plopper et al., 2007). Lung function is decreased in children on days when there was a higher O_3 concentration and children with physician-confirmed asthma experience the greatest decrease in lung function tests (Krzyzanowski et al., 1992; Schmitzberger et al., 1993; Lewis et al., 2005). Childhood asthma diagnoses and the prevalence of symptoms are increased in children living in areas with high ground-level O_3 (Etzel et al., 2002; Sousa et al., 2009). Young children who have been diagnosed with asthma are particularly susceptible to small, short-term increases in O_3 levels (Ko et al., 2007; Yamazaki et al., 2009; Dales et al., 2009) and an increase in emergency room admissions for asthma attacks has been noted on days with elevated O_3 (Boutin-Forzano et al., 2004). Chronic exposure to current ambient O_3 levels contributes to an increase risk of hospitalization for asthmatic children (Villeneuve et al., 2007;

3

Lin et al., 2008; Moore et al., 2008), especially for children under the age of five (Hernández-Cadena et al., 2007). Asthmatic children using maintenance medication are particularly vulnerable to O_3 (Gent et al., 2003). Furthermore, exposure to O_3 reduces the bronchodilation response to short-acting β-agonist prescription medicine in children with asthma (Hernández-Cadena et al., 2009). These results suggest an increased susceptibility to O_3 exposure in early life and childhood.

The Environmental Protection Agency is reconsidering the current national standard for ground-level O_3 of 75ppb on the 8 hour average. However, exposure to O_3 at levels far below the current national standard can be detrimental to pulmonary function. Exposure to 50ppb O_3 showed substantial decreases in pulmonary function in human subjects with cumulative effects of successive exposures (Hackney et al., 1975). In both control and asthmatic subjects, exposure to O_3 can cause significant increases in bronchial responsiveness and airway obstruction (Kreit et al., 1989). O_3 exposure also induces inflammation in human airways, even at levels that are not significant enough to reduce pulmonary function (Koren et al., 1989; Basha et al., 1994). Blunting the airway neutrophilic inflammatory response does not prevent the functional impairment of the airways following O_3 exposure (Vagaggini et al., 2001). Airway hyperresponiveness associated with O_3 exposure might occur prior to the influx of neutrophils in the airway and might be dependent upon local changes (Okazawa et al., 1989). Levels at or below the current EPA standard are detrimental to human health and this study aims to explore a novel mechanism of O_3-induced pulmonary damage, which will provide additional evidence that stricter standards are needed. Furthermore, the mechanism by which O_3 exposure leads to a decrease in pulmonary function remains poorly understood, but it is not a result of early

inflammatory responses (Blomberg et al., 1999). By exploring other mechanisms of action, this study will provide more information for potential clinical treatment.

B. Tracheal Epithelial Cells

Early childhood (6 to 8 years of age) is a critical period of lung development in humans (Burri, 1997). Differentiation of critical cell types and systems, among the most important being the respiratory epithelium and critical immune effector cell populations, occurs during this period (Finkelstein and Johnston, 2004). The airway epithelial cell is known to be particularly sensitive to many inhaled environmental irritants, including O_3 (Bayram et al., 2002). Epithelial cells respond to O_3 exposure in a series of three stages: constitutive cell activation, synthesis and release of chemotactic factors, and eosinophil or monocyte infiltration (Leikauf et al., 1995). Acute O_3 exposure causes epithelial cell injury and early epithelial necrosis in terminal bronchioles and the trachea (Mustafa et al., 1990; Hyde et al., 1992; Pino et al., 1992). Activation of the NK-1 receptor during acute O_3 exposure contributes to epithelial injury and subsequent epithelial proliferation, but does not influence neutrophil emigration into airways (Oslund et al., 2008). Epithelial cell injury and regeneration is greatest when O_3 exposure is combined with another airway irritant, such as dust (Adamson et al., 1999) or diesel exhaust particles (Kafoury & Kelley, 2005). O_3 is thought to modulate airway responses by upregulating the release of inflammatory mediators from bronchial epithelial cells, a mechanism which may be more sensitive in asthmatic patients (Bayram et al., 2001).

C. Nerve Growth Factor (NGF)

Neurotrophins are believed to play a role in the pathogenesis of asthma because serum levels of nerve growth factor (NGF; Bonini et al., 1996) and brain derived neurotrophic factor (BDNF;

Virchow et al., 2005) in serum, platelets, and plasma are significantly increased in patients with asthma. As several animal studies have shown, NGF and BDNF appear to play a part in the development of airway hyperresponsiveness (Renz et al., 2001; Bennedich et al., 2007) and in the increased sympathetic and sensory innervation of the lung (Hoyle et al., 1994). NGF and BDNF have also been shown to promote inflammation (Woolf et al., 1994) and symptoms associated with asthma (Nassenstein et al., 2003). Immune cells in the airway are thought to be particularly sensitive to the effect of neurotrophins (Nassenstein et al., 2003). Furthermore, the effect of neurotrophin release might vary across the lifespan. For instance, there is a significant decrease in the expression of the NGF high affinity TrkA in postnatal day 21 old animal compared to earlier life (Molliver and Snider, 1997). Neurotrophins in the airway are released from many cell types, including inflammatory cells in the bronchial mucosa, fibroblasts, smooth muscle cells, and airway epithelial cells (Freund & Frossard, 2004). In the inflamed lung, airway epithelial cells are a major contributor of NGF and BDNF (Hahn et al., 2006). This study will examine the effect of O_3 exposure on NGF production in a pure population of tracheal epithelial cells.

D. Substance P (SP)

NGF is known to stimulate SP expression in sensory neurons (Miller et al., 1992) and is necessary for SP production and neuronal survival *in vitro* (Adler et al., 1984). SP immunoreactive nerve fibers are increased in tracheal smooth muscle in cultured trachea treated with NGF (Wu and Dey, 2006). SP immunoreactive nerve fibers are also increased in the airways of asthmatic subjects (Ollerenshaw et al., 1991). The airway hyperresponsiveness associated with O_3 exposure is thought to be the result of SP-enhanced acetylcholine release (Wu et al., 1997). SP appears to be released in response to stimulation of primary afferent C-fibers

(Barnes, 2001; Budai and Larson, 1995). SP is increased in lung homogenates and in nerve fibers innervating the airway following allergen challenge (Fischer et al., 1996) and O_3 exposure (Wu et al., 2007; Dey et al., 2009). SP potentially plays a role in the development of neurogenic inflammation (Hsiue et al., 1992; Donkin et al., 2007). O_3 exposure increases the release of SP, suggesting that the mode of action of O_3 results in increased activity of airway sensory nerves followed by an increase in the release of neuropeptides (Schierhor et al., 2002). Depletion of C-fibers reduces the effect of O_3 on increased responsiveness, confirming that O_3 increases reactivity in part through the local effects of C-fibers (Joad et al., 1996). The release of SP from sensory nerves can lead to airway submucosal glands mucus secretion (Khansaheb et al., 2010), microvascular leakage, and bronchoconstriction (Lundberg et al., 1983; Tokuyama et al., 1993) (See Figure 1).
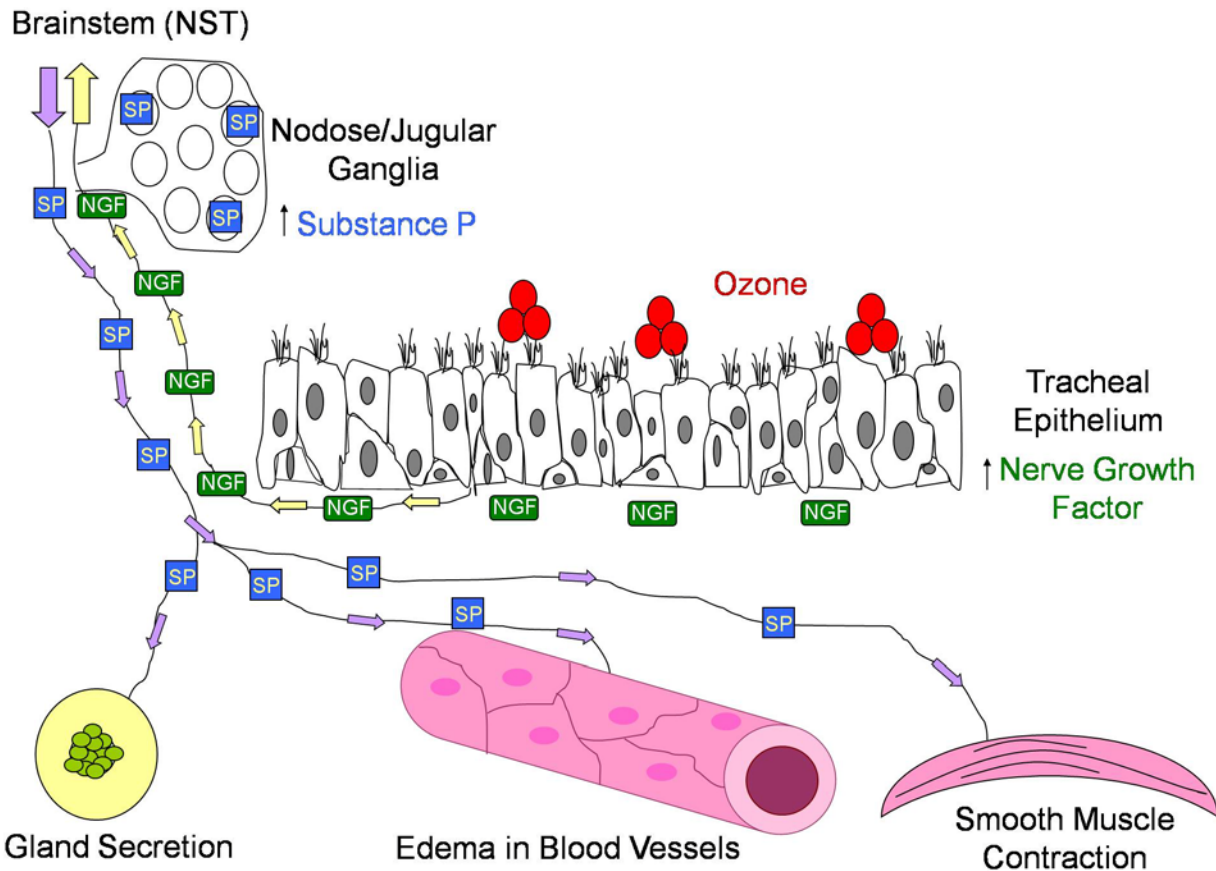
Figure 1. The Role of NGF in O_3 Response. A schematic showing the possible role of NGF release from tracheal epithelial cells in the regulation of O_3 response. NGF released from the tracheal epithelial cells could stimulate the release of Substance P from the Nodose/Jugular ganglia and nucleus of the solitary tract. SP can then stimulate gland secretion, edema in blood vessels, and smooth muscle contraction in the airway.

E. Statement of Problem

When combined, these results suggest an increased sensitivity to O_3 exposure at early postnatal ages leads to an increase in asthma diagnoses and symptoms in part due to SP expression and subsequent neurogenic inflammation. Neurotrophins are known to regulate the expression of SP in airway nerves, particularly those innervating the smooth muscle and blood vessels, which are sites of airway responsiveness and airway inflammation, respectively.

Epithelial cells, which are adjacent to the smooth muscle in the trachea, are the first cells contacted by inhaled O_3 in the airway and one of the major contributors of neurotrophins in the challenged airway. Because NGF is increased in patients diagnosed with asthma and subjects exposed to O_3, it is possible that the early life sensitivity to O_3 exposure could be due to changes in the immediate release of neurotrophins from tracheal epithelial cells.

F. Hypothesis

The central hypothesis of this study is that exposure to O3 will cause an increase in NGF production in rat tracheal epithelial cells. Furthermore, this increase will be age-dependent, such that O_3 exposure at an earlier postnatal day will lead to an increase in neurotrophins while a later exposure will not. Finally, this study hypothesizes that an O_3 exposure during the proposed critical period will increase sensitivity to later O_3 exposures that occur outside of the proposed critical period.

G. Rationale

Previous studies have shown that SP NFD increases in the airway following an early postnatal O_3 exposure. Knowing where the neurotrophins that guide this process are being produced could lead to interventions that eliminate or reduce the response to O_3.

H. Specific Aims

1. Determine the effect of an early postnatal O_3 exposure on the release of NGF from tracheal epithelial cells in an *in vivo* rat model.
2. Define a sensitive period of development during which an exposure to O_3 has an effect on the production of NGF.

3. Examine the effect an O_3 exposure during the proposed critical period has on later exposures to O_3 outside of the critical period.

III. MATERIALS AND METHODS

A. Animal Use and Anesthetics

Pregnant, late gestation Fischer 344 rats (Harlan Laboratories, Inc) were housed one per cage, under controlled light-cycle (12 hr. light/12 hr. dark) and temperature (22-24ºC) conditions, with access to food and water *ad libitum* in the West Virginia University animal facility. Rat pups were housed with their dam until sacrificed. Animals were sacrificed 12 or 24 hours after O_3/air exposure with a lethal intraperitoneal injection of sodium pentobarbital (Nembutal, 200 mg/kg). All procedures were approved through ACUC review under Protocol #10-0401.

B. O_3 Exposure

All *in vivo* O_3 exposures were done at 2000 ppb in a 12x12 inch stainless steel and glass chamber for 3 hours. O_3 was produced by passing hospital-grade air through a drying and high-efficiency particle (HEPA) filter and then through an ultraviolet light source. The O_3 concentration in the chamber was measured by chemiluminescence with a calibrated O_3 analyzer (OA 350-2R model; Forney Corporation; Carrolton, TX). Air control animals were exposed to filtered air using procedures identical to those above, except O_3 was not delivered to the mixing chamber. The O_3 exposure apparatus has been described in detail elsewhere (Wu et al., 2002).

Rat pups were removed from their mother and exposed to O_3/air on postnatal day (PD) 6, 10, 15, 21, or 28. In some experiments, animals were exposed to O_3/air twice to determine the effect of an early postnatal exposure (PD 6) on a second exposure outside of the critical period window (PD 28). See Figure 2 for the critical period experimental design.
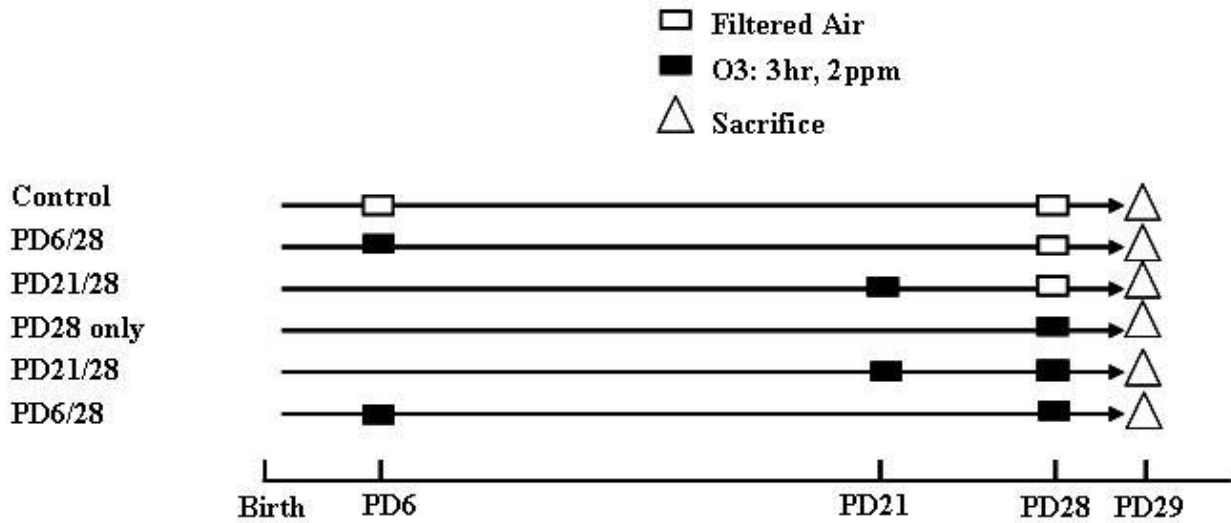
Figure 2. Experimental design showing O_3 exposure paradigm for critical period study. Rat pups were exposed to air or O_3 initially on PD 6 (within the proposed critical period) or PD 21 (outside of the proposed critical period). The pups were reexposed to air or O_3 on PD 28. The animals were sacrificed 12 or 24 hours after the final exposure.

C. Trachea Fixation and NGF Immunohistochemistry

Immunohistochemical staining was used to determine NGF presence in the airway epithelium. Twelve or twenty-four hours after exposure, the tracheas were fixed with picric acid-formaldehyde for three hours. The tracheas were rinsed twice with a 0.1 M phosphate-buffered saline containing 0.15% Triton X-100 (PBS-Tx, pH 7.8) and remained in PBS-Tx overnight at 4°C. The next day, the tracheas were placed on corks, covered with Tissue Tek O.C.T. compound (Sakura, Torrance, CA), frozen in isopentane cooled with liquid nitrogen, and stored in airtight plastic bags at -80°C.

Cryostat sections (12 μm thick) of airway tissue were collected on gelatin-coated cover slips and dried briefly at room temperature. The sections were incubated with rabbit anti-NGF-β monoclonal (1:100) primary antiserum diluted in PBS-Tx + 1% bovine serum albumin (BSA) (PBS-Tx-BSA, pH 7.8) at 4°C overnight. The sections were rinsed three times for 5 minutes

12

each with PBS-Tx-BSA, covered with goat anti-rabbit Alexa 488 (1:100) in PBS-Tx-BSA, incubated at 37°C for 30 minutes, rinsed three times for 5 minutes each 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 a fluorescein filter (excitation 495 nm and emission 520 nm). Controls consisted of testing the specificity of primary antiserum by absorption with 1µg/ml of the specific antigen. Nonspecific background labeling was determined by omission of primary antiserum. Sections of airways were observed (40X magnification) on a Zeiss LSM 510 confocal microscope equipped with an argon laser (excitation 495 nm and emission 520 nm, Zeiss, Oberkochen, Germany).

D. Tracheal Epithelial Cell Extraction

Tracheas were excised and immersed in ice cold Hank's Buffered Salt Solution (HBSS) while excess tissue was removed. Next, the tracheas were placed in 10 mL of pre-warmed (37°C) dissociation medium (0.5% protease in Dulbecco's Modified Eagle Medium (DMEM)-F12/5%Pen-Strep) and incubated at 37°C with agitation for 1 hour. Fetal bovine serum (1ml, FBS) was added to the stop the protease digestion and the tissues was removed and rinsed in 10 mL of culture medium (5% FBS and 4% DNase I in DMEM-F12/5%Pen-Strep). The tracheal pieces were discarded and the culture and dissociation media cell suspensions were pooled. The cell suspension was incubated on anti-IgG antibody coated 100 mm dishes at 37°C for 1 hour to remove immune cells, predominately neutrophils. The cell suspension was decanted and the cells were collected by centrifugation (1,000 rpm/10 minutes).

E. Tracheal Epithelial Cell Immunocytochemistry

  To determine the efficiency of the tracheal epithelial cell extraction, immunocytochemistry was performed. Epithelial cells were extracted from adult rats exposed to O_3 (2000 ppb) for three hours. The density of the extracted cell population was calculated and the appropriate amount of suspension was placed on a subbed slip using a cytospin (400 rpm for four minutes). The slips were dried at room temperature for 30 minutes and placed in a pre-chilled 1:1 methanol/acetone solution for 10 minutes at -20˚C. The slips were dried at room temperature for two minutes and the primary antibody was added. The slips were incubated at 4˚C overnight and washed three times for 5 minutes each in PBS-Tx-BSA. The secondary antibody was added and the slips were incubated at 37˚C for 30 minutes. After incubation the slips were washed three times for 5 minutes each in PBS-Tx-BSA and mounted on slides using Flouromount-G. Slides were analyzed (40X magnification) by a Zeiss LSM 510 confocal microscope equipped with an argon laser (excitation 495 nm and emission 520 nm, Zeiss, Oberkochen, Germany). To confirm that the cells in the population were epithelial cells, the primary antibody used was mouse monoclonal anti-Pan cytokeratin (1:100) with a secondary goat anti-mouse FITC (1:100) antibody. To determine the population of immune cells in the extracted suspension, cells that were treated with IgG antibody coated plates were compared to cells that were not treated with the IgG antibody coated plates. The primary antibody was mouse anti-rat CD45 antibody (1:100) and the secondary antibody was goat anti-mouse FITC-conjugated antibody (1:100). The number of total cells and immune positive cells were counted by two independent researchers.

F. Trypan Blue Exclusion Assay

A Trypan Blue Exclusion Assay was used to determine the viability of the cells following tracheal epithelial cell extraction. The cell density of the cell suspension was determined using a hemacytometer. Immediately following cell extraction and purification, 0.1 mL of 0.4% trypan blue in PBS was added to 1 mL of cell suspension. The cell suspension was loaded on a hemacytometer and examined under a microscope at low magnification. The number of blue stained cells and the number of total cells were counted.

G. Real-Time PCR

Total RNA was extracted from the cells according to the manufacturer's instructions (RNeasy Plus Micro Kit, Qiagen,Valencia, CA). Briefly, cells were lysed and homogenized in a buffer that inactivates RNases. The lysate was passed through a gDNA eliminator column to reduce the risk of genomic DNA contamination. Ethanol was added to the flow-through to promote RNA binding and total RNA was bound by passing the sample through an RNeasy MinElute spin column. Contaminants were washed away and the RNA was eluted in RNase free water. A Nanodrop was used to measure the purity and concentration of the RNA in the sample by determining A260/A280. Total RNA was transcribed into cDNA using the High Capacity cDNA Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The cDNA was then amplified in the 7500 RT-PCR System using the NGF Taq Man gene expression assays (Applied Biosystems). The Relative Quantification Method ($2_{-\Delta\Delta Ct}$) was used to determine the NGF mRNA levels. NGF expression levels were normalized using β-actin expression as an internal control.

H. Rat Tracheal Epithelial Cell Protein Extraction

Following rat tracheal epithelial cell isolation, the cells were washed twice in ice cold PBS. The cell extraction buffer was supplemented with 1 mM PMSF and protease inhibitor cocktail (Sigma) added just before use. The supernatant was removed and discarded. The cells were lysed in 250 µL of Cell Extraction Buffer (Invitrogen) for 30 minutes, on ice, with vortexing for 1 minute at 10 minute intervals. The extract was transferred to microcentrifuge tubes and centrifuged at 13,000 rpm for 10 minutes at 4˚C. The clear lysate was at -80˚C until ready for assay. The total protein level was measured using a nanodrop and was controlled for each sample in the ELISA

I. NGF Enzyme-Linked Immunoassay

The concentration of NGF was assayed using the NGF Emax ImmunoAssay System (Promega, Madison, WI). NGF was detected using an antibody sandwich format in 96 well plates (Figure 2). Each well was initially coated with 100 µl of anti-NGF pAb and incubated overnight followed by 1 hour incubation with blocking buffer (200 µl/well) to prevent non-specific binding. Either 100 µl of cell lysate or 100 µl of NGF standard (7.8-1000 pg/ml) was added to each well. The plate was incubated for 6 hours with agitation followed by an overnight incubation with anti-NGF mAb (100 µl/well). For color development an anti-rat IgG, horseradish peroxidase conjugate antibody was added to each well (100 µl) followed by TMB solution, which reacts with the peroxidase-labeled conjugates. The reaction was stopped after 10 minutes with 1N hydrochloric acid (100 µl/well). 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 lysate sample was calculated from a standard curve. All samples

were run in duplicate, and as a negative control, a cell extraction buffer sample was run with each assay.

J. Statistical Methods

Differences between groups were determined using a two-tailed Student's T-Test using Microsoft Excel software. Animals exposed to O3 were compared to age or time matched air exposed controls. In the critical period experiment, an ANOVA was performed to compare each group to all other groups. In all studies, a p-value less than or equal to 0.05 was considered significantly different. Mean and standard error is reported for each value.

VI. Results

1. NGF Localization in Tracheal Epithelium

A series of experiments was conducted to first determine if NGF is present in rat pup tracheal epithelial cells and to validate the tracheal epithelial cell extraction technique. Rat pups were exposed to air or O_3 (2000ppb) for 3 hours and sacrificed 12 or 24 hours after exposure. The tracheas were fixed, frozen, and sectioned onto cover slips. The sections were treated with rabbit anti-NGF-β monoclonal (1:100) and goat anti-rabbit Alexa 488 (1:100). When viewed under a confocal microscope, NGF fluorescence was observed in the tracheal epithelial layer (see Figure 3). Other studies have also shown that NGF is located in tracheal epithelial cells (Hahn et al., 2006; Pons et al., 2001; Fox et al., 2001).
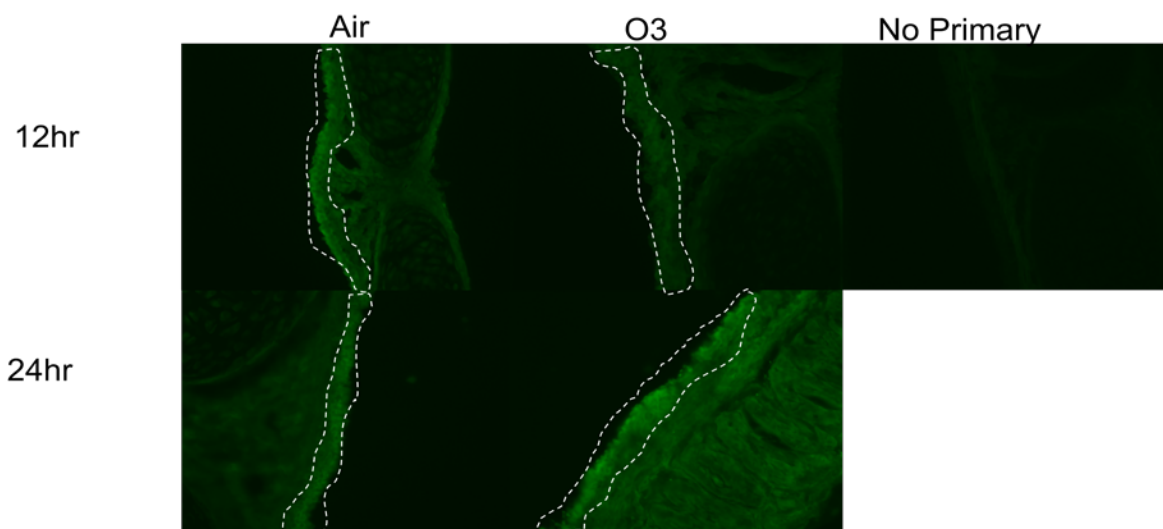
Figure 3. Immunohistochemical staining showing NGF in tracheal epithelial cells from PD 10 rat pups exposed to either air or O_3 (2000 ppb) for three hours. Tracheas were isolated and fixed using PAF 12 or 24 hours after exposure. The primary antibody was rabbit anti-NGF-β monoclonal (1:100). The secondary antibody was goat anti-rabbit Alexa 488 (1:100). The epithelial layer has been outlined in white.

2. Epithelial Cell Extraction

To test the validity of the cell extraction technique, adult Fisher 344 female rats were exposed to O_3 (2000 ppb) for 3 hours and sacrificed 24 hours later. The tracheal epithelial cells were extracted as described and cytospins of the cells were collected on glass slides. The cells were stained with mouse monoclonal anti-pan cytokeratin (1:100) and a goat anti-mouse FITC secondary (1:100) and imaged under a confocal microscope. Fluorescence was observed in the cell population, confirming the presence of epithelial cells. A control sample that was treated with the secondary antibody only showed no staining. (See Figure 4).

(A)

(B)
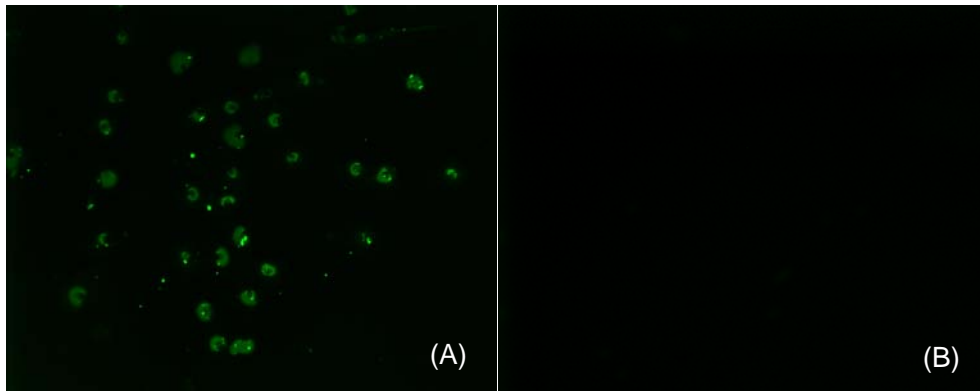
Figure 4. Cytokeratin staining of cells isolated from adult rat trachea showing tracheal epithelial cells. (A) Mouse Monoclonal anti-Pan Cytokeratin; 1:100/Goat anti-Mouse FITC; 1:100. (B) No primary control. These images show that cells isolated from the trachea are epithelial cells.

The treatment of the cells with an anti-IgG antibody was tested to determine the effect this treatment had on the population of immune cells in the cell culture. The cells extracted from adult Fisher 344 female rats that were treated with O_3 were either plated on tissue culture treated plates with an anti-IgG antibody or were plated on plates that had no antibody for one hour. The purpose of this procedure is to remove immune cells from the population by adherence to the antibody on the plate. Cells that were plated with anti-IgG antibody showed a reduction in the number of immune cells in the final population count (See Figure 5). The population that was

treated with the antibody was 96.0% (±1.02632%) pure epithelial cells, while the population that

was not treated was 93.8% (0.49497%) pure epithelial cells (p≤0.05; Figure 5c).

(A)

(B)
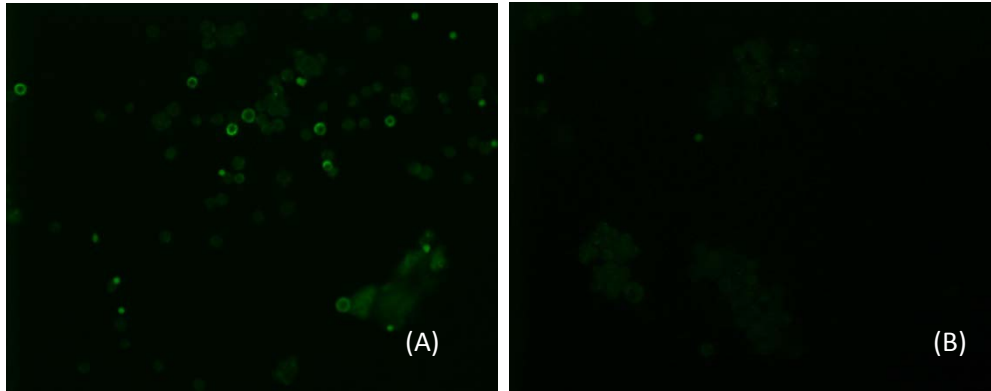
(C)
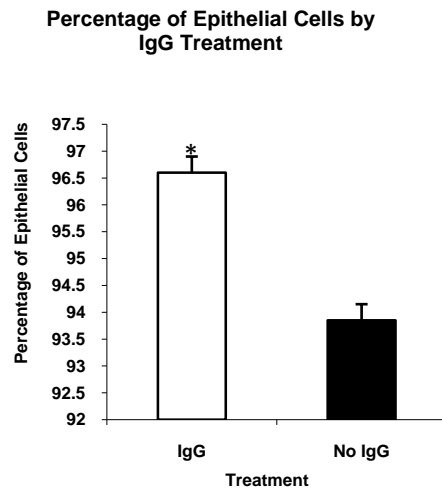
Figure 5. CD45 staining of cells isolated from adult rat trachea showing immune cells (n=3 per group). Mouse anti-Rat CD45;1:100/Goat anti-Mouse FITC;1:00. (A) No IgG treatment was used in the isolation of tracheal epithelial cells. (B) IgG treatment was used to remove immune cells from the cell culture. (C) Percentage of epithelial cells as compared to immune cells for IgG and no IgG treatments (p<0.05). Values are means □□ SE, n=3 for each group and *p<0.05 is significant.

Finally, a Trypan Blue Exclusion Assay was performed on extracted cells to determine

the viability of cells that have been through the extraction procedure. The purpose of this

experiment was to evaluate the potential degradation of RNA during the extraction process, as

compromised cells will take up the blue staining. The cells were treated with Trypan Blue and viewed on a hemocytometer. The number of total cells and the cells that took up the dye were counted. Less than 1% of cells showed Trypan Blue staining (see Figure 6). Beating ciliated epithelial cells were also observed in this experiment.

Figure 6. A Trypan Blue exclusion assay was used to determine cell survival following tracheal epithelial cell isolation. Over 99% of the cells did not show Trypan Blue staining, which suggests that the cells are viable following isolation. Moving ciliated epithelial cells were observed during this experiment.

3. NGF Expression in Rat Tracheal Epithelial Cells Following Early Life O_3 Exposure

Rat pups were exposed to either air or O_3 (2000 ppb) for three hours. The rat pups were sacrificed 1, 6, 12, 18, or 24 hours after exposure. The tracheal epithelial cells were extracted and total RNA was collected and transcribed into cDNA. RT-PCR was performed using an NGF Taq man assay. Rat pups exposed to O_3 and sacrificed 12 hours later showed a significant increase in the amount of NGF mRNA (2.24 ± 0.35) compared to air controls (1 ± 0.252, $p \leq 0.05$). Each

integer represents a fold change in the level of NGF mRNA. Significant differences in the level

of NGF mRNA were not observed at any other time point following exposure. (See Figure 7).

Relative NGF mRNA Expression Across Time Post- Exposure
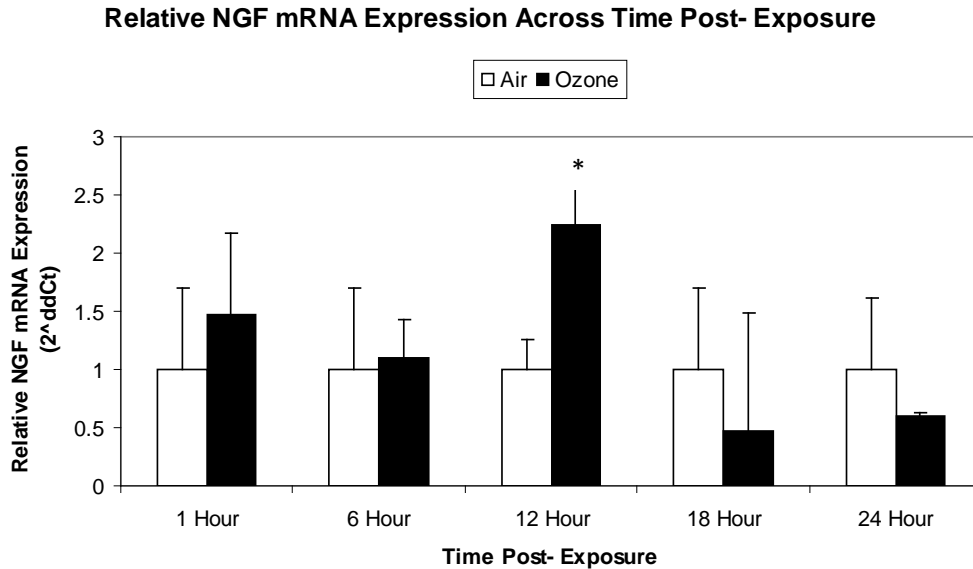
Figure 7. NGF mRNA expression relative to β-actin mRNA expression in PD 6 rat pups 1, 6, 12, 18, and 24 hours following exposure to air or O_3, 2000 ppb (n=3 per group). There was a significant increase in NGF mRNA expression between air and O_3 12 hours post-exposure (p≤0.05). Values are means ±SE, n=3 for each group and ***p<0.05** is significant.

To determine the effect of age at exposure on the level of NGF mRNA in tracheal

epithelial cells, rat pups were exposed to air or O_3 (2000 ppb) on PD 6, 10, 15, 21, or 28 for 3

hours and sacrificed 12 hours later. The level of NGF mRNA was measured as in the previous

experiment. No significant changes were observed at any other age point. A substantial increase

in the total level of NGF mRNA was observed at PD 10 and PD 15 relative to all other ages for

both air and O_3 exposed animals. (See Figure 8).

Relative NGF mRNA Expression in Development
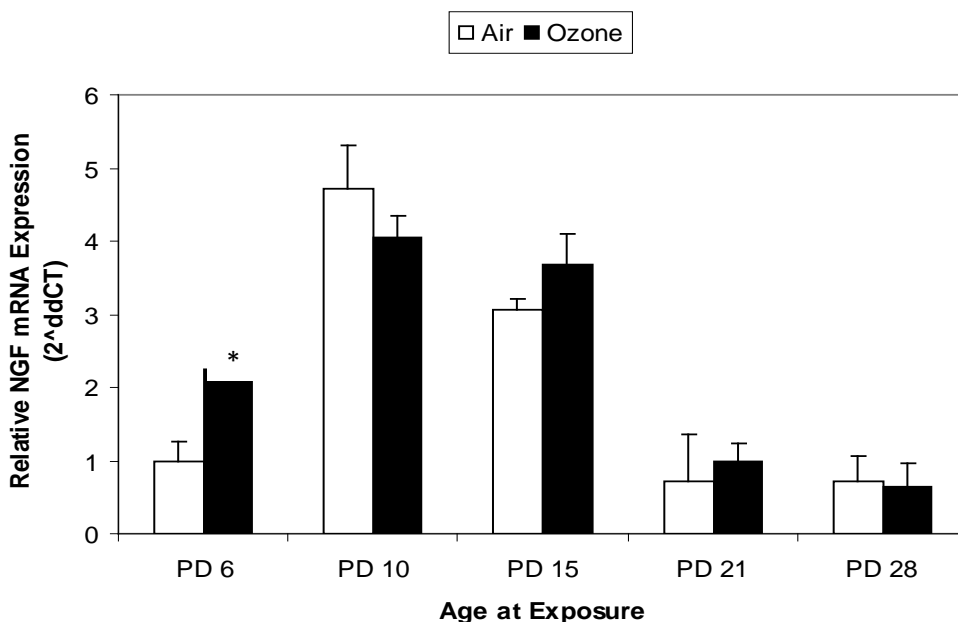
Figure 8. The effect of O_3 exposure on NGF mRNA expression in tracheal epithelial cells over a period of development. NGF mRNA expression relative to β-actin mRNA expression in rat pups exposed to air or O_3 (2000 ppb) for three hours and sacrificed 12 hours later. PD 6 showed a significant difference in NGF mRNA expression between air and O_3 ($p \leq 0.05$). Values are means ±SE, n=6 for each group and *****$p < 0.05$ is significant.

The amount of NGF protein in the isolated rat tracheal epithelial cells was also measured using ELISA to determine the effect of an early postnatal O_3 exposure. NGF protein level was not significantly different from air controls for animals exposed on PD 6, PD 10, or PD 28. NGF protein was significantly different for rat pups exposed on PD 15 (34.11642 ± 6.075263 pg/mL) compared to air control (17.78675 ± 2.396285 pg/mL) and for PD 21 (49.57736 ± 5.933555 pg/mL) compared to control (18.1799 ± 6.962812 pg/mL), $p \leq 0.05$.

Protein in RTEs Following O_3 Exposure
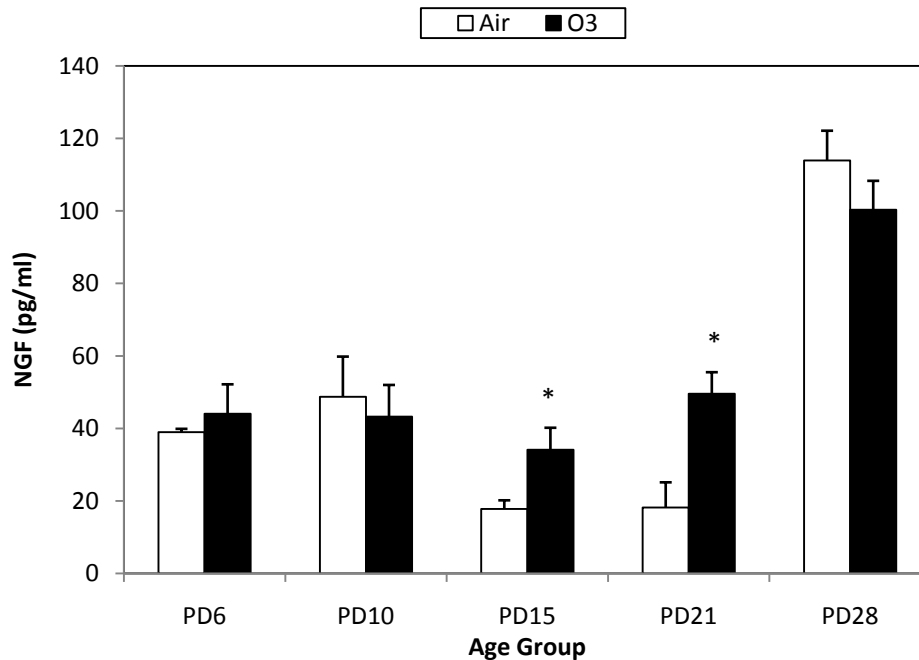
Figure 9. The effect of early postnatal O_3 exposure on the amount of NGF protein in rat tracheal epithelial cells. NGF protein was measured in isolated rat tracheal epithelial cells 24 hours after exposure to determine if O_3 exposure (2000 ppb, 3 hours) had an effect on the level of NGF in the cells. NGF protein was measured by ELISA. Values are means ±SE, n=6 for each group and *****$p<0.05$ is significant compared to air control.

4. NGF mRNA Expression during a Critical Period of Development

To determine the effect of an early O_3 exposure on subsequent exposures, a critical period paradigm was developed (see Figure 2). Rat pups were exposed to air or O_3 on PD 6 or PD 21. The pups were reexposed to either air or O_3 on PD 28 and sacrificed 12 hours later. Control animals were exposed to air or O_3 only on PD 28. The tracheal epithelial cells were extracted as described and NGF mRNA was measured using RT-PCR. NGF mRNA content in the PD 6 and PD 28 O_3 group (2.02 ± 0.10) was significantly higher than the PD 6 O_3 group not re-exposed at PD 28 (1.14 ± 0.19) and the PD 21 and PD 28 O3 group ((1.04 ± 0.24). The NGF expression of the O_3 group exposed on PD 21 and PD 28 (1.04 ± 0.24) was not significantly

different from the control ambient air group (1.00 ± 0.14) and the PD 28 only exposure group

(0.78 ± 0.11).

Relative NGF mRNA during Critical Period Exposure
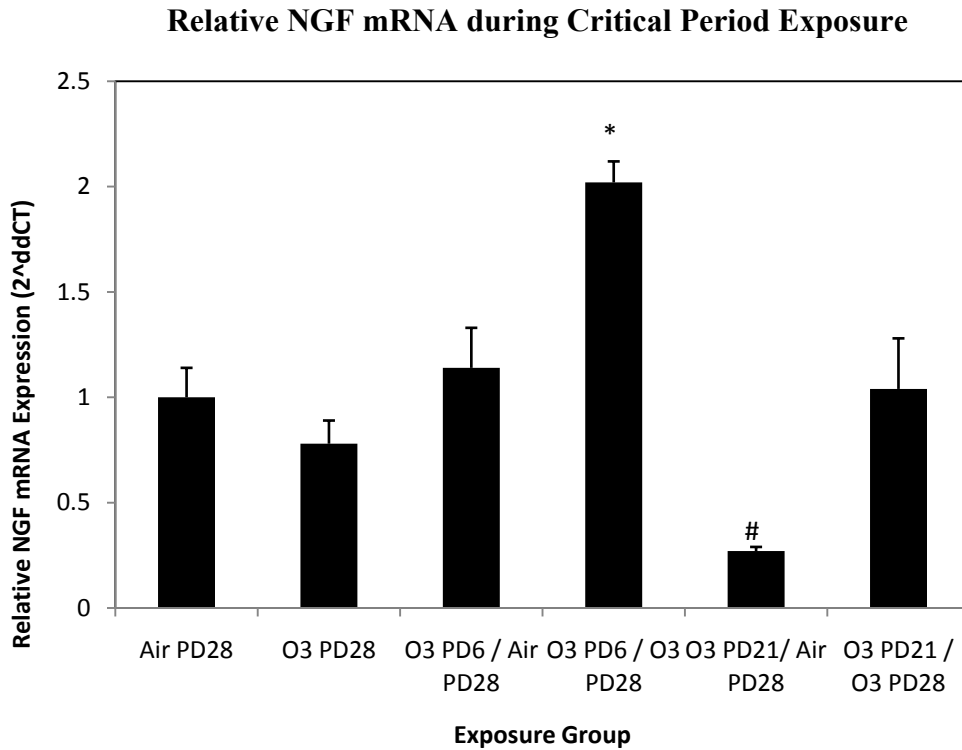
Figure 10. The effect of O_3 exposure on relative NGF mRNA expression in tracheal epithelial cells during a critical period of development. NGF was measured to determine if O_3 exposure (2000 ppb, 3 hours) during a critical period (PD 6) would result in prolonged changes of NGF message upon a second O_3 exposure (PD 28) in comparison to controls and exposures outside the critical period (PD 21). NGF mRNA was measured by real-time PCR relative to β-actin mRNA expression. Values are means $\pm$SE, n=6 for each group and *p<0.05 is significant compared to all groups, #p<0.05 is significant compared to all groups.

V. Discussion

Exposure to ground-level O_3 has been shown to decrease lung function, particularly in vulnerable populations, such as children (Tager et al., 2006). There are increasing concerns that the current EPA standard level of ambient O_3 (75 ppb) is too high to prevent negative human health outcomes (Kim et al., 2011; Schelegle et al., 2009; World Health Organization, 2005). The epithelial cells that line the airway are the first point of contact for inhaled irritants, making the epithelium an important target for O_3 injury. O_3 is thought to modulate airway responses by upregulating the release of inflammatory mediators from airway epithelial cells (Bayram et al., 2001) which in turn alter epithelial cell function (Leikauf et al., 1995). NGF levels are increased in asthmatics (Bonini et al., 1996) and it is thought to play an important role in mediating asthma symptoms. Airway epithelial cells have been shown to produce NGF in response to inflammation (Fox et al., 2001). Increased NGF expression has been observed in patients with allergic inflammatory disease (Bresciani et al., 2009; Sanico et al., 2000) and asthma (Nassenstein et al., 2003; Bonini et al., 1996). NGF has also been shown to upregulate SP expression in C fiber neuronal cell bodies (Malcangio, 1997), increase airway innervation in mice (Hoyle et al., 1998) and induce airway hyperresponsiveness, a hallmark symptom of asthma (Quarcoo et al., 2004; Braun et al., 2001; Braun et al., 1999). Airway epithelial cells have been shown to produce and release NGF in response to inflammatory mediators (Hahn et al., 2006; Fox et al., 2001; Pons et al., 2001). The purpose of this study was to examine the production and release of NGF from tracheal epithelial cells following an acute O_3 exposure during early postnatal life. O_3 exposure is also known to cause an inflammatory response (Hernandez et al., 2010; Aris et al., 1993) and immune cells also produce NGF (Solomon et al., 1998; Noga et al., 2003; Rost et al., 2005). This study developed a novel method for measuring mRNA and protein levels in a population of

isolated tracheal epithelial cells. The experiments used cells in which the immune cells were removed via anti-IgG antibody, resulting in a population of pure epithelial cells. The presence of epithelial cells was confirmed using fluorescent staining for cytokeratin. The presence of immune cells in cell populations with and without the anti-IgG antibody treatment was examined using a fluorescent CD45 antigen antibody. The cells treated with anti-IgG antibody showed a significant reduction in immune cells and enrichment of epithelial cells. Therefore, the results obtained show the effect of O_3 exposure on the production of NGF in tracheal epithelial cells.

Because children appear to be a population that is especially susceptible to inhaled irritants, this study used an early postnatal O_3 exposure model. Previous studies in our lab have shown an increase in SP nerve fiber density in the extrapulmonary epithelium and the intrapulmonary and extrapulmonary smooth muscle in rat pups exposed to O_3 at PD 15 or earlier (Hunter et al., 2010a). In this study, rat pups were exposed to O_3 (2000ppb for 3 hours) at PD 6, 10, 15, 21 or 28 and sacrificed 12 hours later to examine the effect of O_3 on NGF mRNA in the tracheal epithelial cells. Animals were sacrificed 12 hours after exposure because this was the only time point to show a significant difference in NGF mRNA. This is consistent with other studies examining NGF mRNA production (Heumann et al., 1986; Barth et al., 1984). When compared to age-matched air controls, PD 6 rat pups showed a significant two-fold increase in the amount of NGF mRNA extracted from tracheal epithelial cells. Significant differences between air and O_3 exposed animals were not observed at any other age. These results suggest that there is an early life sensitivity to O_3 exposure with regard to epithelial cell interaction, as the earliest age examined was the only one to show a significant change in NGF mRNA.

To further examine the effect of early postnatal O_3 exposure on tracheal epithelial cells, rat pups were exposed to O3 at PD 6, 10, 15, 21 or 28 and the level of NGF protein in the

tracheal epithelial cells was measured 24 hours later, as previous work in our lab has shown an increase in SP nerve fiber density at 24 hours (Hunter et al., 2010a). The early life exposure to O_3 did not lead to an increase in NGF in the isolated rat tracheal epithelial cells at PD 6. It did, however, lead to an increase in NGF at both PD 15 and PD 21. These experiments measured the levels of NGF within the tracheal epithelial cells following an airway irritant exposure. It is possible that the amount of NGF was increased following the O_3 exposure at earlier postnatal days and that this NGF was released from the cells, and therefore not measurable in this set of experiments. The increased NGF protein at PD 15 and PD 21 suggests that this protein was sequestered in the cells and not released at these postnatal ages, but previous work has shown an increase in NGF in bronchoalveolar lavage fluid following an O_3 exposure at PD 10 and PD 15 (Hunter et al, 2010b). As bronchoalveolar lavage fluid measures the total protein in the lung, it is possible that the NGF measurements obtained in that study reflect the contribution of immune cells. Further experiments, potentially *in vitro*, measuring the amount of NGF released from tracheal epithelial cells would provide more information regarding this possible phenomenon.

The increase in NGF mRNA in tracheal epithelial cells at PD 6 did not directly produce an increase in NGF protein. Likewise, the lack of an NGF mRNA increase at PD 15 and PD 21 did not prevent the increase of NGF protein. It is possible that the timing of NGF mRNA production changes with development and that measuring the mRNA at other time points after exposure would have shown an increase in NGF mRNA. It is also possible that the protein was increased at PD 6 and was released from the cells prior to cell extraction and measurement. NGF is produced and secreted, not stored in cells and released on demand (Heumann et al., 1984; Shelton & Reichardt, 1984), so it is possible that the increase in NGF protein occurred at an earlier time point.

Further studies were completed to examine how the effect an O_3 exposure during this early critical or sensitive period of development ($\leq$ PD 6) might affect the response to a second exposure later in life ($\geq$ PD 28). Previous work in our lab has shown that rat pups exposed to O_3 during the critical period show an increase in SP nerve fiber density following a second O_3 exposure outside of the critical period (Hunter et al., 2010a). This effect was not observed when rat pups were initially exposed outside of the critical period or when the initial exposure was followed by an air exposure in later life. The NGF measured in broncoalveolar lavage is also significantly increased in rat pups exposed to O_3 on both PD 6 and PD 28 (Hunter et al., 2010b). The results of the current study correlate with the previous findings. Rat pups were exposed to O_3 within (PD 6) or beyond (PD 21) the proposed critical period. A second exposure to O_3 or air occurred on PD 28. Rat pups that were exposed to O_3 on both PD 6 and PD 28 showed a significant increase in NGF mRNA in the tracheal epithelial cells. Rat pups that were initially exposed to O_3 on PD 21 or that were exposed to O_3 on PD 6 and air on PD 28 did not show any significant change in NGF mRNA from control. The results of this series of experiments suggest a critical period of development during which younger animals are more susceptible to the effects of airway irritants and that this early life exposure could have an effect on the response to later exposures.

The critical period experiments show that the effect of ozone-exposure during the period of early life sensitivity can persistent and have an effect on the response to exposures in later life. These results suggest a novel NGF pathway that contributes to early life sensitivity to inhaled irritants. NGF has been shown to upregulate SP expression in airway nerves (Larsen et al., 2004) through both the TrkA and p75 neurotrophin receptors (Skoff et al., 2006). In guinea pigs, NGF administration increases airway hyperresponsiveness through neurokinin-1 (NK-1), a SP receptor

(de Vries et al., 1999). Increased SP expression has also been shown to increase blood vessel extravasation by binding to endothelial cell NK-1 receptors (Li et al., 2008). Airway hyperresponsiveness and edema are hallmark symptoms of asthma (Bergeron et al., 2010) and these results show a possible role for NGF from tracheal epithelial cells in mediating this asthma symptom. Furthermore, NGF could support the proliferation or branching of nerves in the airway smooth muscle that are typically pruned during development. NGF is known to promote sensory nerve growth and survival (Levi-Montalcini, 1987) and mice that overexpress NGF display increased airway sensory innervation (Hoyle et al., 1998). The increase in NGF in tracheal epithelial cells following an early life O3 exposure might therefore promote innervation. The increase in sensory innervation could lead to increased sensitivity in later responses to O_3 exposure. NGF release plays a role in the recruitment, survival, and activation of inflammatory cells (Freund & Frossard, 2004). The NGF released by tracheal epithelial cells in response to O_3 might therefore contribute to airway inflammation. As proinflammatory cytokines are known to upregulate the release of NGF from airway structural cells, including epithelial cells (Fox et al., 2001), the initial release of NGF from tracheal epithelial cells might lead to a secondary increase in NGF production through inflammation (Leikauf et al., 1995). Blocking the initial increase in NGF could be an important step in reducing the response to airway irritants.

Future studies examining the potential role for NGF in the response to airway irritants need to be conducted. Knocking down or preventing the observed increase in NGF and then measuring SP expression in the airways would show whether or not the increase in NGF leads to the increased SP observed in airway sensory neurons following an O_3 exposure. Currently, there are no selective NGF antagonists that could be used to perform these important studies, but the use of an NGF siRNA could provide more information about NGF's role in the O_3 response.

Further studies could look at the possible role of other neurotrophins, such as brain-derived neurotrophic factor (BDNF), in the response to airway irritants. BDNF has also been shown to be upregulated in asthmatics and associated with clinical severity of asthma symptoms (Lommatzch et al., 2005; Muller et al., 2010). Functional studies examining the role of neurotrophins in response to inhaled irritants are also necessary for a more complete understanding of the clinical significance of these findings.

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Publications

Hunter, D.D., Carrell-Jacks, L.A., Batchelor, T.P., & Dey, R.D. (2010). Role of Nerve Growth Factor in O3-induced neural responses in early postnatal airway development. *Am J Respir Cell Mol Biol.* PMID: 21075861

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OZONE INDUCES NERVE GROWTH FACTOR RELEASE FROM RAT TRACHEAL EPITHELIAL CELLS DURING AN EARLY POSTNATAL CRITICAL PERIOD

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**Thesis submitted to the School of Medicine
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Master of Science
In
Biomedical Sciences**

Department of Neurobiology and Anatomy

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