Interleukin (IL)-1 Regulates Ozone-induced Nerve Growth Factor (NGF) and Substance P (SP) Release in Bronchoalveolar Lavage Fluid (BALF) in Mice

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ABSTRACT

Interleukin (IL)-1 Regulates Ozone-induced Nerve Growth Factor (NGF) and Substance P (SP) Release in Bronchoalveolar Lavage Fluid (BALF) in Mice

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Previous studies show that both IL-1 and NGF increase synthesis of SP in airway neurons after either direct application or ozone exposure. We hypothesize that NGF mediates ozone-induced IL-1 effects on SP, similar to the pathway described for sympathetic neurons in culture. The current study specifically focuses on the effect of ozone on IL-1, NGF, and SP levels in mice BALF and if these mediators can be linked in an inflammatory-neuronal cascade *in vivo*. The experiments demonstrate *in vivo* ozone exposure induces an increase of all three proteins in mouse BALF and that both ozone-induced increases in NGF and SP are mediated by the inflammatory cytokine IL-1. Even though these findings did not definitely link NGF as the mediator of IL-1 effects on SP, it did not conclusively eliminate this possibility. These data did indicate that IL-1 is an early mediator of ozone-induced increase in NGF and SP release in mice *in vivo*.

ACKNOWLEDGEMENTS

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In addition, I would like to acknowledge my mother who has continually provided support during my graduate career and has not only been a great parent but an even better friend.

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I. INTRODUCTION

Although it has been shown in numerous animal models (i.e., mice, rats, guinea pigs and humans) that irritant exposure, specifically ozone, can induce airway inflammation and airway hyperresponsiveness (AHR), the causative mechanism(s) has not been definitely determined. Even so, each of the chemical mediators IL-1, NGF, and SP have been shown to be increased after ozone exposure and have also been shown to separately participate in the resulting ozone-induced AHR.

Exogenous application or increases of any one of these three proteins have been shown to induce AHR by themselves. These mediators have also been shown to exert effects on each other in the airways. For example, the inflammatory cytokine interleukin 1 (IL-1) induces the expression of the neurotrophin Nerve Growth Factor (NGF) and the tachykinin Substance P (SP). Furthermore, exogenous application of NGF increases the synthesis of SP in normal SP-containing neurons and induces SP production in neurons normally devoid of SP. These correlations suggest a possible inflammatory-neuronal cascade that would account for the translation of the ozone insult into the ensuing AHR.

Even though there has been extensive research into the specific down-stream effects of ozone exposure and its mediators including IL-1, NGF, and SP, there has not been an *in vivo* study investigating the possible linking of these mediators. Given the significant role each mediator serves in ozone-induced AHR, this is an important question. The present study attempts to elucidate the *in vivo* causative relationships of these chemical intermediaries. The results demonstrate that the inflammatory cytokine IL-1 mediates the majority of the ozone-induced increases of NGF and SP. The findings suggest that NGF contributes to the ozone-induced

increase in SP, though this needs further investigation. Overall, the findings support the

hypothesis that ozone initiates an inflammatory-neuronal cascade starting with IL-1 production

which triggers NGF release leading to increased SP production.

II. BACKGROUND

A. Ozone (O_3)

As the primary constituent of photochemical smog, ground level ozone is a major urban pollutant and is regulated by the Environmental Protection Agency through the Clean Air Act (United States Environmental Protection Agency, 1990). It is not directly emitted but is formed as a byproduct of fossil fuel combustion from sources such as automobiles, power plants, and the manufacturing industry. The combustion of hydrocarbons releases volatile organic compounds and nitrogen oxides that undergo a photochemical reaction resulting in the ground level matrix of particulate matter and ozone – photochemical smog. Such air pollution is present in virtually all cities but extremely prevalent in very sunny, warm, dry areas with a large number of automobiles, especially where the warm upper air prevents vertical circulation (Baird, 1999). Examples of these ozone-prone areas in the United States are Houston, Los Angeles, Sacramento, San Francisco, and Chicago (United States Environmental Protection Agency, 2006).

Ozone functions as an oxidizing agent and causes clinical symptoms in humans including shortness of breath, chest pain, wheezing, coughing, and the exacerbation of asthma (Menzel, 1984). There is a close correlation between high ozone concentrations and increased hospital visits resulting from respiratory illnesses, specifically asthma (Burnett et al., 1994; Thurston et al., 1992; Romieu et al., 1995; Yang et al., 2003). Effects from exposure to ozone are both transient and prolonged. A recent New England Journal of Medicine article demonstrates a significant increase in respiratory related death rates with increased long-term ozone concentration exposure. The study shows the risk of a respiratory related death is more than

three times as great in the highest ozone concentration metropolitan areas as compared to those with the lowest ozone concentration (Jerret et al., 2009).

The physiological effects of ozone exposure have been shown in numerous animal models including mice (Mustafa et al., 1982), rats (Evans et al., 1988), guinea pigs (Gordon et al., 1984), dogs (Matsui et al., 1991), and in human subjects (Horstman et al., 1990). Pathological changes from ozone exposure include damage to the airway epithelium, pulmonary inflammation and airway hyperresponsiveness (Hazbun et al., 1993; Leikauf et al., 1995; Wu and Lee, 1999; Beckett et al., 1991; Sterner-Kock et al., 2000). The resultant inflammatory response is characterized by neutrophil influx into the trachea and bronchoalveolar space (which is different than eosinophillic influx in allergen induced inflammation) and increased serum protein levels in lavage fluid (Pino et al., 1992; Aris et al., 1993; Basha et al., 1994; Devlin et al., 1996; Krishna et al., 1998; DeLorme et al., 2002). There is also increased smooth muscle response to broncho-constricting agonists, the characteristic feature of airway hyperresponsiveness (AHR) (Hiltermann et al., 1995). The definitive causal relationship between the pulmonary inflammation and the resulting airway hyperresponsiveness is unclear, however much is known about the downstream effects of ozone exposure, including the induction and release of many inflammatory mediators, including cytokines, chemokines, neurotrophins, as well as the release of tachykinins, all of which could produce airway inflammation and AHR.

B. Interleukin-1 (IL-1)

One possible mediator of ozone induced airway hyperresponsiveness and inflammation is the pro-inflammatory cytokine IL-1. Even though the IL-1 family was originally discovered over fifty years ago, it was not discovered until 1985 that IL-1 consisted of two separate entities

– IL-1α and IL-1β (March et al., 1985).   IL-1α is biologically active as both the precursor pro-IL-1α form and as the mature protein (Huising et al., 2004) and is primarily an intracellular mediator (Allan et al., 2005). IL-1β is inactive in its precursor form and is secreted by an undetermined pathway (Thornberry et al., 1992). Even though both are encoded by separate genes, they share high sequence homology and bind to the membrane bound type I IL-1 receptor (IL-1R1) (Sims et al., 1988). The effects of IL-1α and IL-1β can be blocked by a naturally occurring competitive IL-1R antagonist (IL-1Ra) produced and secreted by the same cells that produce IL-1. The IL-1Ra binds to IL-1R1 but does not initiate any signal transduction (Hannum et al., 1990).

IL-1 has been shown to be a major component in irritant induced AHR (Barnes et al., 1998; Park et al., 2004; Johnson et al., 2005) and can induce AHR alone whether from injection in guinea pigs and rats or by *in vitro* application on human isolated bronchi (Frossard et al., 2005). IL-1 has also been shown to increase other possible mediators of AHR, including nerve growth factor (NGF) and substance P (SP). IL-1 has been shown to increases the synthesis and release of NGF from airway structural cells including cultured human airway epithelial cells (Pons, et al 2001; Fox et al., 2001), bronchial smooth muscle cells (Freund et al., 2002; Kemi et al., 2006), and pulmonary fibroblasts (Olgart and Frossard, 2001). Conversely, in cultured human pulmonary fibroblasts (Olgart and Frossard, 2001) and epithelial cells (Pons, et al., 2001) IL-1 mediated increases in NGF can be reduced by application of anti-inflammatory drugs, such as corticosteroids. It has been suggested that IL-1 induction of NGF transcription could be caused by the activation of inflammatory transcriptional factors, like activated protein-1 (AP-1) (Freund et al., 2002), since the AP-1 responsive element is a component of the NGF gene promoter (Lee et al., 1987; Cartwright et al., 1992). Further proof that the AHR effect of IL-1β

may be in part mediated by NGF is the evidence that pre-treatment with an NGF blocking antibody can abolish the IL-1 β-induced AHR in isolated human bronchial smooth muscle strips (Frossard et al., 2005).

Specific effects of IL-1β on airway neurons have also been reported. In ferrets, exogenous IL-1β increases the number of SP-positive neurons of airway ganglia and SP innervation of airway smooth muscle as well as inducing SP production in intrinsic airway neurons which do not normally express it (Wu et al., 2002). In addition, application of IL-1 Ra diminished ozone-enhanced SP levels in the airway and decreases AHR in ferrets (Wu et al., 2008). These studies demonstrate that IL-1 modulates SP expression in airway neurons and participates in ozone-induced AHR.

C. Nerve Growth Factor (NGF)

NGF was originally discovered by Rita Levi-Montalcini and co-workers in the 1960's as a sarcoma-derived substance that stimulated the outgrowth of dorsal root ganglion neurons and is now recognized to be a survival and differentiating factor for neural crest derived sensory neurons, as well as, sympathetic and basal forebrain cholinergic neurons (Levi-Montalcini and Angeletti, 1968; Sofroniew et al, 2001).   Because of this, it is included in the neurotrophin family of peptides, along with brain-derived neurotrophic factor (BDNF), and neurotrophins (NT) -3, -4, -5, and -6 (Frossard, et al 2004), all of which have been established as playing critical roles in vertebrate nervous and cardiovascular development (Tessarollo, 1998; Huang and Reichardt, 2001; Chao, 2003).

NGF is composed of an α, β, and γ subunit with the γ subunit being responsible for its biological activity (Bax, et al., 1993). The actions of NGF are dictated by its binding to one of

two cell surface receptors: (1) the high-affinity tropomyosin-receptor tyrosine kinase A (TrkA), or (2) the low affinity receptor p75, which also interacts with BDNF, NT-3,-4, and -5 (Lindsay and Harmar, 1989; Hempstead, 2002). Of the two receptors, only TrkA uses tyrosine kinase as part of its signal transduction. Among the group of neurotrophins NGF has the highest binding affinity for the TrkA receptor (Ibáñez et al., 1998). Binding of NGF to the TrkA receptor induces its homodimerisation and autophosphorylation, activating many different cell-signaling pathways (Kaplan et al, 1991). The end result is dependent on the specific cell type and subsequent pathways activated and can result in cell proliferation, differentiation, and survival, or apoptosis inhibition (Frossard, et al, 2004). More specifically, NGF has been shown to regulate neuronal phenotypic traits such as stimulus responsiveness (Lewin and Mendell, 1993), response to neuronal injury (Olson et al., 1994) and neuropeptide expression (Kessler and Black, 1980; Otten et al., 1980; Hunter et al., 2000).

Recently, NGF has been shown to not only be a mediator of inflammation resulting in AHR in various *in vitro* and *in vivo* animal models (Woolf, et al, 1994; Braun et al., 1998; Nassenstein, et al, 2004; Bachar, et al, 2004; de Vries, et al 2006) but can specifically cause AHR apparently through direct action on airway smooth muscle (Friberg et al, 2001; de Vries et al., 1999). NGF is produced and released by various inflammatory and structural cells in the airways, including mast cells, eosinophils, neutrophils, monocytes/macrophages, bronchial epithelial and smooth muscle cells, and pulmonary fibroblasts (for review see Frossard et al, 2004). Serum NGF levels are increased in patients with asthma compared to those non-asthmatics (Bonini et al., 1996) and NGF is elevated in bronchoalveolar lavage fluid of asthmatics (Olgart Höglund et al., 2002). Further evidence of NGF's role in AHR has been shown by AHR being induced by tissue specific over-expression of NGF in the airways of

transgenic mice (Hoyle et al., 1998; Päth et al., 2002), and by pre-treatment with anti-NGF antibodies in mice and guinea pigs to reduce allergen induced AHR (Braun et al., 1998; de Vries et al., 2002).

NGF increases the intracellular level and release of SP from a wide range of neurons (Adler et al., 1984; Lindsay and Harmar, 1989; Vedder et al., 1993; Cho et al., 1996; Hoyle, 2003; Malcangio et al., 2000; Skoff et al., 2003;) and causes the phenotypic switch to SP production in airway sensory neurons derived from the nodose ganglia which do not typically express SP (Hunter et al., 2000).

D. Substance P (SP)

The final link between ozone exposure and the subsequent inflammation and AHR could be SP. SP is a member of the tachykinin family and was first discovered over 80 years ago (von Euler and Gaddum, 1931). It primarily binds with high affinity to the neurokinin 1 (NK_1) receptor but does also bind, with low affinity, to the NK_2 and NK_3 receptors (Maggi et al., 1993; Regoli et al., 1994; Severini et al., 2002). The NK_1 receptor is a seven-transmembrane G-protein coupled receptor and activates a phosphatidylinositol-calcium second messenger system. It is important to realize that release of tachykinins from sensory nerves in the airways requires a stimulus of chemical origin.

With regard to airway innervation, SP is synthesized by translation of the preprotachykinin mRNA in nerve cell bodies of sensory neurons located in the nodose and jugular ganglia and, in response to exposure to ozone and other irritants, released from nerve terminals located in the airway epithelium, smooth muscle, and mucosal blood vessels (Lee et al., 1979; Koto et al., 1995; Joad et al., 1996). SP release has also been documented from

intrinsic airway neurons (Wu et al., 2001 and 2003) and has been shown to be a central mediator

of ozone-induced AHR (Lee et al., 1979; Koto et al., 1995; Joad et al., 1996; Wu et al., 1997;

Graham et al., 2001). SP nerves are relatively prevalent in the airways as evident by

radioimmunoassay and immunohistochemistry showing SP nerves beneath and within the

epithelium, around blood vessels, and submucosal glands and within the bronchial smooth

muscle layer (Lundberg et al., 1984; Luts et al., 1993). The NK_1 receptor is expressed in the

airway epithelium, the submucosa, endothelium of blood vessels, goblet cells, surfaces of

inflammatory cells (Chu et al., 2000) and in airway smooth muscle cells (Maghni et al., 2003).

Ultimately, SP induces AHR by increasing the cholinergic sensitivity of airway smooth muscle

(Tanaka and Grunstein, 1984) and has been shown to facilitate acetylcholine release from post-

ganglionic nerves in many species including guinea pigs, rabbits, mouse and humans (Joos et al.,

1988; Hall et al., 1989; Black et al., 1990; Colasurdo et al., 1995; Tournoy et al., 2003).

Blocking the action of SP reverses airway hyperreactivity. Aerosolized neutral

endopeptidase, the enzyme responsible for neutralizing released SP, diminishes ozone-induced

AHR (Nadel, 1991; Murlas et al., 1992). Depletion of SP by capsaicin treatment prior to ozone

exposure prevents AHR, by decreasing the bronchial influx of neutrophils and subsequent

bronchial edema (Tepper et al., 1993; Kaneko et al., 1994; Koto, et al, 1995). Furthermore, it

has been shown *in vivo* that NK_1 receptor antagonist attenuates both the ozone-enhanced airway

responses in ferret trachea (Wu et al., 2001 and 2003) and the NGF-induced AHR in guinea pigs

(de Vries, et al., 1999). This is important in alluding to NGF/TrkA-mediating SP levels in the

airways. Additional proof pointing to this possible cascade is provided by a recent study,

showing an increase in SP as well as trkA in large-diameter neurons in the nodose and jugular

ganglia after injection of NGF in the lower airways of mice, suggesting trkA mediates SP induction of neurogenic airway inflammation (Dinh et al., 2004)

E. Statement of Problem

The chemical mediators IL-1, NGF, and SP are increased after ozone exposure and have been shown to participate in the resulting AHR. In addition, it has also been demonstrated that SP can be induced by both exogenous application of the pro-inflammatory cytokine IL-1 or the neurotrophin NGF and that NGF itself can be induced by exogenous application of IL-1. These findings suggest a possible inflammatory-neuronal cascade that would translate the ozone insult to the ensuing airway pathologies.

F. Hypothesis

The hypothesis that will be studied is that ozone exposure induces airway hyperresponsiveness (AHR) by increasing the level of interleukin-1 (IL-1), which in turn increases the release of nerve growth factor (NGF), which ultimately enhances substance P (SP) release (Figure 1).
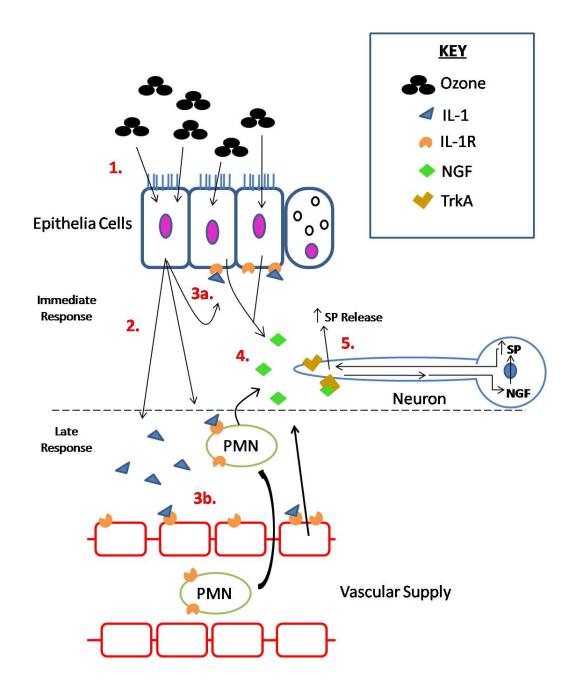
Figure 1. A possible illustration of the proposed inflammatory-neuronal cascade for airway hyper-responsiveness induced by ozone exposure and investigated by this project. Exposure to ozone injures bronchial epithelial cells (**1.**), causing them to release IL-1 (**2.**). This in turn, allows IL-1 to bind to its receptor, IL-1R, on the surface of bronchial (**3a**) and vascular (**3b**) epithelial cells. The binding of IL-1 to the IL-1R of the bronchial epithelial cells releases NGF (**4.**), while the binding of IL-1 to IL-1R on the vascular endothelial cells allows for the passage of polymorhphonuclear cells (PMN) to pass in to the bronchial space, bind IL-1 with their own IL-1R and release NGF (**4.**). NGF from either/both sources binds to trkA receptors on substance P producing neurons, increasing the production and release of substance P (**5.**).

G. Rationale

The rationale of these experiments was two-fold:

(1) Test *in vivo* the above proposed mechanism to determine if an inflammatory-neuron cascade pathway involving IL-1, NGF, and SP is responsible for irritant induced airway hyper-responsiveness

(2) Establish in a murine model the responses we have already characterized using an *in vivo* ferret model. The murine model would provide more cellular manipulations and investigative techniques (i.e., transgenic mice models, small interfering-RNA) for future studies.

H. Specific Aims and Expected Results

1. Determine the effect of ozone on the concentrations of IL-1, NGF, and SP in BALF from an *in vivo* murine exposure model.

2. Determine if IL-1Ra is able to diminish the expected ozone-induced increases of NGF and SP in mouse BALF.

3. Determine if either a tyrosine kinase inhibitor K252a or an anti-NGF antibody will diminish the expected ozone-induced increase of SP in mouse BALF.

The following table is a summary of our experimental design and the expected results:

Experimental Design	Proposed Mechanism Ozone → ↑ IL-1 → ↑ NGF → ↑ SP				Expected Results
	Ozone	IL-1	NGF	SP	
Ozone	+	↑	↑	↑	
IL-1	NA	Added	↑	↑	Expected Results
IL-1Ra + Ozone	+	↑	↓	↓	
K252a + Ozone	+	↑	↑	↓	
Anti-NGF + Ozone	+	↑	↑	↓	

III. MATERIALS AND METHODS

A. Animal use and anesthetics

Adult (8 weeks old) male ICR mice (Harlan Laboratories, Inc) were housed four 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. Mice were anesthetized with a ketamine/xylazine mixture in a single intraperitoneal injection before intratracheal (*i.t.*) instillations. Animals were sacrificed 24 hours after ozone/air exposure or *i.t.* instillations with a lethal dose of sodium pentobarbital (200mg/kg). All procedures were approved through ACUC review under Protocol #06-0501.

B. I.T. instillation of IL-1 or IL-1 receptor antagonist (IL-1Ra)

Mice were anesthetized with a dosage of ketamine/xylazine (25mg/kg and 2mg/kg, respectively) mixture in a single *i.p.* injection. An 21-guage steel tube, 4.7 cm in length connected to a 1 ml tuberculin syringe filled with either 20µl of IL-1β (200µl/ml; Santa Cruz Biotechnology, Inc.), IL-1Ra (200ng/ml; Amgen, Inc.), or saline. The tube was inserted through the oral cavity and pharynx into the trachea and the solution was instilled into the trachea. One hour after the last IL-1Ra or saline instillation, mice were exposed *in vivo* to 2ppm ozone for three hours. The mice were allowed to recover from the anesthetic and returned to the animal facility until the following day.

C. I.T. instillation of the tyrosine kinase inhibitor K252a or anti-NGF antibody

Mice were anesthetized and underwent identical ozone exposure as above. Mice received a 20µl intratracheal (*i.t.*) instillation of anti-NGF antibody (200µg/ml; Santa Cruz Biotechnology, Inc.) or saline one, two, or four hours before ozone exposure. The tyrosine inhibitor K252a (100µg/ml; Alexis Biochemicals; Sand Diego, CA) was administered in two different ways. Initially, the K252a was dissolved in 100% dimethyl sulfoxide (DMSO) and administered to mice by *i.t.* instillation two or four hours prior to ozone exposure. For a second set of experiments, the inhibitor was dissolved in a 10% DMSO in saline solution and then administered by *i.t.* instillation to the mice at four hours prior to ozone exposure. The last experimental design utilized *i.t.* instillation of the K252a DMSO/PBS solution twelve and three hours prior to ozone exposure and then immediately after the exposure; a total of three separate *i.t.* instillations of K252a were given to each mouse.

D. *In vivo* ozone exposure

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

E. Bronchoalveolar lavage fluid (BALF) collection

Twenty-four hours after ozone/air exposure or the final *i.t.* instillation, mice were anesthetized with a single dose of sodium pentobarbital injection (*i.p*). The lungs were lavaged with 3ml PBS (1.5ml, twice) through a tracheal cannula and the BALF was placed into 15ml conical tubes with 30µl of the proteinase inhibitor phosphoramidon (1×10^{-4} µM) to inhibit neutral endopeptidases which degrade SP. The collected BAL fluid was centrifuged at 1,500 rpm for 10-12 min. at 15ºC. The supernatant was aliquoted and frozen at -80ºC for subsequent assays.

F. IL-1 Enzyme-Linked Immunosorbent Assay

The BALF supernatant samples (initial 3ml) were frozen at -80ºC. The concentration of IL-1 (31-100pg/ml) in each sample was assayed using the mouse IL-1β/IL-1F2 DuoSet® ELISA Development System (R&D Systems, Inc; Minneapolis, MN) according to the manufacturer's instructions. IL-1 was detected using an anti-body sandwich format in 96-well plates (Figure 2).

Each well was initially coated with 100µl of rat anti-mouse IL-1β and incubated overnight followed by 1 hr. incubation with blocking buffer (300µl/well) to prevent non-specific binding. Either 100µl of lavage supernatant or 100µl of NGF standard (31-1000 pg/ml) was added to each well. The plate was incubated for 2 hr. followed by 2 hr. incubation with biotinylated goat anti-mouse IL-1β (100µl/well). For color development, streptavidin conjugated to horseradish peroxidase was added to each well (100µl) followed by a TMB solution, which reacts with the peroxidase-labeled conjugates to develop a blue color. The reaction was stopped after 10 min. with 2N sulfuric acid (50µl/well) causing the blue to change to yellow upon acidification. The absorbance of each well was measured at 450nm, with a wavelength correction at 540nm, on a Spectra Max 340pc plate reader (Molecular Devices, Sunnyvale, CA). The concentration of IL-1 in each lavage sample was extracted from an IL-1 standard curve. All samples were run in triplicate, and as a negative control, a PBS sample was run with each assay.

G. NGF Enzyme-Linked Immunosorbent Assay

The BALF supernatant samples (initial 3ml) were frozen at -80ºC. The concentration of NGF (7.8-1000 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 (Figure 2). Each well was initially coated with 100µl of anti-NGF pAb and incubated overnight followed by 1 hr. incubation with blocking buffer (200µl/well) to prevent non-specific binding. Either 100µl of lavage supernatant or 100µl of NGF standard (7.8-1000 pg/ml) was added to each well. The plate was incubated for 6 hr. followed by an overnight incubation with anti-NGF mAb (100µl/well). For color development an anti-rat IgG, horseradish peroxidase conjugate antibody was added to each well (100µl) followed by a TMB solution, which reacts with the peroxidase-labeled conjugates to develop a

blue color. The reaction was stopped after 10 min. with 1N hydrochloric acid (100μl/well)

causing the blue to change to yellow upon acidification. The absorbance of each well was

measured at 450nm 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 triplicate, and as a negative control, a PBS sample was run with each

assay.
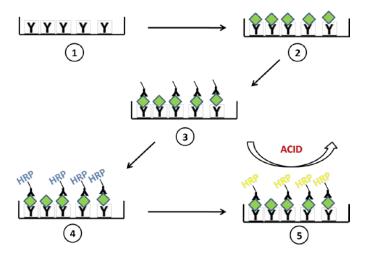
Figure 2. Illustration of a sandwich ELISA. (1) ELISA plate is coated with capture antibody. (2) Sample is added and the respective antigen present binds to capture antibody. (3) Biotin-conjugated secondary detection antibody is added and binds to the antigen captured by the first antibody. (4) Streptavidin-HRP is added and binds to the biotin conjugated detection antibody. (5) Colored product is formed in proportion to the amount of antigen is present in the sample. The reaction is stopped by acid addition and absorbance measured at 450 nm.

H. SP Enzyme-Linked Immunosorbent Assay

The BALF supernatant samples (initial 3ml) were frozen at -80ºC. The concentration of

SP (39-2500 pg/ml) in each sample was assayed using the Parameter Substance P Assay (R&D

Systems, Inc; Minneapolis, MN) according to the manufacturer's instructions. SP was detected

using a competitive binding technique in 96 well goat anti-mouse microplate (Figure 3). Either

50μl of lavage supernatant or SP standard (39-2500 pg/ml) was added to each well. Each well

then received 50μl of the primary antibody solution (mouse monoclonal anti-SP) and 50μl of SP

conjugated with horseradish peroxidase. After a 3 hr. incubation and wash, 200μl of TMB was

added for color development. The reaction was stopped after 30 min. with 2N sulfuric acid

(50μl/well) causing the blue to change to yellow upon acidification. The absorbance of each

well was measured at 450nm, with a wavelength correction at 540nm, on a Spectra Max 340pc

plate reader (Molecular Devices, Sunnyvale, CA). The concentration of SP in each lavage

sample was extracted from an SP standard curve. All samples were run in triplicate, and as a

negative control, a PBS sample was run with each assay.
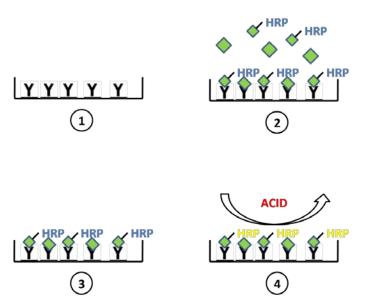
Figure 2. Illustration of a competitive ELISA. (1) ELISA plate is pre-coated with capture antibody. (2) Sample and Tracer antigen (HRP-conjugated SP) are added and compete for a limited number of antigen binding sites present on the capture antibody. (3) Excess sample and tracer antigen are rinsed off. (4) Colored product is formed in an inverse proportion to the amount of antigen present in the sample. The reaction is stopped by acid addition and absorbance measured at 450 nm.

I. Statistical Methods

In all studies, differences among groups were determined using a two-tailed Student's T-Test using Microsoft Excel software. Mean and standard error is reported for each value.

IV. RESULTS

1. Effect of ozone on IL-1, NGF, & SP in BALF

Experiments were conducted to evaluate the effects of ozone on IL-1, NGF, and SP levels in bronchoalveolar lavage fluid (BALF) as compared to control animals exposed to air. BALF was collected 24 hours after ozone/air exposure. Ozone exposure significantly increased levels of IL-1 (210.3 ± 38 pg/ml, n=4), NGF (3230.7 ± 1039 pg/ml, n=6), and SP (4280.3 ± 635 pg/ml, n=4) when compared to air controls (114.6 ± 9.8 pg/ml (n=6), 753.3 ± 217 pg/ml (n=5), and 1488.7 ± 459 pg/ml (n=3), respectively) ($P \leq 0.05$, Figure 4).
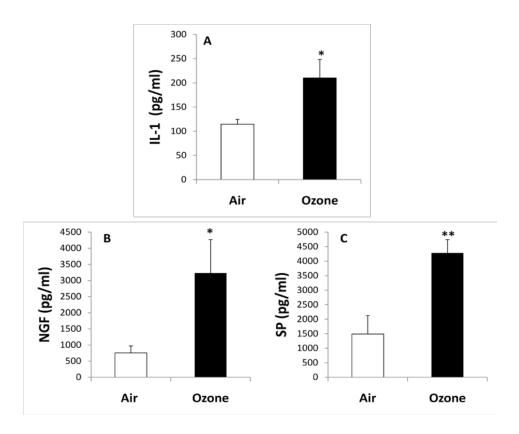
Figure 4. Effect of ozone exposure on the levels of (A) IL-1(air n=6, ozone n=4), (B) NGF (air n=5, ozone n=6), and (C) substance P (air n=3, ozone n=4) in mouse bronchoalveolar lavage fluid as measured by ELISA. Values are means $\pm$ SE. * Significant difference between air and ozone exposed animals, $P = 0.03$. ** Significant difference between air and ozone exposed animals, $P = 0.007$.

2. Effect of exogenous IL-1 on NGF and SP levels in BALF

Experiments were conducted to determine if intra-tracheal (*i.t.*) instillation of exogenous IL-1 would increase NGF and SP in BALF collected 24 hours after instillation and compared to control animals which received a saline *i.t.* instillation. It is important to note that neither the experimental nor control animals were exposed to ozone. NGF levels significantly increased from 1349.7 ± 463 pg/ml in the control animals to 3424.8 ± 915 pg/ml in IL-1 treated animals (n=5 both groups) (P≤0.04, Figure 5A). BALF from IL-1 treated animals also contained a very significantly higher concentration of SP (4970.7 pg/ml, n=6) than BALF collected from saline controls (1557.8 pg/ml, n=6) (P≤0.0005, Figure 5B)
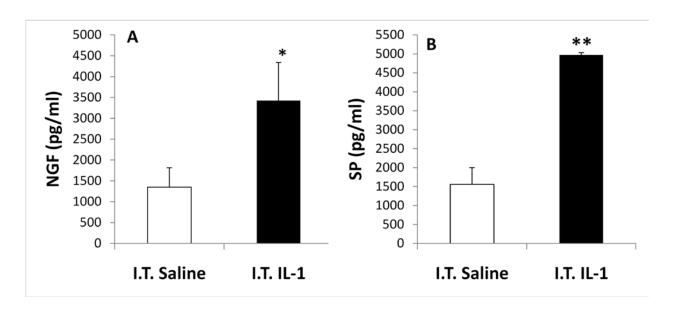
Figure 5. Effect of IL-1 treatment on the levels of (A) NGF (n=5) and (B) substance P (n=6) in mouse bronchoalveolar lavage fluid as measured by ELISA. Values are means ± SE. * Significant difference between treated and untreated animals, P = 0.04. ** Significant difference between treated and untreated animals, P = 0.0005.

3. IL-1 Ra reduces ozone induced NGF and SP levels in BALF

Animals that were administered an *i.t.* instillation of IL-1 Ra prior to ozone exposure had significantly decreased levels of both NGF (2070 ± 371 pg/ml, n=6) and SP (1852.2 ± 567 pg/ml, n=5) in BALF than in control animals that received an *i.t.* instillation of saline prior to ozone exposure (3696.5 ± 681 pg/ml, n=6 and 4959 ± 1038 pg/ml, n=4, respectively). (Figure 6)
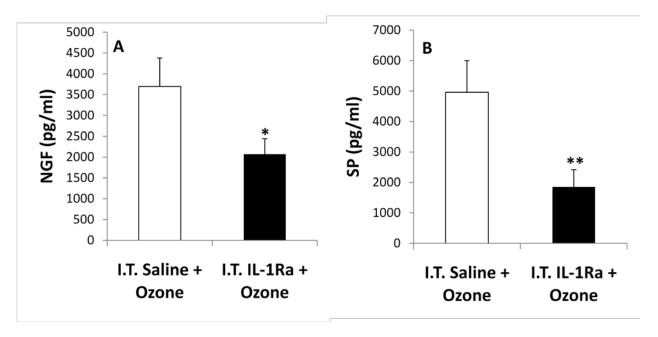
Figure 6. Effect of IL-1 receptor antagonist on the levels of (A) NGF (n=6) and (B) substance P (i.t. saline, n=4, i.t. IL-1Ra n=5) in mouse bronchoalveolar lavage fluid as measured by ELISA. Values are means ± SE. * Significant difference between treated and untreated animals, P=0.03. ** Significant difference between treated and untreated animals, P = 0.01.

4. Tyrosine kinase inhibitor K252a failed to effect SP levels in BALF

K252a was first dissolved in DMSO and administered to mice via an *i.t.* instillation two and four hours before ozone exposure while control groups were administered an instillation of DMSO at the same time prior to ozone exposure. There was no significant difference between

the two hour (4568.8 ± 447.2 pg/ml, n=6) or four hour K252a pre-treatment groups (4405.4 ±

972 pg/ml, n=5) and their saline control groups (4871.4 ± 315.3 pg/ml, n=5 and 3927.7 ± 1515

pg/ml, n=3, respectively) (Figure 7, A and B).

The next two experiments used K252a dissolved in a 10% DMSO in PBS solution.

Initially mice were given an *i.t.* instillation once four hours prior to ozone exposure with control

mice receiving the 10% DMSO in PBS solution only. As before, there was no significant

difference between K252a pre-treated animals (4130.5 ± 1019.6 pg/ml, n=4) and the control

animals (4233.3 ± 2008.3 pg/ml, n=3) (Figure 7C).

In the following experiment, animals received an *i.t.* instillation of the K252a solution 12

hours and 3 hours prior to ozone exposure and then re-administered K252a immediately ***after*** the

ozone exposure. Control animals were given the DMSO/PBS vehicle solution at the same time

prior to and after their ozone exposure. Again, there was no significant difference between the

K252a treated group (5498.7 ± 21.1 pg/ml, n=5) and the vehicle treated group (5560.1 ± 2.3

pg/ml, n=5) (Figure 7D).
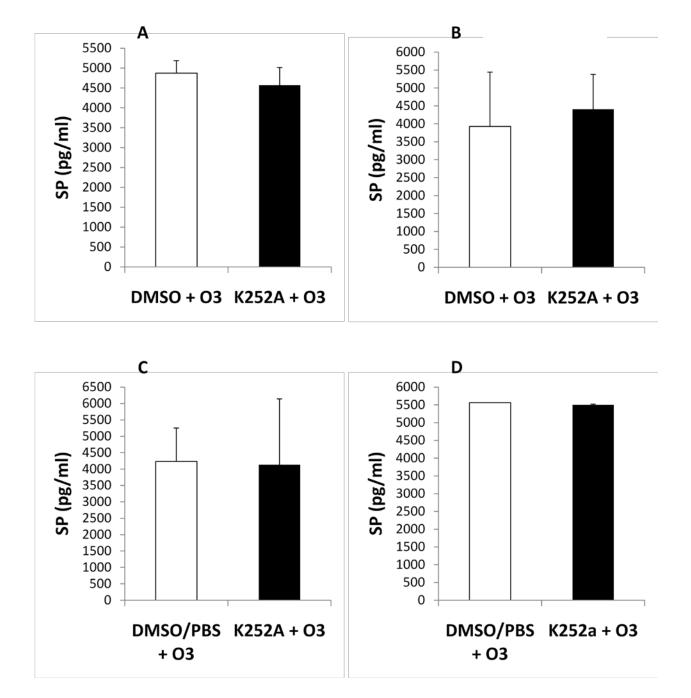
Figure 7. Effect of the tyrosine kinase inhibitor K252a on the levels of substance P in mouse bronchoalveolar lavage fluid as measured by ELISA. (A) K252a instilled two hours prior to ozone exposure (DMSO, n=5, K252a, n=6). (B) K252a instilled four hours prior to ozone exposure (DMSO, n=3, K252a, n=5). (C) K252a in 10% DMSO/PBS delivered four hours before exposure (DMSO/PBS, n=3, K252a, n=4). (D) Instillation of K252a in 10% DMSO/PBS twelve and three hours prior to and immediately after ozone exposure (n=5). Values are means ± SE.

5. Effect of anti-NGF antibody application on SP levels in BALF

Experimental groups of mice were administered an *i.t.* instillation of anti-NGF antibody one, two or four hours prior to ozone exposure. Control animals received a saline *i.t.* instillation at matched times before ozone exposure. Neither the one hour antibody pre-treatment group (3977.6 ± 192.7 pg/ml, n=5) nor the two hour antibody pre-treatment group (5472 ± 172.8 pg/ml, n=5) were significantly different from the saline control groups (3877.5 ± 867.4 pg/ml, n=4 and 5290.5 ± 396.9 pg/ml, n=4, respectively) (Figure 8, A and B). Even though the difference between the four hour antibody pre-treatment (6229.8 ± 67.4 pg/ml, n=5) and the matched saline control group (6505.5 ± 41.4 pg/ml, n=4) appeared to be minimal (275.7 pg/ml), it was significantly different (P=0.01) (Figure 8C).
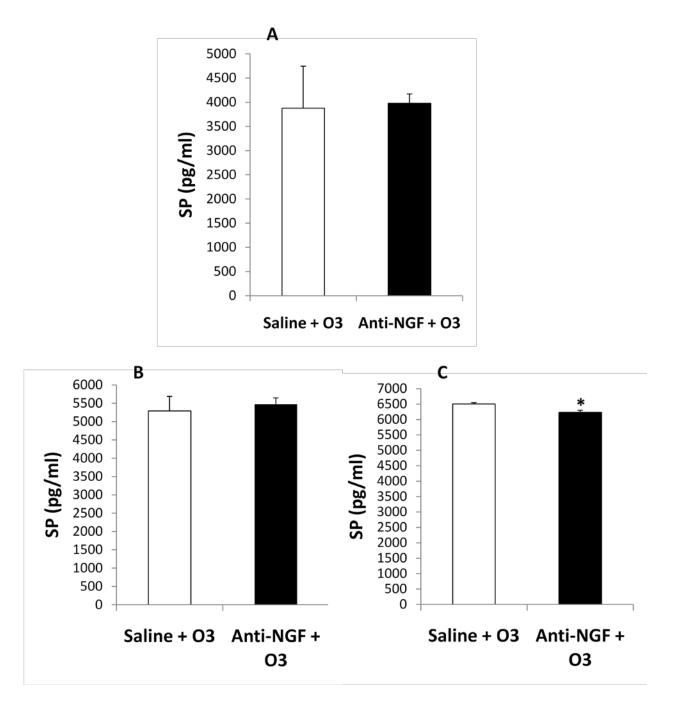
Figure 8. Effect of anti-NGF antibody on the level of substance P in mouse bronchoalveolar lavage fluid as measured by ELISA. (A) Anti-NGF antibody instilled one hour prior to ozone exposure. (B) Anti-NGF antibody instilled two hours prior to ozone exposure. (C) Anti-NGF antibody instilled four hours before exposure. Saline, n=4, Anti-NGF, n=5 for all experiments. Values are means $\pm$ SE. * Significant difference between treated and untreated animals, $P = 0.01$.

V. DISCUSSION

Ozone is an ubiquitous air pollutant and has been shown to cause numerous respiratory effects (Lippmann, 1989; Beckett, 1991). Of these effects, airway inflammation and AHR are the hallmark pulmonary characteristics of ozone exposure. This has been observed in animal models and humans. For example, human subjects who show sensitivity to ozone exposure have bronchial hyperresponsiveness to methacholine challenge not observed in non-sensitive subjects (Hackney et al., 1989; Aris et al., 1991). Asthmatics had increased inflammation markers in nasal lavage fluid after low level ozone challenge (0.12-0.24 ppm) when compared to non-asthmatic control subjects (McBride et al., 1994). The precise mechanism by which ozone exposure causes AHR has not yet been fully elucidated, but previous work has suggested that the airway inflammation seen after ozone exposure is an underlying cause. The current study specifically investigates a possible signaling pathway responsible for translating ozone exposure to airway inflammation through the release of a sensory neuropeptide, substance P, a mediator proven to mediate neurogenic inflammation in the airways (Lundberg et al., 1983).

The study demonstrates *in vivo* that ozone exposure significantly increases the concentrations of IL-1, NGF, and SP in mouse BALF. All three of these mediators have previously been shown to promote airway inflammation, consistent with other animal models of ozone exposure and epidemiological studies (Barnes et al., 2001; Park et al., 2004; Johnson et al., 2005; Woolf, et al, 1994; Braun et al., 1998; Nassenstein, et al, 2004; Bachar, et al, 2004; de Vries, et al 2006; Lee et al., 1979; Koto et al., 1995; Joad et al., 1996; Wu et al., 1997; Graham et al., 2001).

The results showing IL-1 as a key modulator of SP levels in BALF are consistent with our previous *in vivo* and *in vitro* reports in ferret (Wu et al., 2001, 2003) which showed that IL-1

released after ozone exposure enhances airway responsiveness by modulating SP levels. This present study further confirms the role of IL-1 as a key intermediate signaling molecule in the downstream effects of ozone exposure by showing its modulatory effects on NGF. Previous research has shown that IL-1 can modulate NGF expression *in vitro (*Olgart and Frossard, 2001; Pons, et al, 2001; Freund et al., 2002) and increased levels of NGF has been reported in humans, *in vivo*, in situations where IL-1 β is increased, particularly in asthma (Bonini et al., 1996; Olgart et al., 2002; Virchow et al., 1998; Kassel et al., 2001). However, this study is the first report demonstrating the direct manipulation of *in vivo* NGF levels via IL-1, whether exogenously applied or by blockade of the ozone-induced increase of endogenous IL-1.

This study fails in proving NGF is a key mediator of ozone-induced increases in SP, but only in the sense of NGF's action through the TrkA receptor. The inability of K252a or anti-NGF to decrease SP concentration in mouse BALF could be dependent upon the method of blocking NGF effects. K252a blocks the tyrosine kinase pathway and the NGF antibody decreases NGF binding to receptors. However, neither of these approaches would affect the SP already present in neurons that would be *released* during ozone exposure. Another possibility is that SP release could occur through an IL-1 related pathway not involving NGF or the release of unrelated mediators such as TRPV1 ligands, bradykinin, or leukotrienes (Carr and Undem, 2001).

NGF is classified as a neurotrophin but there is increasing evidence suggesting it is involved in a variety of immune functions. NGF has even been implicated as an asthma mediator but direct evidence of inhibiting or blocking NGF induced effects is insufficient, especially *in vivo*. Adding to this, the source and regulation of NGF expression in airways is not fully understood, but a direct action of NGF on neurons may be part of this response since NGF

increases the number of neurons and neuropeptide content in airways (Adler et al., 1984; Lindsay and Harmar, 1989; Vedder et al., 1993; Cho et al., 1995; Hoyle et al., 1998; Malcangio et al., 2000; Hunter et al., 2000; Skoff et al., 2003).

Although most of the data to this point suggests that TrkA mediates the majority of NGF effects, there is growing evidence that p75 is more important than first thought in cellular response to injury. For example, it has been shown that the p75 receptor modulates the NGF excitation of adult rat nociceptive neurons (Zhang and Nicol, 2004). Peripheral tissue inflammation increased p75 mRNA levels in dorsal root ganglia of rats while TrkA mRNA levels remained the same (Cho et al., 1996). Intrathecal infusion of NGF into adult dorsal root ganglia increases the concentration of p75 receptors but not TrkA receptors (Verge et al., 1989). Additionally, local application of NGF in the iris increased p75 mRNA in sympathetic neurons but had no effect on TrkA expression (Miller et al., 1994). Studies with NGF-over expressing mice and p75-NTR knockout mice show that NGF effects are partly mediated by the p75 receptor in allergic airway and enhance the severity of the local inflammatory response (Lommatzsch et al., 2003). Furthermore, Skoff and Adler (2006) demonstrated that *in vitro* NGF up-regulation of SP expression in adult rat dorsal root ganglia sensory neurons requires both the p75 and TrkA receptors. Even though they did not demonstrate the specific contribution of each to the SP increase, they were able to show that blockade of the p75 receptor markedly reduces SP expression. Obviously, the role this receptor has in ozone-induced increases of SP in mouse BALF needs to be investigated before NGF can be ruled out as a key mediator in the proposed transduction pathway.

In conclusion, this study demonstrates that ozone increases the concentrations of all three proteins in mouse BALF, and that IL-1 is a key *in vivo* mediator of the ozone-induced increases

of NGF and SP in mouse BALF. NGF may be a mediator of the ozone-induced increase of SP but these results raise the possibility that a pathway other than the TrkA receptor may transduce ozone mediated signaling through NGF. Further studies involving the precise role the p75 receptor has in this proposed mechanism are needed and could include the blockade of the p75 receptor before ozone exposure or by exposing p75-NTR knockout mice to ozone, then measuring the level of SP in the BALF. These studies may provide the pertinent information for solidifying this inflammatory-neuronal cascade.

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VII. CURRICULUM VITAE

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Publications

Papers and Monographs

Zhu, Y., J. Luo, J. Barker, J. Hochberg, E. Cilento, and F. Reilly. Up-regulation of truncated long interspersed nucleotide element 1 (L1) and myotubularin by rapid intraoperative tissue expansion in mouse skin. Microcirculation. The 7th World Congress for Microcirculation, Monduzzi Editore, Bologna, Italy, pp. 605-609, 2001.

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ABSTRACTS

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