MECHANISMS OF OZONE INDUCED AIRWAY HYPERREACTIVITY CHANGE OVER TIME

by

Kirsten C. Verhein

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

Kirsten C. Verhein

has been approved

Mentor/Advisor

Member

Member

Member

Member

Member

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PUBLICATIONS

Manuscripts:

1. **Verhein KC**, Jacoby DB, Fryer AD. Persistent, but not immediate, airway hyperreactivity is mediated through IL-1 receptors is guinea pigs. *Am J Respir Cell Mol Biol*, 39:730-8, 2008.

2. **Verhein KC***, Hazari MS*, Jacoby DB, Fryer AD. Three days after a single exposure to ozone the mechanism of airway hyperreactivity is dependent upon substance P and nerve growth factor. [Submitted: *Am J Physiol Lung Cell Mol Physiol*, January 2010]. *Co-first authors.

3. **Verhein KC**, Jacoby DB, Fryer AD. Dual p38/JNK mitogen activated protein kinase inhibitors prevent ozone-induced airway hyperreactivity in guinea pigs. [In preparation: *PLoS One*, January 2010].

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5. **Blensly KC**, Jacoby DB, Fryer AD. (2006) Ozone Induced Hyperreactivity Is Mediated by IL-1 in Guinea Pigs. Proceedings of the American Thoracic Society 3:A821.

ABSTRACT

In both humans and animals, ozone induces airway inflammation and airway hyperreactivity: defined as increased airway narrowing in response to irritants or neurotransmitters that would normally have a small or no effect in non-ozone exposed lungs. Ozone-induced airway hyperreactivity is mediated by increased release of neurotransmitters by parasympathetic nerves. In guinea pigs, eosinophils increase acetylcholine neurotransmitter release from parasympathetic nerves one day after ozone exposure via release of major basic protein that blocks neuronal M_2 muscarinic receptors that normally inhibit acetylcholine release. However, three days after ozone neuronal M_2 muscarinic receptor dysfunction and eosinophils no longer contribute to airway hyperreactivity. The hypothesis tested in this thesis is that ozone causes airway hyperreactivity via multiple mechanisms that change between one and three days after exposure from eosinophil mediated hyperreactivity to inflammatory cytokine and neural plasticity mediated hyperreactivity.

Using guinea pigs exposed to ozone and measuring lung inflation pressure, heart rate, mucus production, airway inflammation, and cytokine levels, the involvement of mitogen activated protein kinases, the inflammatory cytokine IL-1, nerve growth factor, and tachykinins were tested in ozone-induced airway hyperreactivity one and three days later. These studies were complimented with ex vivo studies of inflammatory mediators on parasympathetic neural plasticity.

Data demonstrate that mitogen activated protein kinase signaling may precede eosinophil activation and release of major basic protein since inhibition of p38 and JNK mitogen

activated protein kinase prevents M_2 receptor dysfunction one day after ozone (Chapter III). However, IL-1 is not involved in ozone-induced airway hyperreactivity one day after ozone. In contrast, blocking IL-1 with an IL-1 receptor antagonist (Chapter IV) or depleting substance P with an antibody to nerve growth factor (Chapter V) prevents airway hyperreactivity three days after ozone without changing inflammatory cell populations in bronchoalveolar lavage. Thus, mechanisms of ozone-induced airway hyperreactivity change from eosinophils and activity of early kinases, to inflammatory cytokines and neural plasticity.

These findings are important for human health since over half the population of the United States lives in areas with unhealthy levels of ozone. Ozone increases asthma exacerbations and increases hospitalizations and mortality not only the same day ozone levels are high, but also two and three days later. Currently there are no specific therapies available to treat ozone-induced lung complications. Instead, standard asthma therapies that directly dilate airway smooth muscle or non-specifically decrease inflammation are used. Findings presented here demonstrate that different treatments targeting specific mediators may be more beneficial since the mechanisms of ozone-induced airway hyperreactivity change over time.

In summary, this thesis demonstrates that mitogen activated protein kinases are important one day after ozone and that IL-1, tachykinin receptors, and nerve growth factor all contribute to airway hyperreactivity three days, but not one day, after ozone. These data are the first to suggest that hyperreactivity is not mediated by a single mechanism and

that even after a single insult to the lungs, the mechanisms of hyperreactivity and the role of inflammation change with time.

CHAPTER I.

INTRODUCTION

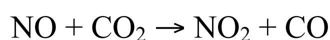
INTRODUCTION

The primary function of the lungs is gas exchange: providing oxygen to and removing carbon dioxide from the circulation. In addition, the lungs are an immune organ essential to host defense in that they serve as both a physical barrier between the external and internal environments and contain resident immune cells, which can quickly mount an inflammatory response. This dissertation focuses on how ozone damage to the lungs changes nerve and inflammatory cell function.

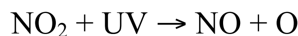
A. OZONE

1. Chemical nature and synthesis

Ozone is both beneficial and harmful in nature. In the upper atmosphere, ozone protects the earth by preventing harmful ultraviolet radiation from reaching the earth's surface. Ground level ozone, however, is a pollutant and is harmful to humans and animals. Ground level ozone is the primary component of smog and is formed by the reaction of sunlight with hydrocarbons and nitrogen from vehicle exhaust.



$$NO + CO_2 \rightarrow NO_2 + CO$$



$$NO_2 + UV \rightarrow NO + O$$

$$O + O_2 \rightarrow O_3$$

The United States Environmental Protection Agency sets the standards for what is an acceptable level of ozone exposure. The National Ambient Air Quality Standard set for ozone is 0.075 ppm (parts per million), averaged over an 8 hour day (epa.gov). Over half

the population of the United States lives in counties with unhealthy levels of ozone exposure as measured by the number of days where ozone concentrations are above the national standard (Association, 2009).

2. Epidemiology

It is well known that humans exposed to ozone have decreased lung function that is particularly severe in children and people with asthma. This has led to a significant body of literature cataloguing the adverse effects and time course of ozone-induced lung damage (Balmes, 1993; Bell *et al.*, 2004; Tatum *et al.*, 2005). However, none have identified a specific mechanism for ozone-induced decreases in lung function.

A number of studies show that adverse effects of ozone persist for days after exposure and in some cases the development of adverse effects lags several days behind the acute exposure (Chan *et al.*, 2005; Lewis *et al.*, 2005; Romieu *et al.*, 1995). Asthmatic children exhibit the most severe clinical symptoms two to three days after ozone when compared to the day of exposure (Lewis *et al.*, 2005; Romieu *et al.*, 1995). In addition to children, lung function in adults declines significantly up to two days following high environmental ozone exposure (Chan *et al.*, 2005). This lag between ozone and adverse effects may be the result of increased airway inflammation.

3. Ozone and lung function

a. Lung function measurements

In humans, common lung function measurements include forced vital capacity and forced expiratory volume in one second (FEV_1). These measurements are simple and non-invasive. Forced vital capacity measures the total volume of air that can forcibly be exhaled after a deep breath. FEV_1 measures how much air can be forcibly exhaled in one second. A normal FEV_1 value is about 80% of the forced vital capacity. Disease states and lung injury affect both FEV_1 and forced vital capacity. For example, in restrictive diseases, where airway resistance increases such as asthma, both forced vital capacity and FEV_1 are reduced. In obstructive airway diseases, such as emphysema, FEV_1 is more significantly reduced than forced vital capacity (West, 2008). Exposure to ozone decreases both FEV_1 and forced vital capacity.

An additional measurement of lung function is airway responsiveness. Airway hyperresponsiveness is exaggerated bronchoconstriction in response to a wide variety of stimuli including pharmacological agents such as methacholine or histamine, and natural stimuli such as exercise or cold air (Hargreave *et al.*, 1986). Hyper-responsiveness is typically assessed by measuring lung function before and after inhalation of increasing concentrations of a stimulus (i.e. methacholine). The more sensitive the airways are, the lower the concentration of agonist that is necessary for decreasing lung function. Normal human lungs constrict in response to inhaled bronchoconstrictors while asthmatic human lungs constrict with a lower concentration of inhaled agonist (Hopp *et al.*, 1985). There

are many mechanisms that contribute to airway hyperresponsiveness, including ozone exposure and airway inflammation, which will be described below.

In animals, measurements of lung function usually only include measuring pulmonary inflation pressure since it is not possible to measure either forced vital capacity or FEV_1. Pulmonary inflation pressure is a measure of the pressure required to inflate the lungs and reflects airway resistance and depends on airflow and the pressure gradient in the airways. Airway diameter has the largest effect on changing airway resistance, for example a decrease in airway lumen radius by half increases airway resistance 16 fold (West, 2008). The major sites of resistance in the airways are the medium-sized bronchi (see section B.1 below for a description of airway branches). Contraction of airway smooth muscle narrows the airways and increases airway resistance, thus increasing pulmonary inflation pressure.

An additional consideration for measurement of pulmonary inflation pressure is airway compliance. Compliance refers to the elasticity of the airways. Lung compliance *decreases* with an increase in airway fibrous tissue, and compliance *increases* with a decrease with alveolar degeneration, commonly seen in pulmonary emphysema (West, 2008). Lung compliance and airway resistance are inversely related: as compliance increases, resistance decreases.

b. Ozone and lung function in humans

Laboratory ozone exposure protocols vary the ozone concentration, length of exposure, amount of exercise, and the amount of time after exposure measurements are performed. Normal humans need exercise in order to have measurable decrements in lung function. This explains why when ozone levels are particularly high, outdoor exercise is not recommended. The "dose" of ozone inhaled is a function of both ozone concentration and exposure time (Gelzleichter *et al.*, 1992). Ozone concentrations between 0.12-0.24 ppm are in the ambient range and while above the daily standard, are often reached in many parts of the country. Concentrations above 0.24ppm are high and are used during controlled exposure studies to reduce the amount of time needed for exposure. See Figure 1.1 for a summary of ozone dose and changes in lung function across species.

Numerous studies have demonstrated that healthy human volunteers exposed to ozone exhibit clinical symptoms of impaired lung function including decreased forced vital capacity and FEV_1, and increased reactivity to exogenous bronchoconstrictors such as histamine and methacholine (Aris *et al.*, 1993; Foster *et al.*, 2000; Golden *et al.*, 1978; Hazbun *et al.*, 1993; Holz *et al.*, 1999; Koren *et al.*, 1989; Krishna *et al.*, 1998; McDonnell *et al.*, 1991; Nightingale *et al.*, 1999; Scannell *et al.*, 1996; Schelegle *et al.*, 2009; Schelegle *et al.*, 1991). In most cases, healthy humans require exercise during ozone exposure in order to have measureable decrements in lung function.

It was hypothesized that ozone may have more profound effects on people with asthma or allergies compared to non-asthmatic individuals, because ozone increases hospitalizations

due to asthma exacerbations. There are a few studies that support this hypothesis. For example, ozone exposure significantly decreases lung function in atopic (allergic)/asthmatic humans compared to healthy controls (Kreit *et al.*, 1989; Scannell *et al.*, 1996), while others suggest there is no difference in lung function or airway reactivity between humans with and without asthma (Holtzman *et al.*, 1979; Nightingale *et al.*, 1999). While these studies used comparable ozone exposures, the Holtzman and Nightingale studies compared atopic and nonatopic individuals whereas the Kreit and Scannell studies compared mild asthmatics to healthy controls. Perhaps ozone enhances airway reactivity more so in asthmatic individuals regardless of atopic status.

Ozone exposure also sensitizes airways to subsequent allergen challenges (Jenkins *et al.*, 1999; Jorres *et al.*, 1996; Molfino *et al.*, 1991). Humans with asthma were exposed to ozone and then subsequently allergen challenged (Chen *et al.*, 2004). Most were not more sensitive to allergen when challenged after ozone exposure compared to challenge after filtered air. However, there was a subset of the asthma group that was sensitized suggesting a subgroup of asthmatics may be highly sensitive to ozone exposure. It may be that subpopulations of allergic asthmatics are most sensitive to adverse effects of ozone.

A potential mechanism for ozone-induced airway hyperresponsiveness involves airway nerves. Early studies with reflex-induced bronchoconstriction demonstrated the parasympathetic nervous system contributes to ozone-induced airway hyperreactivity. Non-asthmatic adults exposed to ozone are hyperreactive to inhaled histamine (Golden *et*

al., 1978; Holtzman *et al.*, 1979) and this hyperreactivity is blocked with the muscarinic antagonist atropine, suggesting involvement of the cholinergic nervous system. Not only are parasympathetic nerves involved in ozone-induced airway hyperreactivity, sensory nerves may play a role as well. The peptide neurotransmitter substance P increases in bronchoalveolar lavage fluid immediately following ozone exposure in humans (Hazbun *et al.*, 1993). However, it is unclear whether substance P plays a physiological role in ozone-induced airway hyperreactivity in humans, or if changes in neurotransmitter release are occurring over the long term.

c. Ozone and lung function in animals

Human studies are limited in that mechanistic questions are hard to answer and results are only correlations between decrements in lung function and inflammatory mediators. The benefits of using animals to tease out the mechanisms of ozone induced lung damage include availability of knock out mice, extensive pharmacological tools, and invasive in vivo procedures. Thus, much work has been done in animals to understand how ozone damages the lungs.

Ozone causes airway hyperreactivity in non-human primates (Schelegle *et al.*, 2003), dogs (Fabbri *et al.*, 1984; Holtzman *et al.*, 1983a; Holtzman *et al.*, 1983b; Lee *et al.*, 1977; Li *et al.*, 1992; Schelegle *et al.*, 1993; Walters *et al.*, 1986), guinea pigs (Gambone *et al.*, 1994; Nogami *et al.*, 2000; Roum *et al.*, 1984; Schultheis *et al.*, 1994b; Yeadon *et al.*, 1992; Yost *et al.*, 1999), rats (DeLorme *et al.*, 2002; Hisada *et al.*, 1999; Ishii *et al.*, 1998; Joad *et al.*, 1993), and mice (Johnston *et al.*, 2007; Kierstein *et al.*, 2008; Park *et*

al., 2004a; Park *et al.*, 2004b; Shore *et al.*, 2001; Williams *et al.*, 2008; Williams *et al.*, 2007a; Williams *et al.*, 2007b). Ozone doses are typically much higher for animal studies than for humans because most small mammals are obligate nose breathers (Green, 1982), thus approximately 50% of the inhaled ozone is filtered by the nose.

FIGURE 1.1. Ozone decreases lung function, causes airway hyperreactivity, and increases airway inflammation in humans and animals.

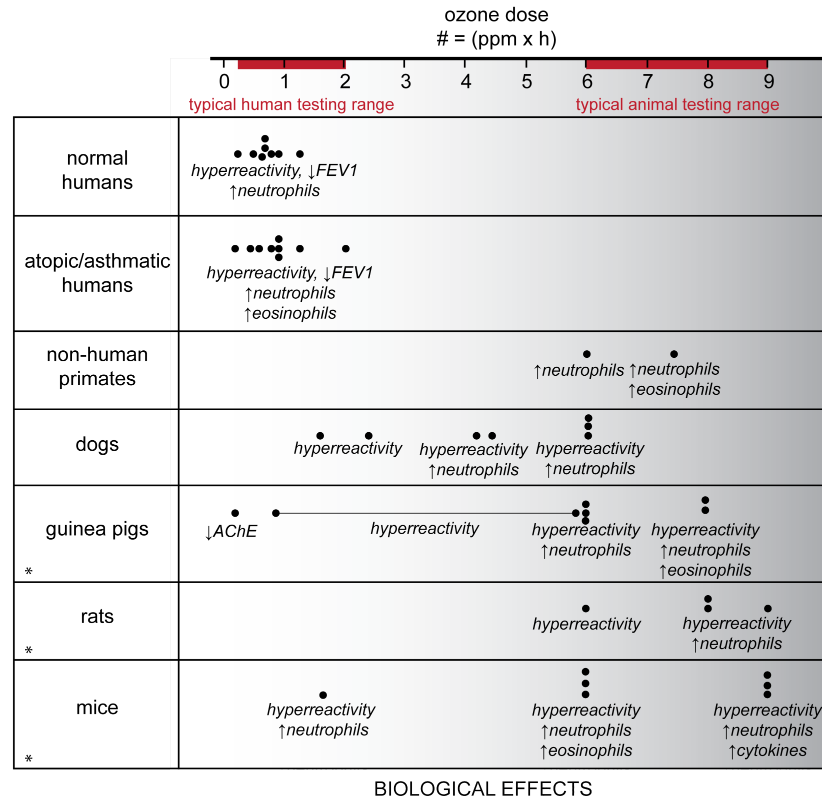


Figure 1.1. Each dot on the above schematic represents one published study on the effects of acute ozone exposure on lung function and/or airway inflammation.

References:
Normal humans: (Aris *et al.*, 1993; Foster *et al.*, 2000; Golden *et al.*, 1978; Hazbun *et al.*, 1993; Holz *et al.*, 1999; Koren *et al.*, 1989; Krishna *et al.*, 1998; McDonnell *et al.*, 1991; Nightingale *et al.*, 1999; Scannell *et al.*, 1996; Schelegle *et al.*, 2009; Schelegle *et al.*, 1991)
Atopic/asthmatic humans: (Bascom *et al.*, 1990; Basha *et al.*, 1994; Hiltermann *et al.*, 1999; Holtzman *et al.*, 1979; Holz *et al.*, 1999; Jenkins *et al.*, 1999; Jorres *et al.*, 1996; Kreit *et al.*, 1989; Newson *et al.*, 2000; Nightingale *et al.*, 1999; Peden *et al.*, 1997; Scannell *et al.*, 1996; Vagaggini *et al.*, 2002)
Non-human primates: (Hicks *et al.*, 2009; Hyde *et al.*, 1992)
Dogs: (Fabbri *et al.*, 1984; Holtzman *et al.*, 1983a; Holtzman *et al.*, 1983b; Lee *et al.*, 1977; Li *et al.*, 1992; Schelegle *et al.*, 1993; Walters *et al.*, 1986)
Guinea pigs: (Gambone *et al.*, 1994; Gordon *et al.*, 1981; Nogami *et al.*, 2000; Roum *et al.*, 1984; Schultheis *et al.*, 1994b; Verhein *et al.*, 2008; Yeadon *et al.*, 1992; Yost *et al.*, 1999)
Rats: (DeLorme *et al.*, 2002; Hisada *et al.*, 1999; Ishii *et al.*, 1998; Joad *et al.*, 1993)
Mice: (Johnston *et al.*, 2007; Kierstein *et al.*, 2008; Park *et al.*, 2004a; Park *et al.*, 2004b; Shore *et al.*, 2001; Williams *et al.*, 2008; Williams *et al.*, 2007a; Williams *et al.*, 2007b)
* 50% of ozone filtered by the nose.

B. AIRWAY STRUCTURE

1. Anatomy of human airways

The respiratory tract can be divided into upper and lower airways. The upper airways include the mouth, nasal passages, larynx, and trachea. These extrapulmonary airway regions are outside of the lungs. The trachea bifurcates at the carina into two main bronchi, one going to each lung. The lower respiratory tract is composed of a series of successively branching tubes, the bronchi, which become smaller and increase in number as they go deeper into the lung (Figure 1.2). The intrapulmonary airways include the bronchi and all generations below them including smaller bronchi, bronchioles, and eventually terminal bronchioles. The bronchi airway generations make up the conducting airways, responsible for leading air to and from the gas exchange regions. Terminal bronchioles continue branching into respiratory bronchioles, alveolar ducts, and finally into alveolar sacs. These latter regions of the lung are the sites of gas exchange and are known as the respiratory zone (West, 2005).

There are approximately 300 million alveoli in human lungs with a surface area of 70-80 square meters (Weibel, 1963). The inner lining of the alveoli is coated with surfactant to decrease surface tension and prevent collapse. The cells of the alveolar wall are connected by tight junctions to limit the movement of ions and water and are only one cell layer thick in order to facilitate gas exchange with capillaries separated from alveoli by a thin basement membrane (Haselton, 1996).

2. Structural components of human airways

Airways from the trachea to the bronchioles are supported by cartilage (Figure 1.2). The trachea has cartilage rings, while cartilage plates support bronchi. Cartilage becomes less abundant as the bronchi branch, ending before bronchioles. Airways also contain smooth muscle that when contracted causes airway shortening and narrowing. Smooth muscle in the trachea is two layers, longitudinal and transverse, while bronchi and bronchioles have circular bands of smooth muscle (Jeffery, 1995) (Figure 1.2).

A layer of epithelial cells line the airways and serves many purposes in the lungs. First, airway epithelial cells provide a physical barrier between the external and internal environments. Second, epithelial cells are often the first to respond to lung insults by releasing inflammatory mediators and recruiting inflammatory cells. In the trachea, epithelial cells are ciliated, pseudostratified, and columnar, though after the major bronchi, epithelial shape becomes simple cuboidal (Jeffery, 1995). Small bronchioles contain Clara cells, which secrete surfactants that reduce alveolar surface tension and prevent airway collapse (Jeffery, 1995).

Interspersed among the epithelial cells are submucosal glands and mucus secreting cells called goblet cells (Jeffery, 1995). Goblet cells contain granules filled with mucin, which are released into the airway lumen. Mucus glands are present from the trachea to the bronchial layer and lie outside the smooth muscle with a duct opening into the bronchial lumen (Haselton, 1996). Mucus is important in airway protection by trapping inhaled particles and pathogens, and then ciliary movement by epithelial cells pushes the particles

trapped in mucus up and out of the airways into the mouth where it is swallowed. Mucus secretion from glands is controlled by parasympathetic, sympathetic, and sensory nerves (nerves described below in section C) (Wine, 2007). Parasympathetic nerves stimulate mucus secretion, as do sensory nerves through release of tachykinins. Sympathetic nerve stimulation in humans only leads to a modest stimulation of mucus secretion (Phipps *et al.*, 1982). Parasympathetic nerves also stimulate mucus secretion from goblet cells although no direct parasympathetic innervation of the airway epithelium has been reported. It is thought cholinergic stimulation of mucus release from goblet cells may be secondary to smooth muscle contraction and physical deformation of epithelial cells (Evans *et al.*, 2009).

Beneath the layer of airway epithelium is the basement membrane, which is a structural boundary between the epithelium and surrounding tissues and serves to regulate movement of cells between the two compartments (Schittny *et al.*, 1989).

FIGURE 1.2. Airway generations.

Zone	Airway	z	Cartilage	Smooth Muscle	Glands	Parasympathetic Nerves
Conducting zone	Trachea	0	rings	longitudinal transverse	yes	yes
	Bronchi	1, 2, 3	plates	circular	yes	yes
	Bronchioles → Terminal bronchioles	4, 5 → 16	—	circular	—	yes
Transitional and respiratory zones	Respiratory bronchioles	17, 18, 19	—	some circular	—	yes
	Alveolar ducts (T_3, T_2, T_1)	20, 21, 22	—	some	—	—
	Alveolar sacs (T)	23	—	no	—	—

Figure 1.2. The conducting zone begins at the trachea and divides into bronchioles and ends at the terminal bronchioles (z=airway generation number). The respiratory zone, where gas exchange occurs, consists of respiratory bronchioles that branch into alveolar ducts and alveoli. Based on (Haselton, 1996) and adapted from (Verbout, 2008).

3. Lung blood supply of human airways

Blood flow through the lungs comes from two systems: pulmonary and bronchial. Pulmonary circulation is the flow of deoxygenated blood from the right ventricle of the heart pumped through the pulmonary artery to respiratory bronchioles and alveoli where gas exchange occurs. Gas exchange occurs mainly in the alveoli where the walls are very thin. Oxygenated blood travels through the pulmonary vein to the left ventricle of the heart to the systemic circulation. The bronchial system supplies oxygenated blood to the bronchi down to terminal bronchioles via a branch of the aorta called the bronchial artery (Staub, 1998). Deoxygenated blood returns to the right atrium of the heart.

4. Differences between species

Guinea pig airway anatomy and physiology closely resembles that of humans (Canning, 2003; Canning *et al.*, 2008; Richardson, 1979; Richardson *et al.*, 1979). Both species have an innermost layer of ciliated airway epithelium (Dalen, 1983) containing goblet cells and mucus glands that release mucus upon stimulation by parasympathetic and sensory nerves (Poblete *et al.*, 1993; Rogers, 2001). In addition to structural similarities, airway innervation of both humans and guinea pigs is very similar where in both species parasympathetic nerves control airway smooth muscle (Canning, 2006; Nadel, 1977; Nadel *et al.*, 1984). Airway nerves will be described below in detail in section C.

In contrast, mouse lungs are structurally different from both humans and guinea pigs in that there is very little smooth muscle, few glands, and major differences in airway innervation (Choi *et al.*, 2000; Persson *et al.*, 1997). Mice appear to have no sympathetic

innervation of airway smooth muscle though they do have β adrenoreceptors on airway smooth muscle (Manzini, 1992; Szarek *et al.*, 1995).

There are also species differences in bronchial circulation. All species larger than a mouse including sheep, dogs, rats, and guinea pigs, have complete functional bronchial vasculature, where mice do not have functional bronchial vasculature except at the trachea and main bronchi (Deffebach *et al.*, 1987; Mitzner *et al.*, 2004).

5. Ozone and epithelial cells

a. General

As mentioned previously, one of the main functions of airway epithelial cells is to provide a barrier between the environment and the rest of the body. As a result, they are often one of the first responders to environmental insults. Ozone is highly reactive and does not pass unreacted through the epithelial layer (Pryor, 1992). Ozone disrupts epithelial integrity and thus increases permeability across the epithelium (Kehrl *et al.*, 1987). After ozone exposure, airway epithelium and alveolar macrophages produce IL-1β and other inflammatory cytokines such as tumor necrosis factor, IL-6, and IL-8 (Arsalane *et al.*, 1995; Cohen *et al.*, 2001; Fakhrzadeh *et al.*, 2004; Pendino *et al.*, 1994; Polosa *et al.*, 2004). Thus, all of the downstream effects of ozone are the result of interaction with components of the epithelial lining fluid, epithelial cells, and inflammatory cells within those two layers.

By reacting with components of these two layers, ozone generates reactive oxygen species and lipid peroxides (Hamilton *et al.*, 1996; Kirichenko *et al.*, 1996). End products of lipid peroxidation and reactive oxygen species activate cell signaling pathways, including the mitogen activated protein kinase pathway (Figure 1.3) (Kumagai *et al.*, 2002).

b. Mitogen activated protein kinases

Mitogen-activated protein kinase signaling pathways are important in many cell processes including differentiation, proliferation, activation, degranulation, and migration. There are four mitogen activated protein kinase subfamilies that have been well characterized: ERK1/2, ERK5, JNK, and p38. The extracellular signal-regulated kinase (ERK) pathway is usually activated by mitogens and growth factors while p38 and c-Jun NH_2 terminal kinase (JNK) pathways are associated with chronic inflammation and are typically activated by inflammatory cytokines, heat shock, and cellular stress (Denhardt, 1996; Kyriakis *et al.*, 1996). All mitogen activated protein kinases are activated by dual phosphorylation of conserved tyrosine and threonine residues by upstream mitogen activated kinase kinases and are inactivated by phosphatases (Figure 1.4). Once phosphorylated, mitogen activated protein kinases translocate to the nucleus and activate transcription factors inducing gene transcription. Activation of mitogen activated protein kinase signaling induces inflammatory cytokine and chemokine production in airway epithelial cells, inflammatory cells, and airway smooth muscle cells (Cui *et al.*, 2002; Kalesnikoff *et al.*, 2001; Peng *et al.*, 2004). Humans with severe asthma have increased activated p38 in airway epithelium compared to mild asthmatics or

healthy controls, as demonstrated by increased immunostaining of phosphorylated p38 in airway biopsies (Liu *et al.*, 2008).

i. p38

In mammals, there are four isoforms of p38 mitogen activated protein kinase encoded by separate genes: α, β, γ, and δ. p38α is the best characterized isoform and is widely expressed in almost every cell type, while p38γ is highly expressed in skeletal muscle and p38δ in testis, pancreas, kidneys and small intestine (Goedert *et al.*, 1997). Based on sequence homology and binding to p38 inhibitors, the four p38 mitogen activated protein kinases can be further divided into two subgroups with p38α and p38β in one group and p38γ and p38δ in the other (Cuenda *et al.*, 1997; Cuenda *et al.*, 2007; Kuma *et al.*, 2005). Inhibitors of p38 usually inhibit both p38α and p38β. p38α in particular is responsible for relaying chemotactic signals, for example migration of C5a stimulated macrophages was inhibited in cells deficient in p38α but not in cells deficient in the other p38 isoforms (Rousseau *et al.*, 2006). p38 signaling induces cytokine and chemokine production in airway epithelial cells, mast cells, and airway smooth muscle cells (Cui *et al.*, 2002; Kalesnikoff *et al.*, 2001; Peng *et al.*, 2004).

In animal models, inhibition of p38 is effective at preventing many aspects of airway inflammatory diseases. Inhibiting p38, either pharmacologically or with antisense oligonucleotides, partially prevents airway hyperreactivity after sensitization to and challenge with antigen in mice (Duan *et al.*, 2005; Nath *et al.*, 2006). In antigen challenged animals, eosinophil influx into bronchoalveolar lavage is prevented by a p38

inhibitor in guinea pigs and mice (Underwood *et al.*, 2000). Blocking p38 not only prevents eosinophil recruitment to the airways, but has direct effects on eosinophils as well. In eosinophils, IL-5 signals through both ERK and p38 mitogen activated protein kinase and inhibition of p38 reduces IL-5 induced cytokine production (Adachi *et al.*, 2000). p38 also plays a role in aspects of airway remodeling in that blocking p38 prevents IL-13 induced mucus metaplasia, the transformation of epithelial cells into mucus producing cells, in human and mouse airway epithelial cells (Atherton *et al.*, 2003; Fujisawa *et al.*, 2008).

ii. JNK

There are three isoforms of JNK mitogen activated protein kinase that are encoded by three separate genes: JNK1, JNK2, and JNK3. JNK1 and JNK2 are expressed in lung, whereas JNK3 is expressed in the brain, cardiac myocytes, and testes (Yang *et al.*, 1997). Activation of the JNK pathway results in expression of inflammatory cytokines such as TNFα, IL-4 and IL-13 (Nath *et al.*, 2005; Yao *et al.*, 1997).

Similar to inhibiting p38, inhibiting JNK mitogen activated protein kinase prevents eosinophil influx into bronchoalveolar lavage and airways after antigen sensitization and challenge in mice (Nath *et al.*, 2005). In rats, the same JNK inhibitor also prevents eosinophil influx into bronchoalveolar lavage, but does not prevent antigen challenge induced airway hyperreactivity (Eynott *et al.*, 2003; Eynott *et al.*, 2004).

iii. Role of mitogen activated protein kinases in ozone

Less is known about the role of the mitogen activated protein kinases in ozone-induced airway hyperreactivity. Inhibiting p38 prevents ozone-induced airway hyperreactivity in mice while inhibiting JNK is partially protective (Williams *et al.*, 2008; Williams *et al.*, 2007a). Ozone-induced increases in inflammatory cells in bronchoalveolar lavage are significantly inhibited in *Jnk1* knockout mice (Cho *et al.*, 2007). Chapter III will address whether both p38 and JNK mitogen activated protein kinase are necessary for ozone-induced airway hyperreactivity in guinea pigs.

FIGURE 1.3. Ozone activates inflammatory cytokine production in airway epithelial cells.

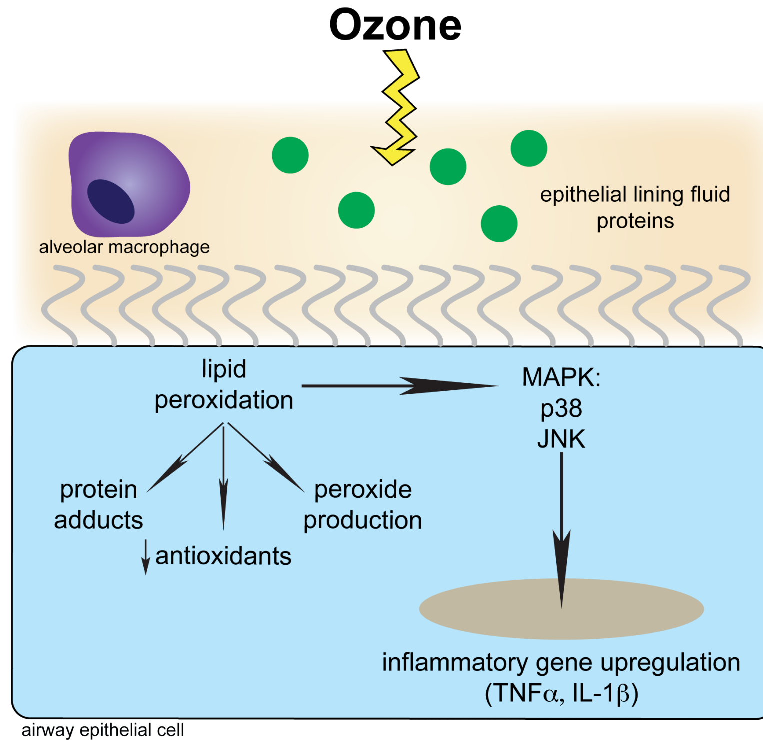


Figure 1.3. Ozone causes reactive oxygen species through lipid peroxidation upon interaction with airway epithelial cells and epithelial cell lining fluid. Reactive oxygen species activate the mitogen activated protein kinase (MAPK) pathway, which then upregulates expression of inflammatory cytokines such as IL-1β.

FIGURE 1.4. Activation of mitogen activated protein kinase pathways.

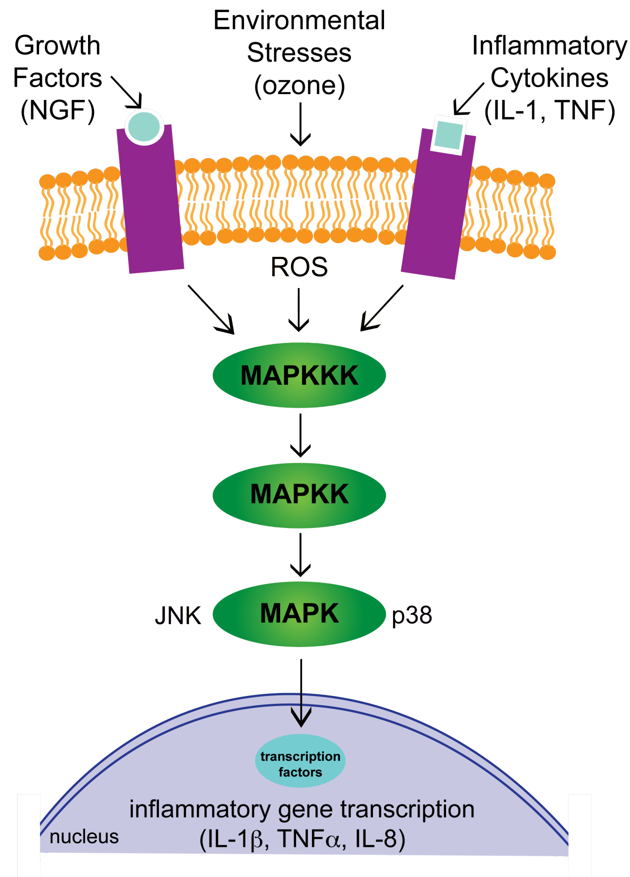


Figure 1.4. Both p38 and JNK mitogen activated kinases (MAPK) are activated by growth factors, environmental stress, through production of reactive oxygen species (ROS), and inflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor (TNF). All of these processes first activate mitogen activated protein kinase kinase kinases (MAPKKK) that then phosphorylate mitogen activated protein kinase kinases (MAPKK), which finally phosphorylate and activate mitogen activated protein kinases (MAPK). p38 and JNK phosphorylate various transcription factors that induce inflammatory gene transcription, including production of IL-1 and TNF.

C. AIRWAY INNERVATION

1. Neuroanatomy

The nervous system is a network of cells that receives and processes internal and external signals. Nerve cells, or neurons, are the functional unit of the nervous system and are composed of a cell body, dendrites, and axon (Figure 1.5). In 1897, Sir Charles Sherrington coined anatomical terms to describe nerves (Sherrington, 1897). The site of contact between two neurons is a *synapse* while contact between a neuron and its target cell is a neuronal *junction*. A group of neuronal cell bodies is called a ganglion and a more than one ganglion are *ganglia*. Nerves with axons that travel toward ganglia are *preganglionic* nerves, and nerves that have cell bodies in ganglia with axons traveling away from ganglia toward target cells are *postganglionic* nerves. Nerves typically signal to each other through chemical neurotransmitters. Terminology used in this thesis is based on Sherrington's definitions.

The nervous system is divided into the central nervous system and peripheral nervous system. The peripheral nervous system can be further divided into the somatic nervous system and the autonomic nervous system. Autonomic nerves regulate body functions not under voluntary control including respiration, digestion, circulation, and metabolism. There are two subdivisions of the autonomic nervous system: the parasympathetic and sympathetic nerves. The airway smooth muscle, glands, and airway epithelium are only innervated by the autonomic nervous system; therefore the nerves of the autonomic nervous system will be the focus of this dissertation and are described in detail below.

FIGURE 1.5. Parts of a neuron.

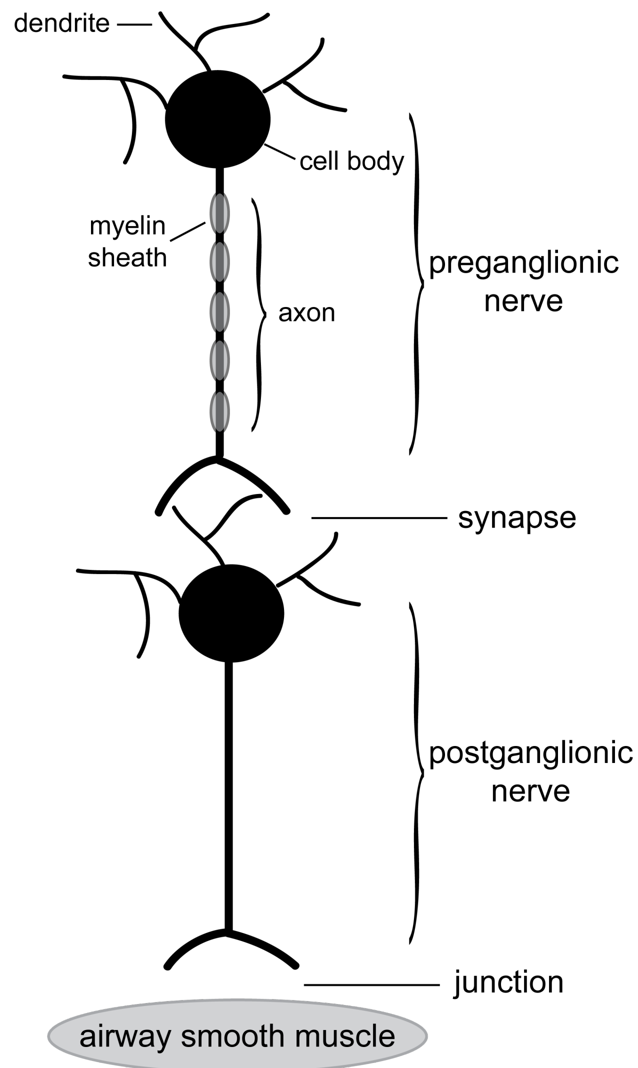


Figure 1.5. Cartoon model of anatomy of neurons, terminology from (Sherrington, 1897).

2. Parasympathetic nerves

a. General

The parasympathetic nerves provide the dominant control of airway smooth muscle tone (Boushey *et al.*, 1980; Nadel, 1977; Nadel *et al.*, 1984; Widdicombe, 1966). Early studies demonstrated pharmacological blockade of muscarinic receptors or vagal sectioning decreased airway constriction. In contrast, sectioning or blocking the sympathetic nerves had no effect on airway smooth muscle tone. Thus, parasympathetic nerves mediate airway tone.

b. Origin

Airway preganglionic parasympathetic nerves originate in the nucleus of the solitary tract in the brainstem and travel to the lungs and heart through the vagus nerves (Figure 1.6). The vagus nerves divide into the superior and recurrent laryngeal nerves to innervate chains of postganglionic parasympathetic neurons in the trachea and main bronchi. (Figure 1.7). Targets of airway parasympathetic nerves include airway smooth muscle, mucus glands, and blood vessels. Parasympathetic nerves induce bronchoconstriction (Cabezas *et al.*, 1971; Nadel, 1977; Woolcock *et al.*, 1969), submucosal gland secretion (Spencer *et al.*, 1964), and dilation of bronchial circulation (Widdicombe, 1963).

c. Acetylcholine synthesis and break down

Parasympathetic nerves primarily use acetylcholine as their neurotransmitter. Sir Henry Dale first characterized acetylcholine in 1914 (Dale, 1914). Studies by Otto Loewi in

1926 provided evidence that acetylcholine was released by vagus nerves using isolated frog hearts.

Acetylcholine synthesis is catalyzed by choline acetyltransferase, which transfers the acetyl group from acetyl-coenzyme A to choline. Acetylcholine is transported into vesicles by the vesicular cholinergic transporter for storage. Acetylcholine is broken down by the enzymes acetylcholinesterase and butyrylcholinesterase, which catalyze the hydrolysis of acetylcholine to choline. Acetylcholinesterase is found in blood, is expressed by neurons and is also highly expressed by airway epithelial cells (Koga *et al.*, 1992). Free choline is taken back up into neurons through the choline transporter for resynthesis of acetylcholine. Acetylcholine binds to two major classes of receptors: nicotinic and muscarinic that will be described in detail in section D.

Although primarily a neuronally produced neurotransmitter, non-neuronal release of acetylcholine has been described. Many lung cells have machinery necessary for acetylcholine synthesis, including epithelial cells and immune cells (Kummer *et al.*, 2008; Wessler *et al.*, 2003), though these cells lack the components necessary for acetylcholine storage. Acetylcholine from non-neuronal sources is thought to act in an autocrine manner; however, the role of non-neuronal acetylcholine in vivo is not well understood. Luminally released acetylcholine may activate epithelial cells to increase cilia beat frequency and enhance mucociliary clearance, while basolaterally released acetylcholine may modulate sensory nerve fibers (Kummer *et al.*, 2008). Experiments in

this thesis do not measure release of acetylcholine from non-neuronal sources, but they must be considered when examining the effects of acetylcholine in the lung.

FIGURE 1.6. Innervation of the airways.

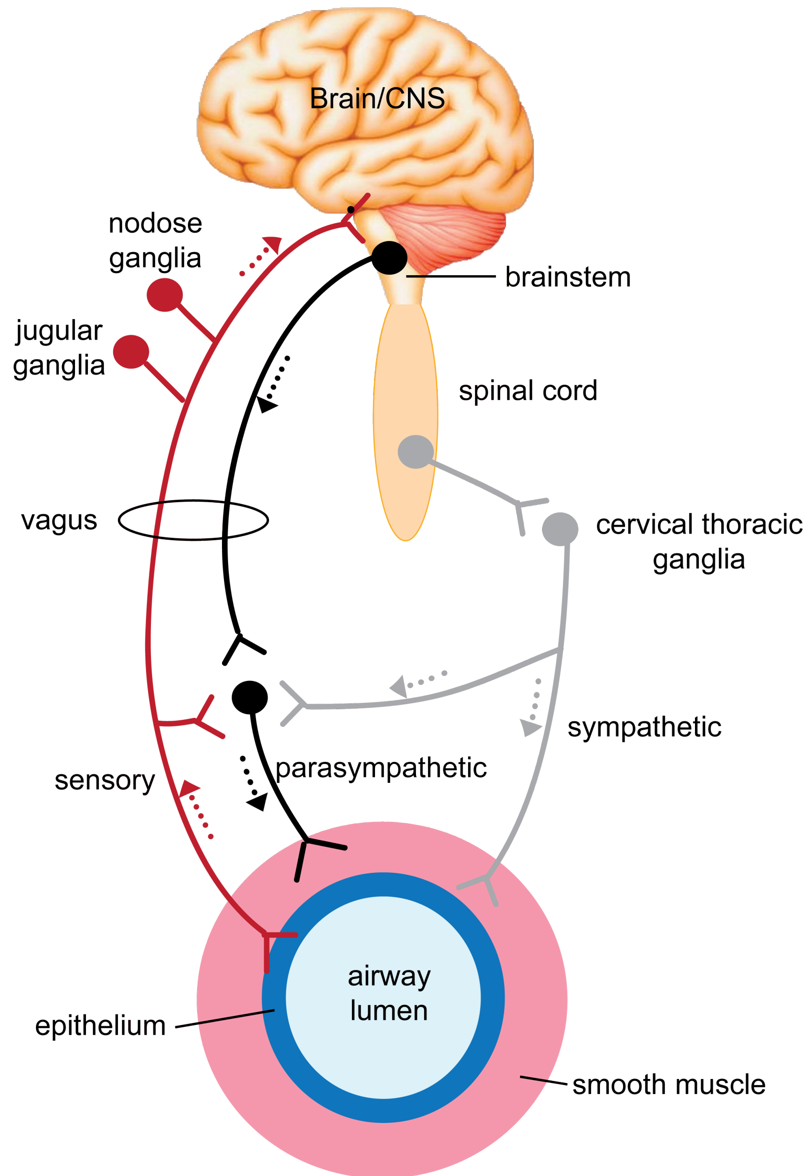


Figure 1.6. Parasympathetic innervation (in black) originates in the brainstem and nerves travel to the airways through the vagus along with the sensory nerves (in red) from the nodose and jugular ganglia. In humans, sympathetic nerves (gray) do not directly innervate airway smooth muscle, while in guinea pigs they do.

FIGURE 1.7. Vagus nerve innervation of parasympathetic ganglia in ferret trachea.

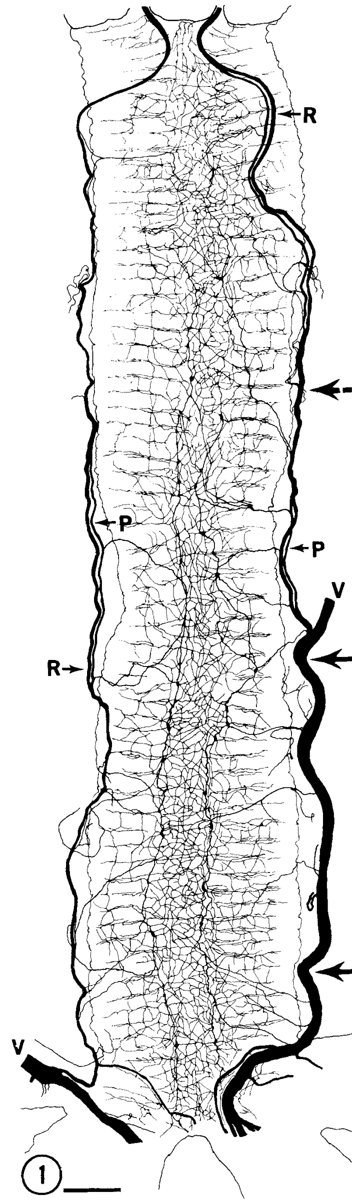


Figure 1.7. Parasympathetic nerves in ferret trachea (black) were visualized with horseradish peroxidase and diaminobenzidine staining. The vagus nerve (V) branches into the recurrent laryngeal nerves (R) and pararecurrent laryngeal nerves (P) and forms connections with chains of parasympathetic ganglia. Figure from (Baker *et al.*, 1986).

3. Sympathetic nerves

a. Origin

Sympathetic nerves are also efferent autonomic nerves. Preganglionic sympathetic neurons synapse onto postganglionic sympathetic neurons in the cervical thoracic ganglia (Figure 1.6). In humans, sympathetic nerves target mucus glands, blood vessels and parasympathetic ganglia, but do not innervate airway smooth muscle directly (Mann, 1971; Richardson *et al.*, 1976). In other species, sympathetic innervation differs, with guinea pigs (O'Donnell *et al.*, 1978), dogs (Knight *et al.*, 1981), and cats (Dahlstrom *et al.*, 1966) receiving direct smooth muscle sympathetic innervation. Rats and mice appear to have no relaxant innervation of airway smooth muscle though they do have β adrenoreceptors on airway smooth muscle that mediate relaxation (Manzini, 1992; Szarek *et al.*, 1995). Stimulation of sympathetic nerves results in relaxation of pre-contracted airway smooth muscle.

b. Norepinephrine synthesis and break down

Sympathetic nerves release the neurotransmitter norepinephrine. Norepinephrine is synthesized from tyrosine. Tyrosine is converted to 3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase, which is then converted to dopamine by decarboxylation. Finally, dopamine β-hydroxylase converts dopamine to norepinephrine. Norepinephrine signaling is terminated by diffusion away from the synapse, breakdown by monoamine oxidase, and reuptake by neurons through the norepinephrine transporter.

4. Non-adrenergic non-cholinergic nerves

The importance of non-adrenergic non-cholinergic (NANC) nerves in human health has not been established. Evidence for the existence of these nerves has been found in many species including humans and guinea pigs (Li *et al.*, 1991; Linden, 1996; Palmer *et al.*, 1986). Their cell bodies are thought to reside in airway parasympathetic ganglia and the esophagus. There are reported to be both excitatory and inhibitory NANC nerves, with regard to smooth muscle contraction. However, this nomenclature is not sufficient to describe their functions, since the same neurotransmitter that causes airway smooth muscle contraction also causes vascular smooth muscle relaxation. Both nitric oxide and vasoactive intestinal peptide (VIP) are thought to mediate the smooth muscle relaxation effects of NANC nerves (Li *et al.*, 1991; Takahashi *et al.*, 1995). Peptide neurotransmitters substance P and neurokinin A are thought to be the NANC neurotransmitters that regulate smooth muscle contraction. It is not well understood how these transmitters are released because many NANC nerves co-express multiple peptide neurotransmitters.

5. Sensory nerves

a. Origin

Approximately 80% of nerves in the vagus nerve are sensory nerves (Agostoni *et al.*, 1957). The majority of afferent sensory nerves arrive in the airways via the vagus nerves (Agostoni *et al.*, 1957), although some sensory nerves originate in dorsal root ganglia and arrive in the lungs with spinal sympathetic nerves. Sensory nerve cell bodies are located in the nodose and jugular ganglia at the base of the skull; sensory nerve targets include

airway epithelium, submucosal layers, and smooth muscle (Figure 1.6) (Brouns *et al.*, 2006). There are two main classes of sensory nerves, A-delta fibers and C fibers that are classified by size of their cell body, conduction velocity, and myelination. A-delta fibers have large cell bodies, fast conduction velocities, and are myelinated. In contrast, C fibers have small cell bodies, slow conduction velocities, and are unmyelinated.

Subsets of A-delta fibers are rapidly adapting receptors that respond to stretch. In the airways, these nerves monitor changes in lung compliance and induce broncho-constriction by reflex activation of parasympathetic nerves (Pack *et al.*, 1983; Wagner *et al.*, 1999).

Sensory nerves innervate airway smooth muscle, glands, airway epithelium, and airway blood vessels (Brouns *et al.*, 2006; Lundberg *et al.*, 1984; Luts *et al.*, 1993). Afferent sensory nerve stimulation induces smooth muscle contraction, mucus secretion, and vasodilation. In isolated human bronchi, sensory nerves innervate parasympathetic ganglia and airway smooth muscle (Sheldrick *et al.*, 1995). Sensory nerves release neurotransmitters in the lungs as well as initiating reflex responses via the parasympathetic nerves that induce cough, bronchoconstriction, and mucus secretion (Coleridge *et al.*, 1994).

b. Neurotransmitters/Tachykinins

Sensory nerves primarily release peptide neurotransmitters. These include members of the tachykinin family: substance P, neurokinin A, neuropeptide K, neuropeptide γ, and

neurokinin B. Neuropeptide K and neuropeptide γ are amino-terminally extended forms of neurokinin A. Other peptide neurotransmitters include VIP and calcitonin gene related peptide (CGRP). Different subsets of sensory nerve populations release different peptide neurotransmitters. This dissertation will focus on the tachykinin family of peptide neurotransmitters, in particular substance P, which is released by sensory C fibers (Holzer, 1988; Hua *et al.*, 1985). As with other neurotransmitters, substance P is not exclusively a neuronal product and is made by epithelial and inflammatory cells, including macrophages and eosinophils (Bost *et al.*, 1992; Killingsworth *et al.*, 1997; Weinstock *et al.*, 1988).

Substance P and neurokinin A are excitatory neurotransmitters in both the central and peripheral nervous system (Almeida *et al.*, 2004; Holzer, 1988; Maggi *et al.*, 1988). Neurokinin B is almost exclusively expressed in the central nervous system (Almeida *et al.*, 2004; Moussaoui *et al.*, 1992). Neuropeptide K has been found in the airways but is not a very potent inducer of bronchoconstriction (Martling *et al.*, 1987). Substance P and neurokinin A are the most extensively studied tachykinins and since substance P is highly expressed in the lungs, substance P will be the focus of this dissertation.

c. Substance P synthesis and break down

In 1931 Von Euler and Gaddum first discovered substance P in horse brain as a potent contractile agent (V. Euler *et al.*, 1931). Substance P and most other tachykinins are synthesized from the preprotachykinin-A gene (Krause *et al.*, 1987) with the exception of neurokinin B is synthesized from the preprotachykinin-B gene. Alternative splicing of the

preprotachykinin-A gene leads to four distinct mRNAs: αPPT-A, βPPT-A, γPPT-A and δPPT-A. All four mRNAs encode for substance P, while βPPT-A also encodes neurokinin A and neuropeptide K (Figure 1.8) (Carter *et al.*, 1990). Substance P is an 11 amino acid peptide that is identical across mammalian species (Hoyle, 1998) and is synthesized by ribosomes in the nerve cell body, packaged into storage vesicles, and transported to nerve terminals (Brimijoin *et al.*, 1980; Harmar *et al.*, 1982). Metabolism of substance P occurs primarily through enzymatic breakdown by neutral endopeptidase, which is highly expressed by airway epithelial cells and airway smooth muscle (Cohen *et al.*, 1996; Devillier *et al.*, 1988).

FIGURE 1.8. Preprotachykinin gene mRNA splice variants.

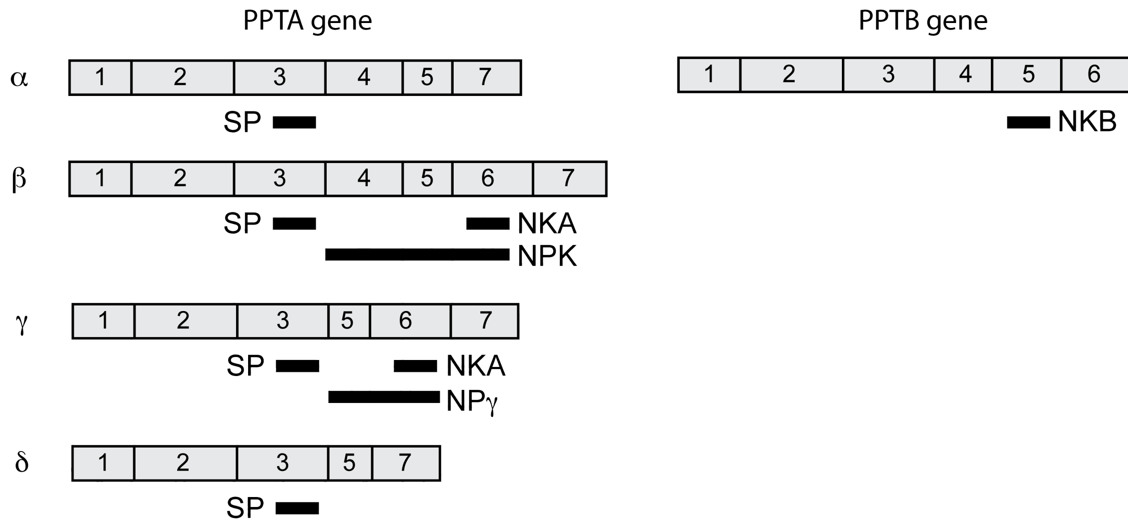


Figure 1.8. Schematic representation of the mRNA splice variants for the preprotachykinin-A (PPTA) and preprotachykinin-B (PPTB) genes. Substance P (SP); neurokinin A (NKA); neuropeptide K (NPK); neuropeptide γ (NPγ); neurokinin B (NKB). Adapted from (Almeida *et al.*, 2004).

D. KEY RECEPTORS IN PARASYMPATHETIC AND SENSORY CONTROL OF LUNGS

1. Acetylcholine receptors

There are two classes of acetylcholine receptors, nicotinic and muscarinic. Acetylcholine receptors were named by Sir Henry Dale in 1914 because he observed they are selectively activated by either nicotine or muscarine (Dale, 1914). Both receptor subtypes are expressed in the lungs. Nicotinic receptors are present on parasympathetic ganglia and inflammatory cells, while muscarinic receptors are expressed by almost every cell in the airways including epithelial cells, parasympathetic nerves, airway smooth muscle, and inflammatory cells.

a. Nicotinic receptors

Nicotinic receptors are non-selective cation channels and are found in the airways on parasympathetic nerves, macrophages, epithelial cells, eosinophils, neutrophils, and mast cells (Blanchet *et al.*, 2007; Iho *et al.*, 2003; Racke *et al.*, 2004; Su *et al.*, 2007; Sudheer *et al.*, 2006; Wang *et al.*, 2003). Nicotinic receptors are composed of 5 subunits that together form a channel through the cell membrane through which ions pass. There are four nicotinic receptor subunits (α, β, γ, and δ) with many different isoforms of each subunit, and the subunit combination varies depending on cell type. Activation of nicotinic receptors depolarizes postganglionic nerves and produces action potentials.

b. Muscarinic receptors

There are 5 subtypes of muscarinic receptors (M_1-M_5), all of which are coupled to G-proteins (Peralta *et al.*, 1987). G-proteins are classified by their α subunits and are broadly classified as α_s, α_i, or α_q. Gα subunits are coupled to β and γ subunits and these complexes bind to G-protein coupled receptors. Ligand binding to the receptor causes a conformational change thus releasing the G protein complex. The exchange of GTP for GDP on the α subunit separates the α subunit from the $\beta\gamma$ dimer, thus initiating signaling through second messenger systems.

M_1, M_3, and M_5 receptors are all coupled to Gα_q proteins (Figure 1.9). Gq proteins activate membrane bound phospholipase enzymes; the best characterized is phospholipase C, which generates inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate. Release of IP_3 and DAG increases intracellular calcium, leading, for example, to smooth muscle contraction.

M_2 and M_4 receptors are coupled to Gα_I, which inhibit adenylyl cyclase that decreases cyclic adenosine monophosphate (cAMP) (Ritter *et al.*, 2009). The $\beta\gamma$ subunits are active in signaling as well and couple to potassium channels that decrease cellular excitability. In general, odd muscarinic receptors, (M_1, M_3, and M_5) are considered excitatory, while even muscarinic receptors (M_2 and M_4) are considered inhibitory.

There are no selective agonists that discriminate between muscarinic receptor subtypes. There are some selective antagonists including pirenzipine, gallamine and

methoctramine. Atropine is the classic antagonist that blocks all muscarinic receptor subtypes equally.

FIGURE 1.9. Muscarinic receptor and tachykinin receptor coupling to G-proteins.

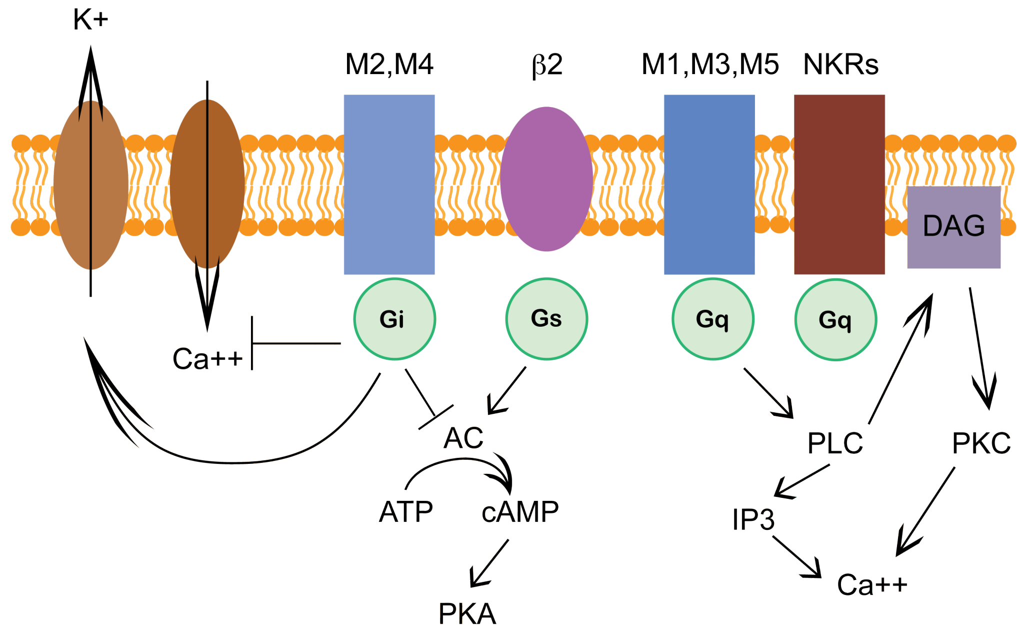


Figure 1.9. Muscarinic M_1, M_3, and M_5 receptors and neurokinin receptors (NKRs) couple to Gq, which activates phospholipase C (PLC) that generates diacylglycerol (DAG) and inositoltriphosphate (IP3). DAG activates protein kinase C (PKC) and this increases intracellular calcium (Ca++). M_2 and M_4 muscarinic receptors couple to Gi, which inhibits adenylyl cyclase (AC), inhibits calcium channels, and activates potassium (K+) channels. For comparison, β2 receptors are coupled to Gs, which activates AC and converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) activating protein kinase A (PKA).

i. Muscarinic receptor distribution in the lung

Almost every cell in the lungs expresses muscarinic receptors, from structural cells to inflammatory cells (Figure 1.10). M_1 receptors facilitate neurotransmission and are found in airway parasympathetic ganglia (Beck *et al.*, 1987; Bloom *et al.*, 1987; Lammers *et al.*, 1989; Yang *et al.*, 1991). M_1 receptors are also present in the peripheral lung and on airway epithelium (Casale *et al.*, 1988; Gies *et al.*, 1989; Mak *et al.*, 1992) where their function is unknown.

The majority of muscarinic receptors on airway smooth muscle are M_2 receptors (Haddad *et al.*, 1991; Roffel *et al.*, 1988). There are species differences in M_2 receptor content in airway smooth muscle with 50% being M_2 in humans, and about 80% in guinea pigs (Haddad *et al.*, 1991). M_2 receptor activation on airway smooth muscle inhibits β agonist-induced activation of adenylyl cyclase thus inhibiting bronchodilation (Fernandes *et al.*, 1992; Sankary *et al.*, 1988).

M_2 muscarinic receptors are also located on airway parasympathetic nerves where they inhibit release of acetylcholine. The autoinhibitory function of these receptors was first described in guinea pig lung (Fryer *et al.*, 1984). Dysfunction of neuronal M_2 muscarinic receptors increases acetylcholine release and bronchoconstriction leading to airway hyperreactivity in humans with asthma (Minette *et al.*, 1989) and animal models of asthma (Belmonte *et al.*, 1998; Evans *et al.*, 1997; Larsen *et al.*, 2000; Yost *et al.*, 1999; Zhang *et al.*, 1999). Mechanisms of neuronal M_2 receptor dysfunction will be described below (section F.2.f).

M_3 muscarinic receptors are found on airway smooth muscle where they mediate smooth muscle contraction and bronchoconstriction (Roffel *et al.*, 1990). Activation of M_3 receptors also increases mucus and water secretion from glands (Borson *et al.*, 1980; Lopez-Vidriero *et al.*, 1975) and increases ciliary beat frequency on airway epithelium (Klein *et al.*, 2009; Mak *et al.*, 1992; Wong *et al.*, 1988).

Muscarinic receptors are also expressed on inflammatory cells but their physiological function is not well understood. Ex vivo, macrophages have M_3 receptors through which acetylcholine stimulates release of leukotriene B_4 (Sato *et al.*, 1998). Mast cell release of histamine is inhibited by acetylcholine (Reinheimer *et al.*, 1997; Reinheimer *et al.*, 2000). M_3, M_4 and M_5 receptor mRNA have been found in human neutrophils and eosinophils (Bany *et al.*, 1999; Verbout, 2006). What muscarinic receptors do on inflammatory cells in vivo is unknown. They may be responding to acetylcholine release from airway nerves, or to non-neuronal sources of acetylcholine such as airway epithelium.

ii. Muscarinic receptor regulation of bronchoconstriction

In the lungs, parasympathetic nerves provide the dominant autonomic control over airway smooth muscle tone through release of acetylcholine onto M_3 muscarinic receptors (Boushey *et al.*, 1980; Nadel *et al.*, 1984; Roffel *et al.*, 1990). Control of acetylcholine release is mediated by neuronal M_2 muscarinic receptors (Fryer, 1995; Fryer *et al.*, 1984). When neuronal M_2 receptors are inhibited ex vivo with the non-selective antagonist atropine, acetylcholine release is increased 5-8 fold (Baker *et al.*, 1992). In vivo,

inhibition with the M_2 selective antagonist, gallamine, increases bronchoconstriction in response to electrical stimulation of the vagus nerves 8-10 fold (Fryer *et al.*, 1984). Thus, M_2 muscarinic receptors are important regulators of acetylcholine release and bronchoconstriction.

FIGURE 1.10. Muscarinic receptors in the airways.

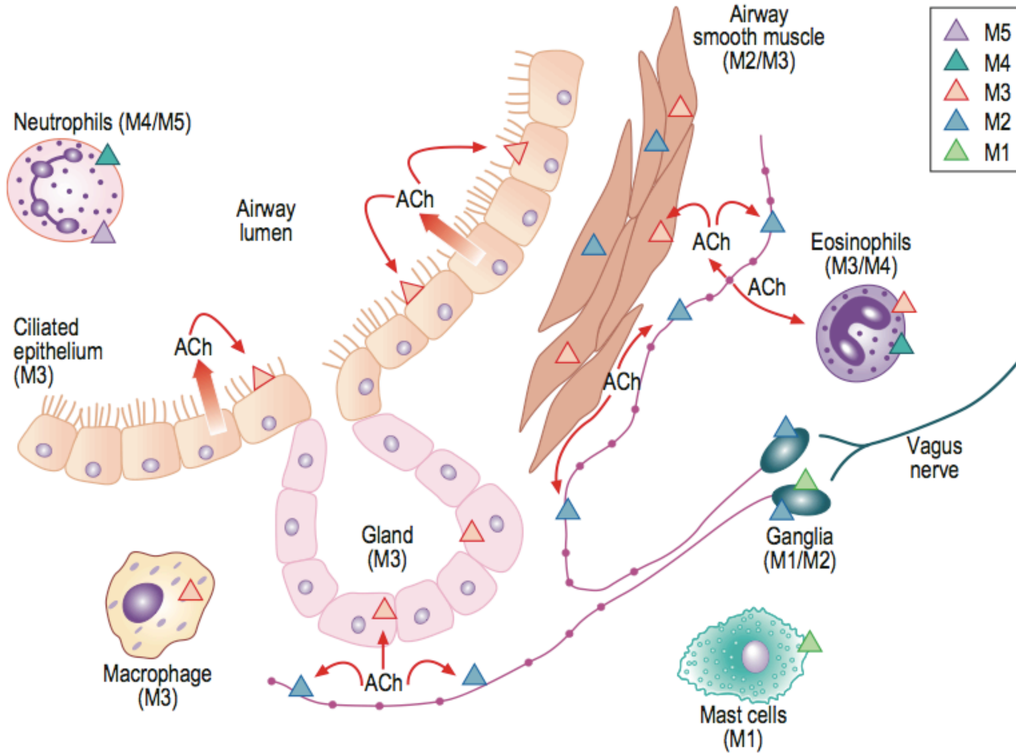


Figure 1.10. All cells in the airways express muscarinic receptors. Acetylcholine (ACh). Figure from (Fryer, 2008).

2. Tachykinin receptors

In 1973, Erspamer and colleagues first proposed the existence of multiple receptors for substance P and other tachykinins based on differences in potencies in different bioassays (Erspamer *et al.*, 1973). This observation was followed by functional data with rank order potencies confirming the presence of multiple receptors provided by Iversen's group in the early 1980s (Iversen *et al.*, 1982; Lee *et al.*, 1982; Watson *et al.*, 1984). It is now understood that tachykinins bind to three distinct neurokinin receptors (NK_1, NK_2, and NK_3), which are all coupled to the same G-protein: $G\alpha_q$ (signaling described above in section D.1.b). Substance P preferentially binds NK_1 receptors, neurokinin A preferentially binds NK_2, and neurokinin B preferentially binds NK_3, but all three tachykinins are full agonists at all neurokinin receptors (see Table 1.1) (Ingi *et al.*, 1991; Mussap *et al.*, 1993).

a. Tachykinin receptor distribution and function in the airways

Parasympathetic nerves express all three neurokinin receptors (Figure 1.11) (Mapp *et al.*, 2000; Myers *et al.*, 2005). Activation of NK_3 receptors increases acetylcholine release by depolarizng postganglionic parasympathetic nerves and NK_2 receptor activation also increases release of acetylcholine from airway nerves (Canning *et al.*, 2002; Grumann-Junior *et al.*, 2000; Hall *et al.*, 1989). Thus, substance P enhances acetylcholine release through tachykinin receptors on parasympathetic nerves.

Airway smooth muscle expresses both NK_1 and NK_2 receptors that induce smooth muscle contraction (Castairs *et al.*, 1986; Naline *et al.*, 1989). Tachykinin-induced

bronchoconstriction is predominantly mediated by NK_2 receptors since an NK_2 selective agonist potently constricts isolated human bronchi, while NK_1 and NK_3 receptor agonists have very little effect (Naline *et al.*, 1989). Further evidence for a dominant role of NK_2 receptors is neurokinin A is the most potent tachykinin for smooth muscle contraction (Sheldrick *et al.*, 1995).

NK_1 receptors are also present on submucosal glands, blood vessels, and airway epithelium (Bai *et al.*, 1995; Chu *et al.*, 2000; Coles *et al.*, 1984; Strigas *et al.*, 1996). Activation of NK_1 receptors on glands induces mucus secretion (Rogers *et al.*, 1989), and on epithelial cells induces release of mediators such as nitric oxide and prostaglandins (Figini *et al.*, 1996; Szarek *et al.*, 1998).

b. Tachykinin receptors on inflammatory cells

NK_1 receptors are expressed by alveolar macrophages, eosinophils, neutrophils, and mast cells (Bardelli *et al.*, 2005; El-Shazly *et al.*, 1996; Gallicchio *et al.*, 2008; Kulka *et al.*, 2008; Pinto *et al.*, 2004; Sun *et al.*, 2007; Xu *et al.*, 2008). NK_2 receptors are also present on eosinophils, mast cells and macrophages (Mapp *et al.*, 2000; Pinto *et al.*, 2004; Renzi *et al.*, 2000). Function of tachykinin receptor activation on inflammatory cells is unknown except in mast cells where activation leads to production of inflammatory mediators such as RANTES and IL-8 (Kulka *et al.*, 2008; Xu *et al.*, 2008).

TABLE 1.1. Tachykinin receptors and agonist rank order potencies.

Nomenclature	NK_1	NK_2	NK_3
Other names	Substance P	Substance K	Neurokinin B, neuromedin K
Ensembl ID	ENSG00000115353	ENSG00000075073	ENSG00000169836
Principal transduction	$G_{q/11}$	$G_{q/11}$	$G_{q/11}$
Rank order of potency	SP > NKA > NKB	NKA > NKB >> SP	NKB > NKA > SP
Selective agonists	SP methylester, [Sar^9,$Met(O_2)^{11}$]SP, [Pro^9]SP, septide	[β-Ala^8]NKA-(4-10),[Lys^5,Me-Leu^9,Mle^{10}] NKA-(4-10), GR64349	Senktide, [$MePhe^7$]NKB
Selective antagonists	Aprepitant (10.7; Hale *et al.*, 1998), SR140333 (9.5), LY303870 (9.4), CP99994 (9.3), RP67580 (7.6)	GR94800 (9.6), GR159897 (9.5), MEN10627 (9.2), SR48968 (9.0), MEN11420	SR142802 (9.2), SB223412 (9.0; Sarau *et al.*, 1997), PD157672 (7.8)

Table 1.1. Neurokinin 1 (NK_1); neurokinin 2 (NK_2); neurokinin 3 (NK_3); substance P (SP); neurokinin A (NKA); neurokinin B (NKB). From (Alexander, 2009).

FIGURE 1.11. Neurokinin receptor expression in the airways.

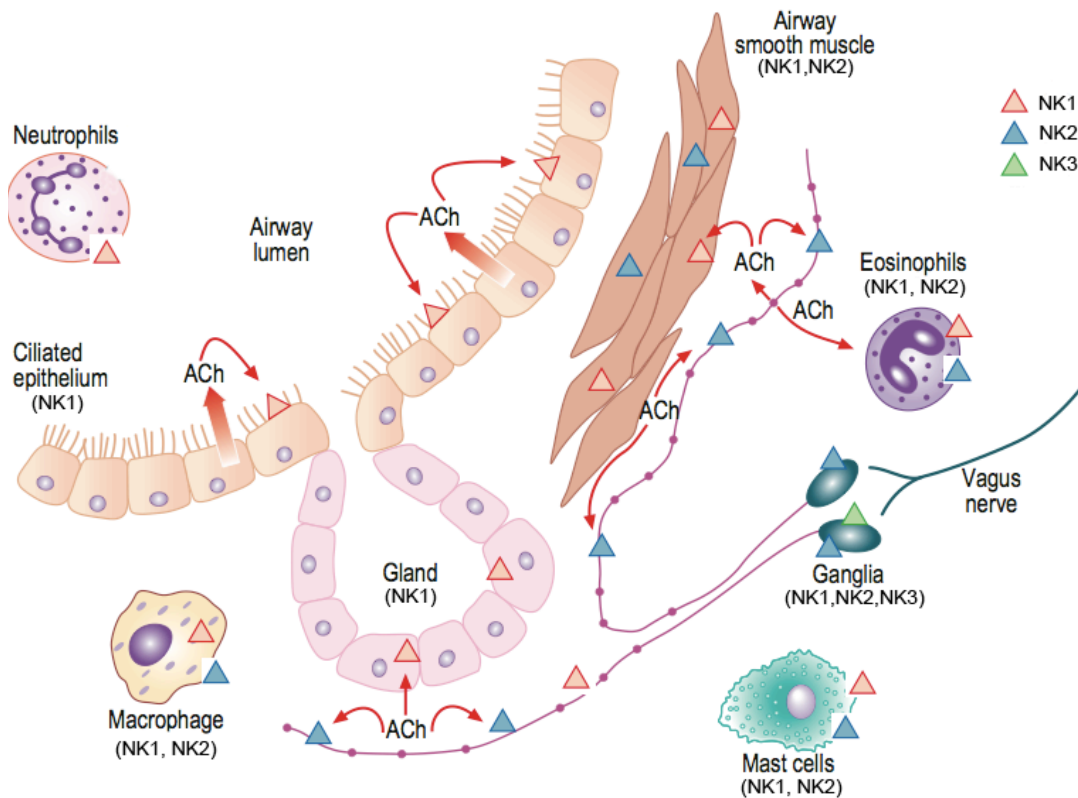


Figure 1.11. Neurokinin receptors are present on many airway cells. See text for references. Neurokinin 1 (NK1); Neurokinin 2 (NK2); Neurokinin 3 (NK3). Figure adapted from (Fryer, 2008).

E. NEURAL PLASTICITY

1. General overview

Neural plasticity is the phrase loosely used to describe any change in neurons including morphology, neurotransmitter release and content, receptor and channel expression, and synaptic strength. These changes can be acute, or long-term, and occur in the brain during normal development, in processes of learning and memory, and also in the periphery, especially under injury and inflammatory conditions.

During development, neural plasticity is common and employs both morphological changes and changes in neurotransmitter expression first observed in motor nerves during development. Initially, multiple motor nerve axons innervate each skeletal muscle end plate, however after birth, each motor end plate ends up innervated by only one motor nerve through selective axon retraction (Brown *et al.*, 1976). Neural plasticity also occurs in peripheral sympathetic nerves. A classic example of plasticity in neurotransmitter expression is in sympathetic neurons that innervate sweat glands. These sympathetic nerves convert from producing and releasing norepinephrine to acetylcholine as their principal neurotransmitter between 7 and 14 days postnatal in rats (Landis, 1983; Landis *et al.*, 1983). The switch to acetylcholine as the main neurotransmitter occurs when nerves stop producing norepinephrine and begin to express acetylcholinesterase.

Plasticity in neurotransmitter content occurs during inflammatory states as well as during development. After nerve injury or allergen challenge in mice, sensory A-fibers begin to produce substance P, which is not normally expressed by A-fibers (Chuaychoo *et al.*,

2005; Ruscheweyh *et al.*, 2007). These observations suggest plasticity in peripheral sensory nerves where A-fibers take on a sensory C-fiber phenotype.

Neural plasticity also includes changes at individual synapses termed synaptic plasticity. Synapse strength changes with increasing or decreasing stimulation from presynaptic nerves and mechanisms include increasing or decreasing presynaptic neurotransmitter release or postsynaptic neurotransmitter content. Changes in synaptic strength are important in the central nervous system in processes of learning and memory.

2. Mediators of neural plasticity

Many proteins contribute to various aspects of neural plasticity, from nerve growth to synaptic plasticity. These include inflammatory cytokines, neurotrophins, and chemokines (Abbadie *et al.*, 2009; Boulanger, 2009). This thesis will focus on the neurotrophin family of neural plasticity mediators because they are highly expressed in the lungs and by inflammatory cells recruited to the airways following ozone exposure.

3. Neurotrophins

One family of proteins important in neural plasticity is the neurotrophin family. Nerve growth factor (NGF) is the prototypical member of the neurotrophin family of structurally related proteins involved in nerve growth, survival, and differentiation. Rita Levi-Montalcini discovered NGF in experiments in which a mouse sarcoma tumor was transplanted into a chick embryo and significant increases in embryonic sympathetic and sensory nerve growth were observed (Levi-Montalcini *et al.*, 1954). She hypothesized the

tumors were releasing a diffusible factor since implantation of the tumors away from direct contact with embryonic tissue still caused significant nerve growth (Levi-Montalcini, 1953). The factor was named nerve growth factor and was isolated and characterized from mouse salivary glands (Cohen, 1960). Since its discovery, NGF has been shown to play wide-ranging roles in nerve development and neural plasticity in adult neurons including increasing nerve growth and neurotransmitter expression.

All neurotrophins are synthesized as precursors called pro-neurotrophins. NGF is synthesized as pro-NGF and is cleaved into mature NGF by intracellular furin or pro-convertases (Edwards *et al.*, 1988a; Edwards *et al.*, 1988b). There are three subunits of NGF: α, β, and γ. The β subunit of NGF forms a dimer with itself that is responsible for biological activity (Fahnestock, 1991).

Other members of the neurotrophin family include brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4. In addition to NGF, these neurotrophins also play a significant role in neural development and plasticity. During development, neurotrophin-3 knockout mice lose about 30% of sensory neurons in dorsal root ganglia (Tessarollo *et al.*, 1994). These data suggest separate populations of sensory nerves are sensitive to either NGF or neurotrophin-3 during development. In adult animals, a significant role in neural plasticity has been described for brain derived neurotrophic factor in the context of learning and memory (Minichiello, 2009). This dissertation will focus on the effects of NGF on airway parasympathetic nerves because NGF is highly expressed in the airways and its effect on peripheral parasympathetic nerves is not well understood.

a. Neurotrophin receptors

There are two types of neurotrophin receptors: the selective tyrosine kinase receptors (Trk), and the nonselective p75 receptor. NGF preferentially binds TrkA, neurotrophin-3 binds TrkB, and neurotrophin-4 binds TrkC, while all mature neurotrophins bind to p75 with low affinity (Figure 1.12). Pro-neurotrophins also have biological activity and bind the p75 receptor with high affinity (Lee *et al.*, 2001). When neurotrophins bind, Trk receptors dimerize and cross-phosphorylate each other leading to recruitment of signaling molecules including activators of the mitogen activated protein kinase pathway (described above in section B.5), and proteins involved in survival such as phosphatidylinositol 3-kinase. Neurotrophins also bind to p75 causing receptor dimerization and activation of the mitogen activated protein kinase pathway, the transcription factor NF-κB, and proteins involved in cell death.

Neurotrophin receptors are located on many different and diverse airway cells. All three Trk receptors are present on human and rat vascular smooth muscle (Donovan *et al.*, 1995) and TrkA and p75 are present on parasympathetic nerves (Hazari *et al.*, 2007; Ricci *et al.*, 2004). Alveolar cells and glands express TrkA and Trk C, but not p75 (Ricci *et al.*, 2004).

NGF receptors are also present on inflammatory cells. TrkA receptors are found on mast cells, lymphocytes, macrophages, basophils, and eosinophils (Barouch *et al.*, 2001; Burgi *et al.*, 1996; Ehrhard *et al.*, 1993; Hamada *et al.*, 1996; Tam *et al.*, 1997; Torcia *et al.*, 1996). Macrophages and eosinophils also express p75 receptors (Caroleo *et al.*, 2001).

Activation of NGF receptors leads to differentiation and proliferation of inflammatory cells and release of inflammatory mediators.

FIGURE 1.12. Neurotrophin receptors and signaling pathways.

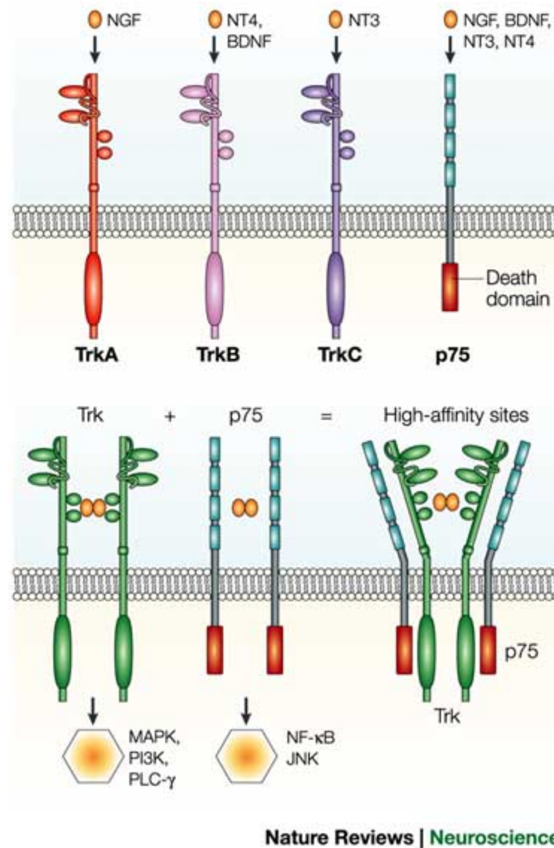


Figure 1.12. Nerve growth factor (NGF) selectively binds TrkA receptors, neurotrophin-4 (NT4) and brain derived neurotrophic factor (BDNF) bind TrkB, and neurotrophin-3 binds TrkC. All neurotrophins bind the p75 receptor with low affinity and all pro-neurotrophins bind p75 with high affinity. Trk receptors signal through the mitogen activated protein kinase pathway (MAPK), and activate phosphotidylinositol 3-kinase (PI3K) and phospholipase C-γ (PLC-γ). The p75 receptor activates the transcription factor NF-κB and JNK mitogen activated protein kinase. p75 receptors can be a co-receptor for Trk receptors to form high affinity binding sites for mature neurotrophins. Figure from (Chao, 2003).

b. Sources of NGF in the airways

Many airway cells are capable of synthesizing and releasing NGF including mast cells (Leon *et al.*, 1994), macrophages (Braun *et al.*, 1998), eosinophils (Noga *et al.*, 2003; Solomon *et al.*, 1998), smooth muscle (Freund *et al.*, 2002), and epithelial cells (Fox *et al.*, 2001; Hahn *et al.*, 2006). In addition, both mast cells and eosinophils store NGF (Leon *et al.*, 1994; Noga *et al.*, 2003; Solomon *et al.*, 1998). Airway smooth muscle and airway epithelium produce NGF after stimulation with IL-1β (Fox *et al.*, 2001; Freund *et al.*, 2002).

Eosinophils from allergic individuals make and release more NGF than eosinophils from non-allergic humans (Noga *et al.*, 2003). This may be important in allergic individuals exposed to ozone since they have an increased eosinophilic response compared to non-allergic humans (described below in section F.3).

c. NGF in the airway

In addition to NGF's role as a neurotrophic factor, it has been implicated as a mediator of airway inflammation and asthma. Increased levels of NGF are found in the airways in bronchoalveolar lavage fluid and in the circulation of humans with asthma (Bonini *et al.*, 1996; Kassel *et al.*, 2001; Sanico *et al.*, 2000; Virchow *et al.*, 1998). NGF also increases in bronchoalveolar lavage and in airway macrophages in antigen-sensitized mice (Braun *et al.*, 1998). Mice overexpressing NGF in the lungs have increased eosinophils in the airways and increased ovalbumin specific IgE antibody titers after antigen challenge (Path *et al.*, 2002; Quarcoo *et al.*, 2004).

d. NGF and neural plasticity

NGF has been shown to have multiple effects on neural plasticity in terms of both neural development and by increasing cholinergic transmission and substance P expression. NGF production by target tissues is required for growth and survival of many sensory and sympathetic nerves. In NGF knockout mice, there is a significant reduction of sensory nerves in dorsal root ganglia and sympathetic nerves in superior cervical ganglia both due to apoptosis of neurons, and also failure to innervate target tissues (Crowley *et al.*, 1994; Glebova *et al.*, 2004). These observations confirmed reports of significant sensory and sympathetic nerve loss in animals treated with an antibody against NGF (Johnson *et al.*, 1980; Pearson *et al.*, 1983). In 1977, Robert Campenot developed a three-chamber culture system to study the effects of NGF on cultured sympathetic nerves (Campenot, 1977). In this system, nerve cell bodies are cultured in a central chamber while neurites can grow in two separate chambers where fluids are kept separate. Using this system, he demonstrated NGF induced significant neurite growth only when present in the same chamber as the neurites and not the cell bodies. Thus, NGF production by target organs is crucial to the proper growth of developing sensory and sympathetic neurons.

In addition to promoting and targeting neuron growth, NGF affects neurotransmitter expression. There are a number of studies demonstrating NGF increases gene expression and protein expression of substance P in sensory nerves (Kessler *et al.*, 1980; Lindsay *et al.*, 1989; MacLean *et al.*, 1988; Mingomataj *et al.*, 2008; Vedder *et al.*, 1993). NGF induces hyperreactivity to electrical stimulation of airway parasympathetic nerves that is blocked by an NK_1 receptor antagonist, suggesting NGF induces airway hyperreactivity

by increasing substance P in ferret trachea (Wu *et al.*, 2006). In addition to ferrets, NGF increases substance P expression in the lungs of both rabbits (Larsen *et al.*, 2004) and mice (Hoyle *et al.*, 1998) and increases neurokinin A and substance P expression in dorsal root ganglia (Vedder *et al.*, 1993).

Enhanced airway inflammation in NGF overexpressing mice is partly mediated by tachykinins, as a dual NK_1/NK_2 receptor antagonist prevents the influx of eosinophils (Quarcoo *et al.*, 2004). In antigen sensitized and challenged mice, pretreatment with an antibody to NGF prevented the influx of eosinophils into bronchoalveolar lavage (Path *et al.*, 2002). The reduction of eosinophils with antibodies to NGF is important since NGF has been shown to enhance eosinophil survival although which neurotrophin receptor is responsible for NGF-induced survival is unknown (Hamada *et al.*, 1996).

Less is known about the role of NGF and parasympathetic nerves. NGF is not thought to be necessary for development of peripheral parasympathetic nerves, but in the adult there is evidence that NGF induces changes in neurotransmitter release and content. NGF increases nerve excitability in vitro and dendrite outgrowth in vivo in airway parasympathetic nerves (Hazari *et al.*, 2007). NGF also increases expression of choline acetyltransferase, the enzyme responsible for production of acetylcholine (Venero *et al.*, 1996). Increased expression of choline acetyltransferase could increase acetylcholine content, and perhaps in the absence of regulated acetylcholine release (for example when neuronal M_2 receptors are dysfunctional) NGF would lead to increased bronchoconstriction by increasing acetylcholine.

NGF induces hyperinnervation of cultured mouse tracheal segments (Bachar *et al.*, 2004). The authors presumed NGF was either enhancing survival or inducing new growth of parasympathetic neurons because they were unable to stain with sensory nerve markers for substance P and CGRP. It is not surprising the authors were unable to stain cultured trachea sections for neuronal substance P or CGRP since the tracheas were cultured ex-vivo for 4 days during which time, it is likely any sensory fibers would have died off since their cell bodies are not located in the trachea. Thus, it is likely NGF increases growth of parasympathetic nerves, but growth of sensory nerves has not been excluded.

NGF's role in neural plasticity suggests it could play a role in airway hyperreactivity by directly increasing parasympathetic neurotransmission, or by increasing substance P expression in sensory and parasympathetic nerves. Whether NGF plays a role in ozone-induced airway hyperreactivity and neural plasticity will be addressed in chapter V.

F. AIRWAY INFLAMMATION

1. The inflammatory cascade

Airway inflammation occurs in a number of airway diseases, including asthma and chronic obstructive pulmonary disease, and in response to environmental pollutants like ozone. In the context of ozone, the inflammatory cascade begins with damage to alveolar macrophages and airway epithelial cells (described above in section B.5). Injury to these cells induces production and release of inflammatory cytokines, which are small glycosylated proteins released during inflammatory states that have multiple effects on

target cells. There are both pro- and anti-inflammatory cytokines. In addition to release of cytokines, macrophages and epithelial cells release chemotactic factors that recruit additional inflammatory cells to the airways, including eosinophils and neutrophils. Protein chemotactic factors called chemokines bind to G-protein coupled receptors on immune cells. Activation of chemokine receptors induces trafficking from the blood stream along a concentration gradient, from low to high, towards the cells producing the chemokines. All airways cells have been shown to release chemokines such as epithelial cells, airway smooth muscle, nerves, and inflammatory cells (Dokic *et al.*, 2006; Fryer *et al.*, 2006; Hirst *et al.*, 2002).

2. Inflammatory cells

a. General

Inflammatory cells will be discussed with an emphasis on cells shown to have a direct role in ozone-induced airway inflammation: macrophages, neutrophils, and eosinophils. Others will be discussed briefly. Specific roles of inflammatory cells following ozone exposure will be discussed after a general introduction to each specific cell type.

b. Lymphocytes

Both B and T lymphocytes are found in airways and are important regulators of allergic airway disease. B cells are responsible for production of antigen specific antibodies IgG, IgA, and IgG. There are multiple subclasses of T cells found in the airways, including T helper 1 and T helper 2 (Th1 and Th2). T cells release many inflammatory mediators in response to a variety of stimuli and it's thought that in asthma there is a predominance of

the Th2 phenotype of lymphocytes. Th2 cytokines (IL-4, IL-5, IL-13) promote airway eosinophilia and airway hyperreactivity (Cohn *et al.*, 1998; Mattes *et al.*, 2002).

c. Mast cells

Mast cells are associated with allergic airway diseases and are classically activated by antigen crosslinking of receptor molecules on their surface (Brightling *et al.*, 2002; Stanworth, 1971). The primary mediator mast cells release is histamine, which causes bronchoconstriction directly (Drazen *et al.*, 1978) and indirectly through neurally mediated reflex bronchoconstriction (Benson *et al.*, 1977; Ellis *et al.*, 1992). Mast cell activation also induces release of other mediators including leukotrienes and prostaglandins (Boyce, 2003).

d. Neutrophils

Neutrophils increase in the airways during inflammatory conditions and following exposure to environmental pollutants including ozone (Basha *et al.*, 1994; Fahy *et al.*, 1995; Scannell *et al.*, 1996). Activation of neutrophils releases inflammatory cytokines such as IL-1, IL-6, IL-8, tumor necrosis factor, granulocyte-monocyte colony stimulating and factor (Monteseirin, 2009; Roberge *et al.*, 1994) and also mediators of airway remodeling, such as matrix metalloproteinases (Cundall *et al.*, 2003). In addition, neutrophil elastase has been implicated in goblet cell degranulation (Agusti *et al.*, 1998; Nogami *et al.*, 2000; Takeyama *et al.*, 1998). Neutrophils are typically recruited early after an inflammatory insult by the chemokine IL-8 (Gosset *et al.*, 1997; Schelegle *et al.*,

1991). The role of neutrophils in ozone-induced inflammation is described below in section F.3.

e. Macrophages

Macrophages are the dominant resident inflammatory cell found in the airways. Macrophages reside primarily in the airway lumen where they are a first line of defense against external pathogens and environmental insults. Macrophages are phagocytes that ingest pathogens, inhaled particles, and apoptotic cells. They are also important in maintaining the redox state of the airways by scavenging and generating reactive oxygen species.

Activation of macrophages leads to release of many inflammatory cytokines (IL-1β, TNFα) (Arsalane *et al.*, 1995; Borish *et al.*, 1992; Dinarello, 1996). A detailed description of the effects of IL-1β is found below. Chemokines important for macrophage recruitment are increased in humans with asthma (Rozyk *et al.*, 1997) and depletion of macrophages with liposome-encapsulated clodronate prevents virus-induced and organophosphate pesticide-induced airway hyperreactivity in guinea pigs (Lee *et al.*, 2004; Proskocil, 2008). Thus, macrophages are important regulators of immune function in the lung.

i. IL-1β in airway inflammation

Interleukin 1β (IL-1β) is a potent cytokine mediator of inflammation that increases granulocyte (neutrophils and eosinophils) production in bone marrow (Hestdal *et al.*,

1994), induces expression of chemokines and adhesion molecules (Birdsall *et al.*, 1992; Dinarello, 1996; Jedrzkiewicz *et al.*, 2000), and activates eosinophils (Gounni *et al.*, 2000; Okada *et al.*, 1995). IL-1β is increased in bronchoalveolar lavage of humans with asthma compared to healthy controls, and is produced by epithelial cells and alveolar macrophages from humans with asthma (Arsalane *et al.*, 1995; Borish *et al.*, 1992; Jarjour *et al.*, 1995; Sousa *et al.*, 1996). IL-1β induces airway hyperresponsiveness in isolated human bronchi that is blocked by an antibody to NGF (Frossard *et al.*, 2005).

IL-1β stimulates substance P synthesis in myenteric nerves in rat intestine (Hurst *et al.*, 1993), and parasympathetic nerves in ferret trachea (Wu *et al.*, 2002). IL-1β may also contribute to neural plasticity by inducing production and release of NGF from airway smooth muscle and airway epithelial cells (Fox *et al.*, 2001; Freund *et al.*, 2002; Frossard *et al.*, 2005; Pons *et al.*, 2001). Thus, IL-1β contributes to both airway hyperreactivity and airway inflammation through multiple mechanisms.

The discovery of IL-1 began with observations that inflammatory cells release proteins that induce fever (Beeson, 1948). Throughout the next 30 years, research continued on protein mediators released by inflammatory cells that appeared to have multiple physiological effects, from fever induction, joint disintegration, and synthesis of other protein mediators. IL-1 went by many names early in its discovery, including leukocyte endogenous mediator, lymphocyte activating factor, and endogenous pyrogen, to name a few. In 1978, Kampschmidt demonstrated macrophages released a protein that induced fever in rabbits and induced synthesis of proteins that increase early after an

inflammatory stimulus (Kampschmidt, 1978; Kampschmidt *et al.*, 1978). At the same time in the field of rheumatoid arthritis a factor released by monocytes was shown to increase collagenase expression (Dayer *et al.*, 1977). There was skepticism in the scientific community that such a diverse range of actions was due to one molecule until IL-1β was cloned in 1984 (Auron *et al.*, 1984; Lomedico *et al.*, 1984). It is now recognized there are multiple members in the IL-1 family found on different genes including the agonists IL-1α and IL-1β, and the antagonist called IL-1 receptor antagonist (IL-1Ra). IL-1α and IL-1β are both synthesized as precursor proteins. Pro-IL-1α is biologically active and is cleaved to its mature form by membrane associated cysteine proteases (Kobayashi *et al.*, 1990) and is not typically found in circulation except during severe disease (Watanabe *et al.*, 1994). Pro-IL-1β has very little biological activity (Jobling *et al.*, 1988) and is cleaved to its mature active form by the enzyme IL-1β converting enzyme, also called caspase-1 (Black *et al.*, 1988).

1. IL-1 receptors

There are multiple IL-1 receptors including the active IL-1 receptor type I, the decoy receptor type II, and the soluble type II receptor (Figure 1.13) (Dinarello, 1997). Only binding to the type I receptor activates the target cell. The presence of multiple inactive IL-1 receptors probably evolved because of the potency of IL-1β. Multiple levels of control developed for regulating IL-1 signaling including decoy and soluble receptors that sequester IL-1β from binding to the active receptor. IL-1β is so potent that as little as 1ng/kg of intravenous infusion in humans produces symptoms such as fever (Tewari *et al.*, 1990). IL-1 receptor type I activation signals through multiple pathways including

p38 mitogen activated protein kinase (Freshney *et al.*, 1994) and activation of the transcription factor NF-κB (Stylianou *et al.*, 1992). Activation of IL-1 receptors leads to increased production of itself, and other inflammatory genes including tumor necrosis factor, IL-6, granulocyte-monocyte colony stimulating factor, eotaxin, and intracellular adhesion molecule-1 (Birdsall *et al.*, 1992; Dinarello, 1996, 2002; Jedrzkiewicz *et al.*, 2000; Rosenwasser, 1998; Wuyts *et al.*, 2003b). Both IL-1α and IL-1β bind to the same receptors with different affinities (see Table 1.2 for binding affinities).

2. IL-1 receptor antagonist

The endogenous IL-1 receptor antagonist was identified in the mid 1980s as the first endogenous protein antagonist (Balavoine *et al.*, 1986; Seckinger *et al.*, 1987) and binds to IL-1 receptors without biological activity (Dripps *et al.*, 1991). Similar to IL-1α and IL-1β, the IL-1 receptor antagonist is made by inflammatory cells, in particular macrophages and neutrophils (Matsushime *et al.*, 1991; Poutsiaka *et al.*, 1991; Roberge *et al.*, 1994). There is evidence that the ratio of IL-1β and the IL-1 receptor antagonist correlates with disease severity. In Lyme arthritis, patients with more severe disease had higher levels of IL-1β than the IL-1 receptor antagonist and patients who recovered more quickly had higher levels of the IL-1 receptor antagonist (Miller *et al.*, 1993). There is little sequence homology (26%) between IL-1β and the IL-1 receptor antagonist, but high structural homology between the two. Currently, a recombinant form of the IL-1 receptor antagonist is available as a treatment for rheumatoid arthritis (Cohen *et al.*, 2002). This drug is called anakinra and is available as a daily subcutaneous injection.

FIGURE 1.13. IL-1 receptors.

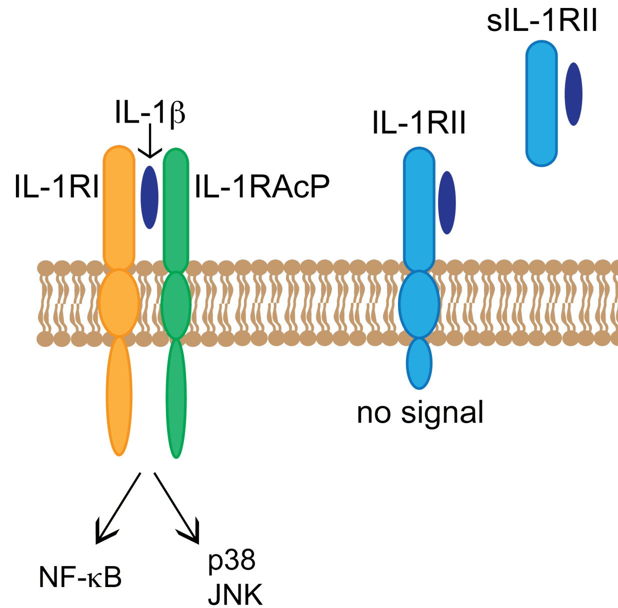


Figure 1.13. The IL-1 receptor type I (IL-1RI) binds IL-1β and the co-receptor IL-1 receptor accessory protein (IL-1RAcP) and signals through the transcription factor NF-κB, and the mitogen activated protein kinases p38 and JNK. The decoy receptor IL-1 receptor type II (IL-1RII) binds IL-1β without signaling. The type II receptor can be cleaved into the soluble IL-1 receptor type II (sIL-1RII), which binds IL-1β and prevents signaling through the IL-1 receptor type I.

TABLE 1.2. Binding affinities for IL-1α and IL-1β.

Ligand	Receptor		
	IL-1RI	IL-1RII	sIL-1RII
IL-1β	500 pM-1 nM	100 pM	500 pM
IL-1α	100-300 pM	30 M	10 nM

Table 1.2. IL-1 receptor type I (IL-1RI); IL-1 receptor type II (IL-1RII); soluble IL-1 receptor type II (sIL-1RII). Data from (Arend *et al.*, 1994; Dinarello, 2002; Slack *et al.*, 1993).

f. Eosinophils

Credit for the discovery of eosinophils is given to Paul Ehrlich who in 1879 described a white blood cell that stained with a negatively charged compound called eosin. Since then, eosinophils have been recognized to contribute to a number of disease states besides the classically defined role as the cell responsible for defense against parasites. Eosinophils are graulocytes produced in the bone marrow and are approximately 8μm in diameter. Eosinophils have a bi-lobed nucleus and are filled with spherical and ovoid granules that contain proteins released by a process known as degranulation (Figure 1.14) (Giembycz *et al.*, 1999; Rothenberg *et al.*, 2006).

Eosinophils are highly associated with chronic allergic airway inflammation, in particular in humans with asthma (Jacobsen *et al.*, 2007; Yamada *et al.*, 2000) and animal models of airway inflammation and airway hyperreactivity (Elbon *et al.*, 1995; Jose *et al.*, 1994). It was demonstrated in 1975 that eosinophils in blood were inversely correlated with airway function in humans (Horn *et al.*, 1975). Increased airway eosinophils are also correlated with decrements in lung function (Bousquet *et al.*, 1990), and eosinophils cluster around airway nerves in humans who have died of fatal asthma (Costello *et al.*, 1997).

Eosinophils are recruited to the airways by multiple chemotactic factors. The only mediators implicated in selective recruitment of eosinophils are IL-5 and eotaxin (Yamada *et al.*, 2000). For example, blocking the receptors for eotaxin prevents eosinophil recruitment to the airways of antigen challenged guinea pigs (Fryer *et al.*,

2006). Once recruited to the airways, eosinophils release many preformed proteins that are stored in granules including major basic protein, inflammatory cytokines, and neurotrophic factors such as nerve growth factor (Table 1.3) (Giembycz *et al.*, 1999; Kobayashi *et al.*, 2002; Noga *et al.*, 2003; Zheng *et al.*, 1999).

FIGURE 1.14. Guinea pig peritoneal eosinophil.

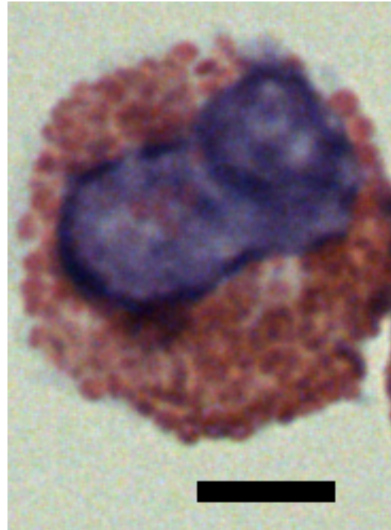


Figure 1.14. Photograph of a guinea pig peritoneal eosinophil stained with hemacolor. Scale bar is 10μm.

TABLE 1.3. Mediators released by eosinophils.

Granule Proteins	Cytokines	Chemokines	Lipids	Neural Mediators
MBP	IL-1α	GM-CSF	LTB4	NGF
ECP	IL-2	Eotaxin	LTC4	BDNF
EDN	IL-3	MCP-1	PAF	NT3
EPO	IL-4	MCP-3	PGE2	Substance P
	IL-5	MCP-4		LIF
	IL-6	MIP-1α		
	IL-8	RANTES		
	IL-10			
	IL-11			
	IL-12			
	IL-13			
	IL-16			
	IL-17			
	TNFα			

Table 1.3. Abbreviations: MBP, major basic protein; ECP, eosinophil cationic protein; EDN, eosinophil derived neurotoxin; EPO, eosinophil peroxidase; IL, interleukin; TNF, tumor necrosis factor; GM-CSF, granulocyte/macrophage colony stimulating factor; MCP, monocytes chemotactic protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T-cell expressed and secreted; LTB, leukotriene B; PAF, platelet activating factor; PGE, prostaglandin E; NGF, nerve growth factor; BDNF, brain derived neurotrophic factor; NT3, neurotrophin 3; LIF, leukemia inhibitory factor. List compiled from (Giembycz *et al.*, 1999; Hogan *et al.*, 2008; Rothenberg *et al.*, 2006).

i. Role of eosinophils in neural plasticity

The role of eosinophils in neural plasticity has not been widely studied. Eosinophils may play a role in neural plasticity due to their presence around airway nerves (Costello *et al.*, 1997) and they release a variety of factors known to affect neurons (see Table 1.3). The primary protein eosinophils release is major basic protein. Eosinophil major basic protein is an allosteric antagonist of neuronal M_2 muscarinic receptors (Jacoby *et al.*, 1993) and inhibition of M_2 receptors on airway parasympathetic nerves increases acetylcholine release that increases bronchoconstriction and leads to airway hyperreactivity. In addition to effects on parasympathetic nerves, major basic protein and other eosinophil-derived cationic proteins increase the sensitivity of sensory neurons by increasing capsaicin-evoked inward currents and the number of action potentials (Gu *et al.*, 2008). Thus, eosinophil major basic protein contributes to airway hyperreactivity by increasing acetylcholine release from parasympathetic nerves and by sensitizing sensory nerves.

Similar to airway nerves, eosinophils are associated with nerves in the intestine after parasitic infection and in the nose with allergic rhinitis (Fischer *et al.*, 2005; O'Brien *et al.*, 2008). Following parasitic infection in rats, innervation of the Peyer’s patches in rat intestine significantly increased, as did the presence of eosinophils (O'Brien *et al.*, 2008). Intestinal nerves were stained for GAP-43 and neural cell adhesion molecule, both markers of neural plasticity that significantly increased after parasitic infection. In nasal biopsies from humans with allergic rhinitis, eosinophils around airway nerves were significantly increased, as were peptide neurotransmitters in nasal mucosa nerves (Fischer *et al.*, 2005). Although neither of these studies conclusively links the co-

localization of eosinophils and nerves with the increase in neural plasticity markers, they suggest a direct link between the two may be possible.

Eosinophils release NGF, which has many effects on nerves (Kobayashi *et al.*, 2002; Noga *et al.*, 2003). Supernatant from eosinophils stimulated with immune complexes and IL-5 stimulates neurite growth in PC-12 cells differentiated to resemble sympathetic neurons (Kobayashi *et al.*, 2002). Neurite extension was prevented with an antibody to NGF, suggesting NGF released by eosinophils can have direct effects on nerve growth. The other numerous effects of NGF on neural plasticity are described in detail in section E.3. Whether eosinophils play a role in neural plasticity in parasympathetic nerves will be addressed in chapter IV.

3. Ozone and airway inflammation

a. Ozone and airway inflammation in humans

Ozone exposure induces an inflammatory response in lungs, in particular an early increase in neutrophils (Aris *et al.*, 1993; Koren *et al.*, 1989; Krishna *et al.*, 1998; Schelegle *et al.*, 1991; Seltzer *et al.*, 1986). Neutrophil influx into bronchoalveolar lavage peaks between 6 and 24 hours after exposure (Schelegle *et al.*, 1991). Not only neutrophils, but also inflammatory markers are upregulated in bronchoalveolar lavage including IL-6, IL-8 and products of arachidonic acid metabolism such as prostaglandin E2 and thromboxane B2 (Aris *et al.*, 1993; Koren *et al.*, 1989; Krishna *et al.*, 1998; Seltzer *et al.*, 1986).

Unlike lung function, there does appear to be a difference in the inflammatory response in the lungs of asthmatic/atopic humans compared to non-asthmatic/non-atopic. When exposed to ozone, asthmatics have increased numbers of neutrophils and total protein in bronchoalveolar lavage, as well as an increase in the inflammatory cytokine IL-8 when compared to non-asthmatics exposed to the same ozone conditions (Scannell *et al.*, 1996). An increase in total protein concentrations in bronchoalveolar lavage is indicative of an increase in epithelial permeability due to epithelial damage. Even when ozone concentrations are not high enough to reduce lung function, there is still an inflammatory response characterized by neutrophil infiltration and inflammatory cytokines IL-6 and IL-8 in bronchoalveolar lavage in asthmatic humans, suggesting mechanisms of early loss of lung function may be distinct from the inflammatory response (Basha *et al.*, 1994). Epithelial biopsies from atopic humans express more IL-5, GM-CSF, and IL-8 by

antibody staining than epithelium from non-atopic humans following ozone exposure (Bosson *et al.*, 2003).

Perhaps the main difference between atopic/asthmatic humans and healthy controls in their response to ozone exposure is that eosinophils are recruited to the lungs in atopic/asthmatics (Bascom *et al.*, 1990; Peden *et al.*, 1997; Peden *et al.*, 1995). Challenge with inhaled allergen potentiates the effects of ozone in asthmatic subjects. Mild asthmatics exposed to inhaled allergen and then ozone have increased eosinophils in sputum and decreased lung function measurements when compared to challenge followed by filtered air exposure (Vagaggini *et al.*, 2002). Thus, ozone exacerbates the eosinophilic response in humans with asthma. Even when eosinophils themselves are not present, or not measured, eosinophil cationic protein increases following ozone exposure in people with asthma, suggesting airway eosinophils are degranulating in response to ozone (Hiltermann *et al.*, 1999; Newson *et al.*, 2000).

b. Ozone and airway inflammation in animals

With animal studies, the role of individual inflammatory cells or inflammatory mediators in ozone-induced lung damage can be studied more thoroughly than in humans. Similar to humans, neutrophils increase in bronchoalveolar lavage and airway tissues early after ozone exposure in many species including dogs, guinea pigs, rats and mice (Hicks *et al.*, 2009; Holtzman *et al.*, 1983a; Hyde *et al.*, 1992; Johnston *et al.*, 2007; Kierstein *et al.*, 2008; Park *et al.*, 2004a; Park *et al.*, 2004b; Shore *et al.*, 2001; Williams *et al.*, 2008; Yost *et al.*, 2005). Increased neutrophilia after ozone exposure may be due to NGF since

mice overexpressing NGF in the airways have hyperinnervation of sensory nerves and increased infiltration of neutrophils after ozone exposure (Graham *et al.*, 2001).

Although neutrophils increase significantly and early after ozone exposure their exact role in ozone-induced lung damage is unknown. Neutrophil depletion with an antiserum did not prevent epithelial cell damage in ozone-exposed rats (Pino *et al.*, 1992). In dogs, prevention of neutrophil migration into the lungs with an antibody to the cell adhesion molecule CD11b/CD18 or pharmacologically with an inhaled steroid did not prevent ozone-induced airway hyperreactivity (Li *et al.*, 1992; Stevens *et al.*, 1994). There is some evidence neutrophil infiltration increases mucus production after ozone exposure (Cho *et al.*, 2000; Nogami *et al.*, 2000). Thus, neutrophils may contribute more to airway remodeling and mucus production than airway hyperreactivity after ozone exposure.

Eosinophils are also significantly increased in the lungs of guinea pigs, mice, rats, dogs and primates after ozone exposure (Fabbri *et al.*, 1984; Hyde *et al.*, 1992; Ishii *et al.*, 1998; Park *et al.*, 2004a; Schultheis *et al.*, 1994a; Tan *et al.*, 1992; Yost *et al.*, 2005). The role of eosinophils in ozone-induced airway hyperreactivity in guinea pigs is described in detail below.

Ozone also directly activates alveolar macrophages since they are found in the airway lumen where they come in direct contact with ozone. Macrophages isolated from bronchoalveolar lavage of ozone-exposed rats express more IL-1 and TNFα than macrophages from air exposed controls (Arsalane *et al.*, 1995; Ishii *et al.*, 1997; Manzer

et al., 2008; Pendino *et al.*, 1994). Thus far, no experiments have selectively depleted macrophages prior to ozone exposure, however many studies have investigated the contribution of macrophage produced inflammatory cytokines in ozone-induced lung damage and airway hyperreactivity. For example, tumor necrosis factor receptor knockout mice are protected from ozone-induced airway hyperreactivity (Cho *et al.*, 2001; Shore *et al.*, 2001). IL-6 knockout mice have reduced airway inflammation following ozone exposure, but are still hyperreactive (Johnston *et al.*, 2005). Although many cells and mediators have been demonstrated to play a role in ozone-induced airway inflammation and hyperreactivity, the relationship between them is still unclear.

c. Mechanisms of ozone-induced airway hyperreactivity in guinea pigs

Acutely, ozone-induced airway hyperreactivity in guinea pigs is mediated by recruitment and activation of eosinophils at airway nerves (Yost *et al.*, 1999). Activated eosinophils release preformed proteins including eosinophil major basic protein, which is an endogenous antagonist for M_2 muscarinic receptors (Evans *et al.*, 1997; Jacoby *et al.*, 1993). In the lungs, M_2 muscarinic receptors normally limit acetylcholine release from parasympathetic nerves (Fryer *et al.*, 1984). Eosinophils, via release of major basic protein, inhibit neuronal M_2 function resulting in increased acetylcholine release, increased bronchoconstriction, and vagally mediated airway hyperreactivity (Evans *et al.*, 1997; Fryer *et al.*, 1992). One day after a single exposure to ozone, neuronal M_2 receptor dysfunction and airway hyperreactivity are prevented or reversed by depleting eosinophils (Figure 1.15), preventing eosinophil migration into the lungs, and by blocking eosinophil major basic protein (Yost *et al.*, 1999). Therefore, one day after

ozone airway hyperreactivity is mediated by eosinophils, neuronal M_2 muscarinic receptor blockade, and subsequent increased acetylcholine release from parasympathetic nerves. What is not known is how ozone exposure results in stimulation of major basic protein release from tissue eosinophils.

In contrast, three days after ozone exposure airway hyperreactivity is mediated by different mechanisms. Although neuronal M_2 muscarinic receptors are dysfunctional at this time point, protecting or restoring M_2 function does not prevent airway hyperreactivity (Yost *et al.*, 2005). The role of eosinophils in hyperreactivity three days after ozone has changed from causative to protective, in that depleting eosinophils worsens airway hyperreactivity three days after ozone exposure (Figure 1.15) (Yost *et al.*, 2005). Hyperreactivity three days after ozone exposure is mediated by tachykinins. Blocking either neurokinin 1 or neurokinin 2 receptors completely prevents ozone-induced airway hyperreactivity three days after exposure (Figure 1.16) (Hazari, 2005). What is not known is the source of tachykinins three days after ozone exposure. The work in this thesis will explore this time course and the role of inflammatory mediators and tachykinins in ozone-induced airway hyperreactivity.

FIGURE 1.15. Depletion of eosinophils prevents ozone-induced airway hyperreactivity one and two days after ozone, and potentiates hyperreactivity three days after ozone.

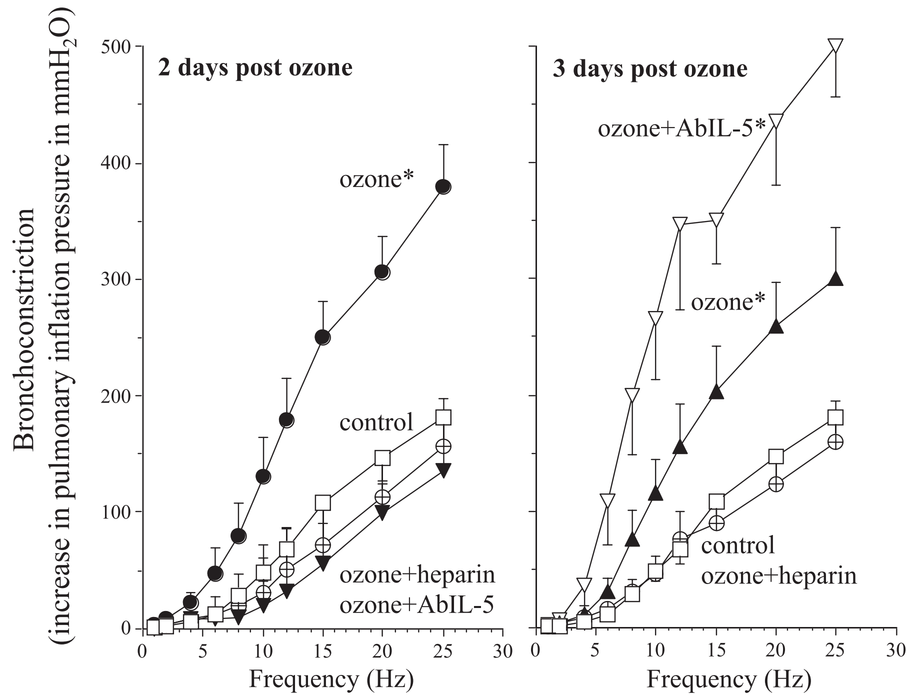


Figure 1.15. Eosinophils were depleted with an antibody to IL-5 (AbIL-5), a cytokine necessary for eosinophil activation and migration into tissues. A single ozone exposure caused airway hyperreactivity two (A closed circles) and three (B closed triangles) days after exposure measured as an increase in pulmonary inflation pressure in anesthetized guinea pigs compared to air exposed controls (open squares). Depletion of eosinophils one day (data not shown) and two days after ozone (A inverted closed triangles) prevented ozone-induced airway hyperreactivity. Depletion of eosinophils three days after ozone (B open inverted triangles) made hyperreactivity worse than ozone alone. Data from (Yost *et al.*, 2005).

FIGURE 1.16. Blocking substance P receptors prevent ozone-induced airway hyperreactivity three days after ozone.

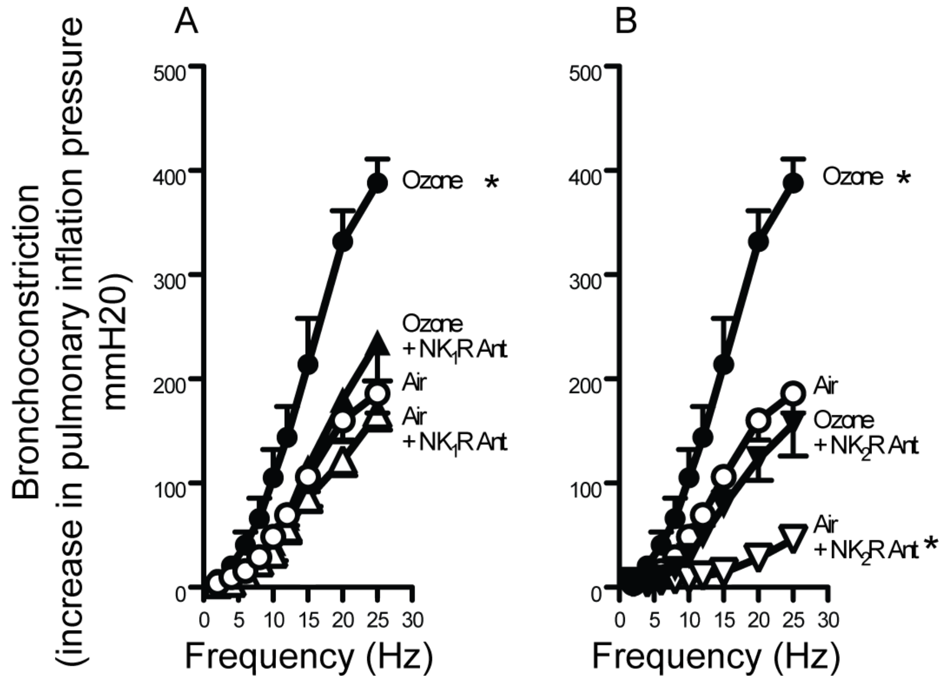


Figure 1.16. Guinea pigs were exposed to ozone and three days later airway hyperreactivity was measured as an increase in pulmonary inflation pressure in anesthetized animals. Ozone exposure significantly potentiated bronchoconstriction mediated by stimulation of the vagus nerves (closed circles) compared to air exposed animals (open circles). Both a neurokinin 1 (NK_1, A filled triangles) and a neurokinin 2 (NK_2, B filled inverted triangles) receptor antagonist completely prevented ozone-induced airway hyperreactivity. Data from (Hazari, 2005).

G. HYPOTHESIS AND RESEARCH AIMS

Currently, the majority of the population of the United States lives in areas with unhealthy levels of ozone. It is well know that exposure to ground level ozone causes airway hyperreactivity that persists over several days. Despite much research using animal and human exposures, the precise mechanism for how ozone actually causes airway hyperreactivity is still largely unknown. Confusion in the field as to the mechanism of ozone-induced airway hyperreactivity might be due to the existence of more than one mechanism.

One day after ozone exposure eosinophils are found in close proximity to airway nerves and mediate airway hyperreactivity in guinea pigs by releasing major basic protein that inhibits neuronal M_2 muscarinic receptors leading to increased acetylcholine release and increased smooth muscle contraction. Three days after ozone exposure airway hyperreactivity is mediated by tachykinins and not by release of major basic protein from eosinophils.

Ozone activates airway epithelial cells through reactive oxygen species and this leads to airway inflammation by stimulating recruitment of neutrophils and eosinophils, and also induces production and release of inflammatory mediators such as IL-1β. IL-1β induces NGF production and both these mediators induce substance P, which also increases in the airways after ozone exposure. *The overall hypothesis tested in this thesis is that ozone causes airway hyperreactivity via multiple mechanisms that change over time from eosinophil mediated to inflammatory cytokine and neural plasticity mediated.*

Specifically, these studies will determine signaling pathways involved in ozone-induced airway hyperreactivity one day after exposure and how inflammatory cytokines and eosinophils affect airway hyperreactivity and neural plasticity three days after exposure (Figure 1.17). A three-day time course will be used here because this is the time point where the role of eosinophils has changed from detrimental to beneficial.

FIGURE 1.17. Overall model of hypothesis tested in this thesis.

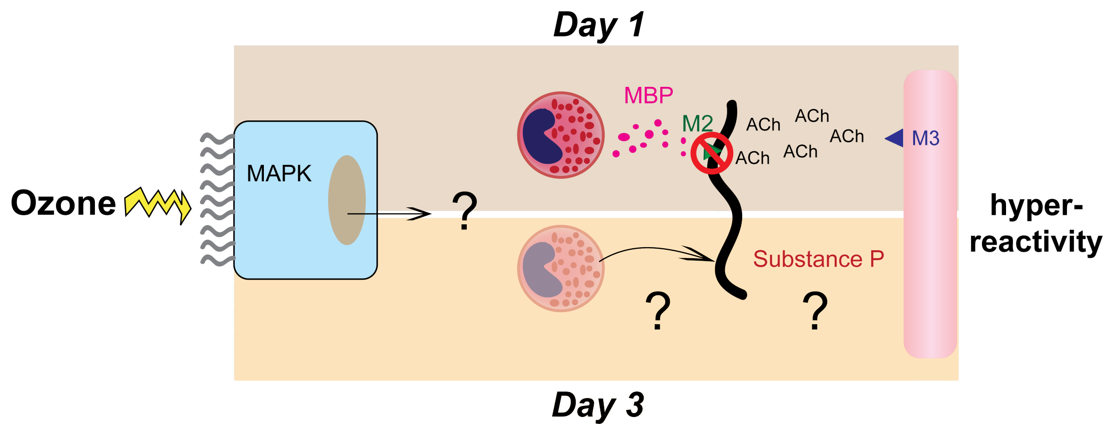


Figure 1.17. One day after ozone exposure, eosinophils release major basic protein (MBP) onto airway nerves inhibiting neuronal M_2 muscarinic receptors, increasing acetylcholine (ACh) release onto M_3 muscarinic receptors on airway smooth muscle resulting in airway hyperreactivity. Three days after ozone, the mechanisms have changed and are not mediated by eosinophil inhibition of M_2 receptors. This thesis will test whether ozone-induced airway hyperreactivity is mediated by release of inflammatory mediators, possibly from airway epithelial cells, which then induce neural plasticity in airway nerves. The arrows in the diagram are suggestive of the order of events but may not include all cells or mediators in between.

CHAPTER II.

GENERAL METHODS

A. CHOICE OF ANIMAL MODEL

Many different animal models are used to study ozone-induced airway hyperreactivity and inflammation. For the studies in this dissertation, the guinea pig was chosen as the animal in which to study ozone-induced airway changes. Guinea pigs are the ideal small mammals for studying airway hyperreactivity for many reasons. The gross anatomy of a guinea pig lung is very similar to humans in that there is an innermost layer of ciliated airway epithelium (Dalen, 1983) containing goblet cells and mucus glands that release mucus by neuronal stimulation (Poblete *et al.*, 1993; Rogers, 2001). Guinea pig and human airways also both have airway smooth muscle that contracts and relaxes with the same agonists. Airway receptor pharmacology in guinea pigs more closely resembles humans than other small animals, such as mice (Ressmeyer *et al.*, 2006). Most importantly, the autonomic innervation of both humans and guinea pigs is very similar. Sensory nerves innervate airway epithelium (Canning, 2006; Canning *et al.*, 2006) and parasympathetic nerves innervate airway smooth muscle (Canning, 2006). Parasympathetic nerves in both species induce smooth muscle contraction by activating M_3 muscarinic receptors on airway smooth muscle, and acetylcholine release is controlled by inhibitory M_2 muscarinic receptors located on the nerves (Fryer *et al.*, 1984; Ten Berge *et al.*, 1993). Similar to humans, guinea pigs exposed to ozone have airway inflammation (Yost *et al.*, 2005) and airway hyperreactivity that persists over several days (Schultheis *et al.*, 1994b).

B. IN VIVO MEASUREMENT OF AIRWAY PHYSIOLOGY

1. Guinea pigs

Specific pathogen-free female Hartley guinea pigs were used (350-550g Elm Hill Breeding Labs, Chelmsford, MA) for measurement of airway physiology *in vivo*. Guinea pigs were shipped in filtered crates, housed in high efficiency particulate filtered air, and fed a normal diet. Animals were handled in accordance with the standards established by the United States Animal Welfare Act set forth in National Institutes of Health guidelines. All protocols in this thesis were approved by Oregon Health & Science University Animal Care and Use Committee.

2. Ozone exposure

Guinea pigs were exposed to 2ppm ozone or filtered air for 4 hours as described previously (Yost *et al.*, 1999). This concentration of ozone exposure is higher than the environmental limit set by the Environmental Protection Agency because guinea pigs are obligate nose breathers, which means 50% of inhaled ozone reacts with nasal epithelium and does not reach the lungs (Green, 1982). Additionally, exposure is based on time and concentration; with a higher concentration the time of exposure is reduced (Gelzleichter *et al.*, 1992). Animals were placed in individual wire cages in a 700-liter stainless steel exposure chamber with laminar airflow (Figure 2.1). Guinea pigs had access to food and water in ceramic bowls. Ozone was generated by passing oxygen through an ultraviolet light generator (Orec, Glendale, CA) at a rate of 2L/min. Chamber ozone concentrations were monitored (model 1008 AH, Dasibi Environmental, Glendale, CA) and the air

supply was replaced at a rate of 20 times per hour. Following exposure, animals were returned to the animal care facility until physiological measurements were made.

3. Pretreatments

10-30 mg/kg recombinant human IL-1 receptor antagonist (Kineret) (Park *et al.*, 2004b; Sato *et al.*, 1999) or vehicle (6.5 mM sodium citrate, 140 mM NaCl, 48 mM EDTA, 1 mg/ml polysorbate 80) was diluted in sterile phosphate buffered saline, and given intraperitoneally 30 minutes prior to ozone or air exposure, and once daily thereafter.

Guinea pigs were given 30 mg/kg intraperitoneally of dual p38 and JNK mitogen activated protein kinase inhibitors V-05-013, V-05-014, or V-05-015 (Vertex Pharmaceuticals, Cambridge, MA) one hour prior to ozone exposure. Inhibitors were dissolved in 25% DMSO in phosphate buffered saline (PBS). Vehicle exposed control animals were given 25% DMSO in PBS one hour before exposure. Ki information for the dual mitogen activated protein kinase inhibitors is in Chapter III.

Guinea pigs were treated with either an antibody to nerve growth factor (AbNGF, 10μg/kg ip) or goat IgG (10μg/kg ip), one hour prior to ozone or air exposure. Company information and antibody concentrations are located in Table 2.3.

4. Surgical preparation

Resting physiological parameters for guinea pigs are in Table 2.1. One or three days after exposure to ozone, guinea pigs were anesthetized with 1.9 g/kg urethane

intraperitoneally. Complete anesthesia was achieved when both the foot and eye blink reflexes were absent. Both jugular veins were cannulated for intravenous administration of drugs and the right carotid artery was cannulated and connected to a pressure transducer to measure heart rate and blood pressure (Figure 2.2 and 2.3). Both vagus nerves were isolated, cut, and the distal ends placed on platinum electrodes submerged in mineral oil. Animals were tracheostomized and ventilated with positive pressure and constant volume (1 ml/100 g body weight, 100 breaths per minute). Animals were paralyzed to prevent spontaneous breathing with a constant infusion of succinylcholine (10 μg/kg/min iv). They were also chemically sympathectomized with guanethidine (5 mg/kg iv) to prevent release of norepinephrine from sympathetic nerves. A sidearm of the trachea cannula was connected to a pressure transducer for measurement of pulmonary inflation pressure, the pressure required to inflate the lungs.

5. Measurement of bronchoconstriction and fall in heart rate

Electrical stimulation of both vagus nerves (10V, 0.2 ms pulse width, 1-25 Hz, 5 sec duration at 1 minute intervals) produced frequency dependent bronchoconstriction, measured as the increase in pressure over basal inflation produced by the ventilator, and bradycardia, measured as a fall in heart rate, due to release of acetylcholine onto muscarinic receptors (Figure 2.4). Blockade of vagally induced bronchoconstriction by atropine (1mg/kg iv) at the end of each experiment was used to confirm that bronchoconstriction was mediated by acetylcholine release from parasympathetic nerves. M_3 muscarinic receptor function on airway smooth muscle was tested by measuring bronchoconstriction to 1-10 μg/kg acetylcholine iv in vagotomized guinea pigs.

TABLE 2.1. Guinea pig resting physiological parameters.

Measurement	Values
Respiratory frequency (breaths/min)	90-150
Tidal volume (ml)	1.0-4.0
Mean heart rate (beats/min)	155 (130-190)
Arterial blood pressure (mmHg)	90/56
Blood volume; total (ml/kg bodyweight)	75
Blood volume; terminal exanguination (ml/kg bodyweight)	35
Blood volume; safe maximum single sample (ml/kg bodyweight)	7

Table 2.1. Values obtained from (Green, 1982).

FIGURE 2.1. Ozone chamber.

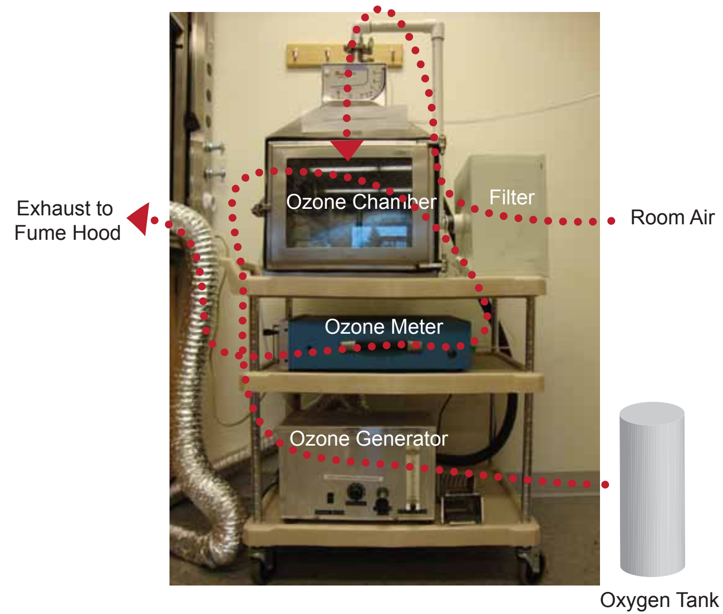


Figure 2.1. Guinea pigs are placed in a stainless steel wire mesh cage in the chamber with access to food and water. Oxygen is pumped through the ozone generator and ozone is mixed with filtered room air to maintain a concentration of 2ppm, measured by the ozone meter. Ozone exhaust leaves through a fume hood.

FIGURE 2.2. Anesthetized and ventilated guinea pig.

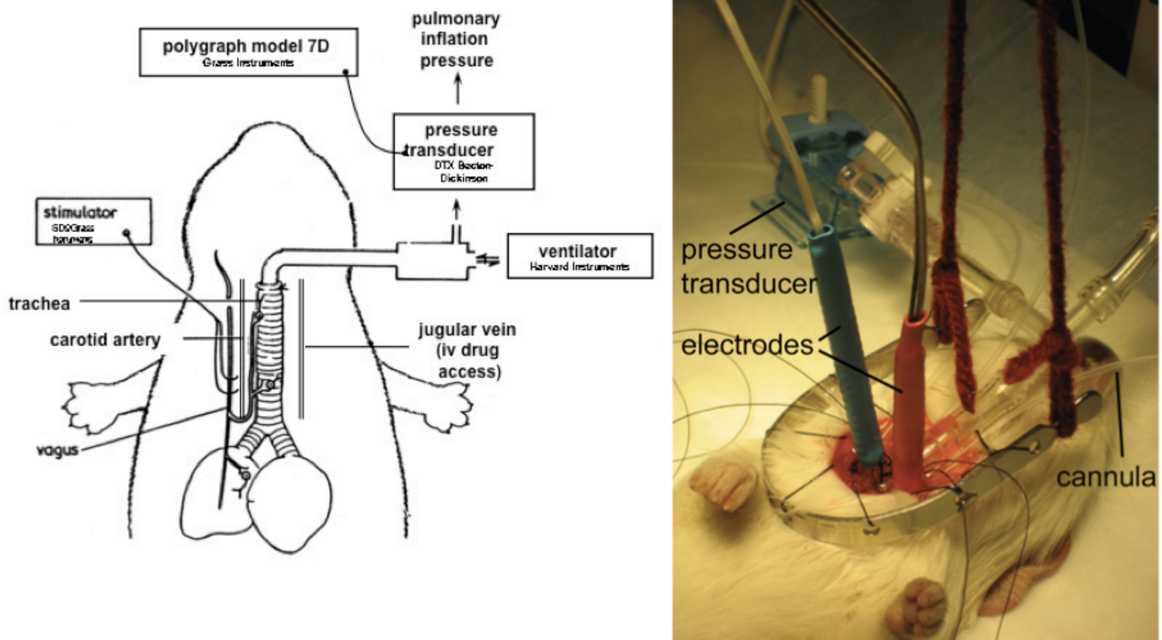


Figure 2.2. Guinea pigs are anesthetized with urethane (1.9 g/kg, ip), both jugular veins are cannulated for intravenous drug access, the carotid artery is cannulated to measure heart rate and blood pressure, and the trachea is cannulated to measure pulmonary inflation pressure while the animals are mechanically ventilated. Both vagus nerves are isolated, cut at the distal end, and attached to electrodes for electrical stimulation. The left panel is a schematic representation of the surgical procedure, while the panel on the right is a photograph of the final surgical set up of an anesthetized guinea pig. Schematic adapted from (Fryer, 1986).

FIGURE 2.3. Cannulation of a jugular vein.

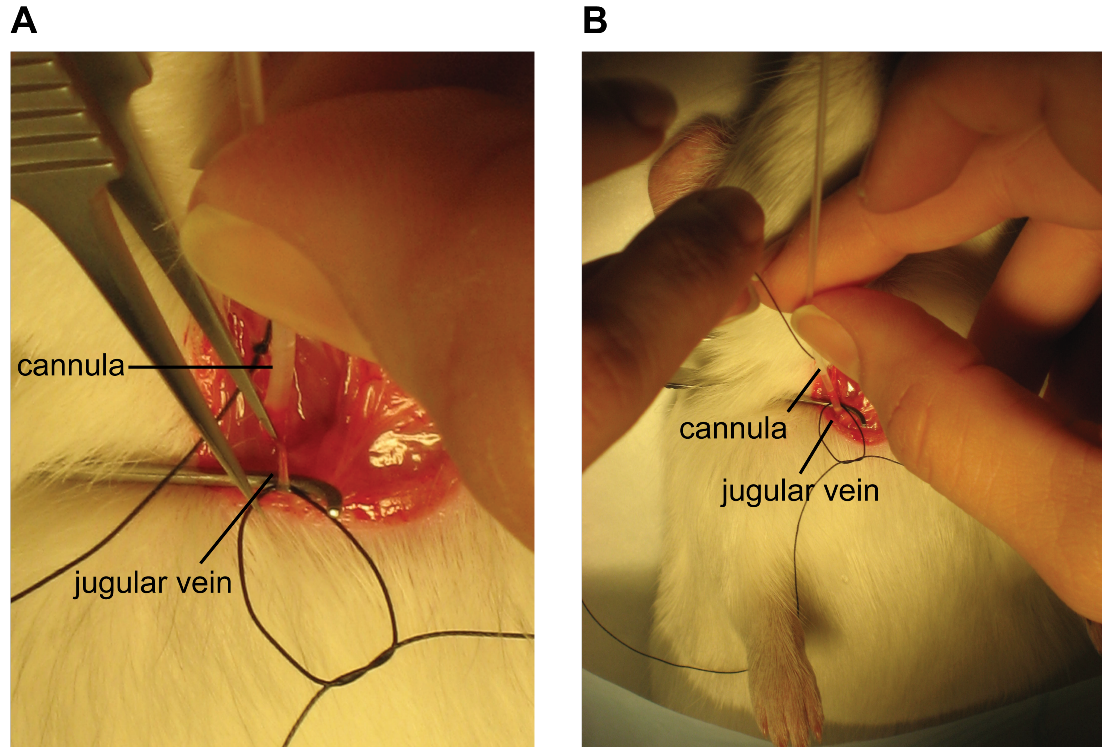


Figure 2.3. Guinea pigs were anesthetized and the neck was exposed. In these photographs, the right jugular vein was isolated and cleaned of any connecting tissue. Suture was tied tightly around the rostral end of the vein and loosely around the caudal end. A small hole was cut in the vein below the rostral suture and the cannula (PE90 tubing) was inserted through this hole towards the heart (A). In B, the cannula was slid into the vein and the caudal suture was tied around the cannula and vein.

FIGURE 2.4. Effects of vagal stimulation in anesthetized guinea pigs.

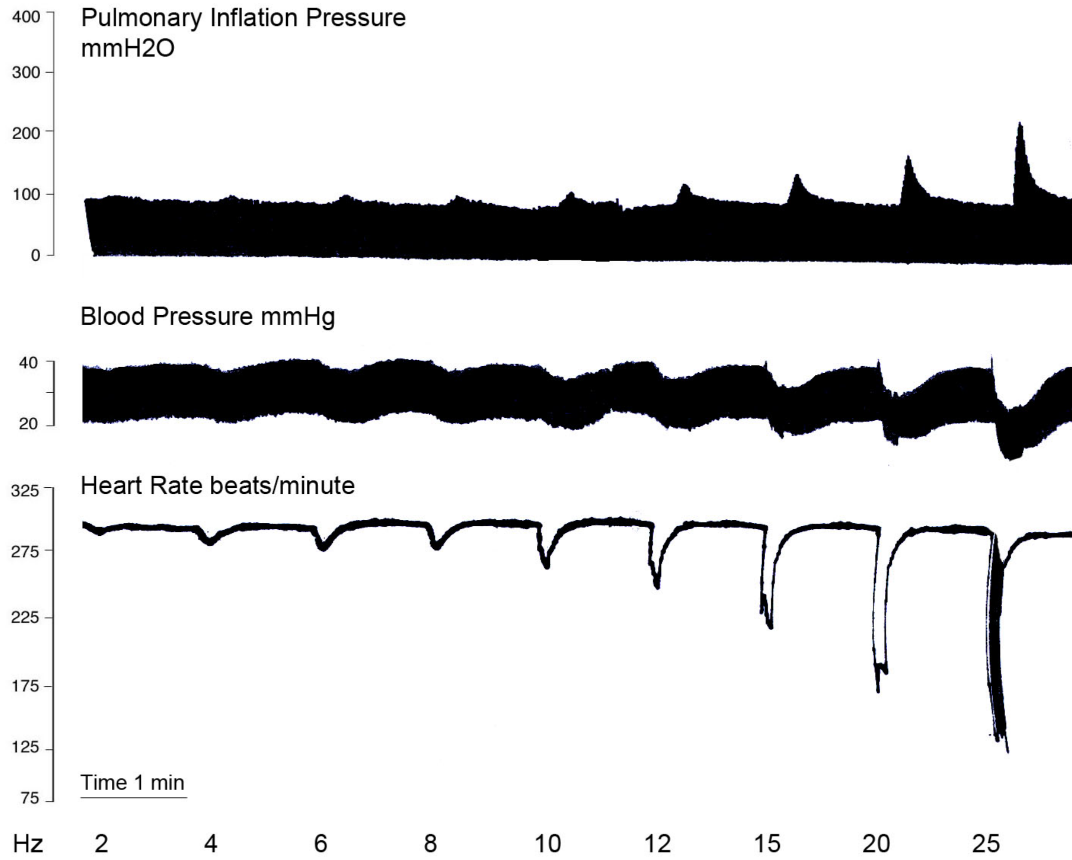


Figure 2.4. Shown is an example trace recorded on a Grass Polygraph. The top trace is pulmonary inflation pressure (mmH_2O), the center trace is blood pressure (mmHg) and the bottom trace is heart rate. Stimulation of both vagus nerves (2-25Hz, 10V) increases pulmonary inflation pressure and decreases heart rate.

6. Measurement of M_2 receptor function

Activation of M_2 muscarinic receptors on postganglionic parasympathetic nerves limits further release of acetylcholine (Fryer *et al.*, 1991b). Function of M_2 receptors was tested following administration of gallamine (0.1-30 mg/kg, iv), an M_2 receptor antagonist. Baseline bronchoconstriction was established by electrically stimulating the vagus nerves (15Hz, 3-30V, 0.2 ms pulse width, 3 sec duration at 1 minute intervals). Voltage was adjusted to achieve a baseline bronchoconstriction between 10-20 mmH_2O. Data are presented as ratios of bronchoconstriction before gallamine administration over bronchoconstriction after each dose of gallamine. If M_2 receptors are functional, gallamine causes an increase in bronchoconstriction because the nerve has lost the ability to inhibit acetylcholine release. In contrast, if M_2 muscarinic receptors are already inhibited, gallamine will not cause potentiation of bronchoconstriction.

7. Bronchoalveolar lavage and peripheral blood analysis of leukocytes

At the end of each experiment, the lungs were lavaged with five 10 ml aliquots of phosphate buffered saline (PBS) containing 100 μg isoproterenol. Cells were centrifuged (300xg 10 minutes), resuspended in PBS and counted using a hemocytometer. Cells were cytospun and stained with Hemacolor for differential cell counts. Whole blood was taken from the carotid artery cannula and lysed with 0.1N HCl. Total peripheral white blood cells were counted with a hemocytometer and cell differentials obtained from a blood smear stained with Hemacolor.

C. HISTOLOGY

1. Lung fixation

Following physiological measurements, lungs were isolated and fixed for histological analysis. The lungs were removed from the chest cavity and the left lobes were tied off at the main bronchi. The left lobes were removed below the suture and fixed in 4% formaldehyde overnight at 4°C, then cryoprotected in 18% sucrose, then 9% sucrose and 50% optimal cutting temperature (OCT). The right lobes were inflated through the trachea with zinc buffered formalin and fixed overnight at 4°C, then transferred to 70% ethanol. Lungs fixed in formaldehyde were frozen in OCT and sectioned on a cryostat (12μm, cut at 10-14°C), and right lobes were fixed in zinc buffered formalin and sectioned from paraffin blocks (6μm). Paraffin sections were stored at room temperature, and frozen sections were stored at -80°C.

2. Quantification of eosinophils in lungs and around nerves

Paraffin sections were dewaxed in xylene, followed by decreasing concentrations of ethanol in water. Slides were treated with antigen unmasking solution for 10 minutes at 90°C and then blocked with 10% normal goat serum for 1 hour at 37°C. Nerves were detected using rabbit polyclonal antibody to protein gene product 9.5 at 4°C overnight. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol. Sections were blocked with CAS Block, and incubated with secondary antibody, biotinylated goat anti-mouse IgG diluted at 1:400 in 5% normal goat serum in PBS at 37°C for 90 minutes. Slides were incubated with avidin-linked horseradish peroxidae (HRP). PGP9.5 staining was visualized with diaminobenzidine and nickel substrate. Eosinophils were stained

with 1% chromotrope 2R for 45 minutes. Slides were dehydrated in graded ethanol solutions followed by xylene and mounted with Cytoseal-60.

Eosinophils were quantified in 6-8 airways per guinea pig using four to six animals per group. Airways were photographed and airway area measured using MetaMorph software (version 6.2, Universal Imaging). Total area of the conducting airways between basement membrane and alveoli, including airway smooth muscle but excluding cartilage and blood vessels was measured and eosinophils within this area were counted and expressed as eosinophils per mm^2 of airway. Eosinophils within 8 μm of any airway nerve (as identified with PGP9.5 staining) were also counted. 8 μm was the distance chosen because it is approximately the diameter of an eosinophil (Sokol *et al.*, 1988).

3. Measurement of MBP deposition in lungs

Eosinophil major basic protein (MBP) was detected in guinea pig lung paraffin sections using a rabbit monoclonal antibody against guinea pig MBP (Lewis *et al.*, 1976) as previously described (Verbout *et al.*, 2007). Lung sections were dewaxed, treated with antigen unmasking solution, and blocked as for PGP staining. Slides were incubated with antibody to MBP, diluted 1:1000 in goat serum, overnight at 4°C, and staining detected with Alexa Fluor 555 goat anti-rabbit IgG (Molecular Probes). Slides were mounted with aqueous media containing DAPI (4',6-diamidino-2-phenylindole) to counterstain cell nuclei. Negative control slides were treated as above without primary antibody.

Slides were coded and analysis performed blinded. Airways were photographed under identical conditions and photographs analyzed using MetaMorph software. Area was calculated as for eosinophils in lungs, and deposition of extracellular MBP (intact eosinophils as identified by solid spheres of MBP staining greater than or equal to 8 μm in diameter were excluded) was quantified using a technique adapted from Tuder et al. (Tuder *et al.*, 2003). Background fluorescence intensity was measured in a noncellular region in the lumen of every airway and values were averaged and subtracted from each image. A threshold for MBP staining intensity was measured using the negative control of the airway with the strongest MBP signal. A serial section containing this airway was stained without primary antibody and average fluorescence measured. This threshold value set the lower limit of the calibration scale for all subsequent measurements. Only fluorescence above background and above MBP intensity threshold was measured. Total intensity was calculated by multiplying mean fluorescence intensity by the area containing positive MBP signal. Average fluorescence per μm^2 was determined by dividing total intensity by the total area of the airway. Data are means of 4-6 animals per group and 4 airways per animal.

4. Measurement of substance P in airway nerves

Initially, paraffin embedded lung sections were used to measure substance P expression in airway nerves. For this protocol, slides were dewaxed in xylene and graded ethanol solutions as above, incubated in antigen unmasking fluid for 10 minutes at 90°C followed by 10 minutes at room temperature, and then blocked with 10% NGS in PBS for 30 minutes at 37°C. Primary antibodies were incubated overnight at 4°C (mouse monoclonal

anti-PGP 1:200, and rabbit anti-substance P at 4 different dilutions (1:1000, 1:5000, 1:10,000, and 1:15,000). Primary antibodies were rinsed off and secondary antibodies incubated for 2 hours covered at room temperature (goat anti-mouse Alexa 488 1:1000 and goat anti-rabbit Alexa 555 1:2000). Slides were rinsed again in PBS and then coverslipped with aqueous mounting medium for fluorescence with DAPI. This protocol did not work to stain substance P in paraffin sections because there was no specific substance P staining at any primary antibody concentration (Figure 2.5).

FIGURE 2.5. Representative experiment during development of substance P staining protocol in guinea pig lung sections.

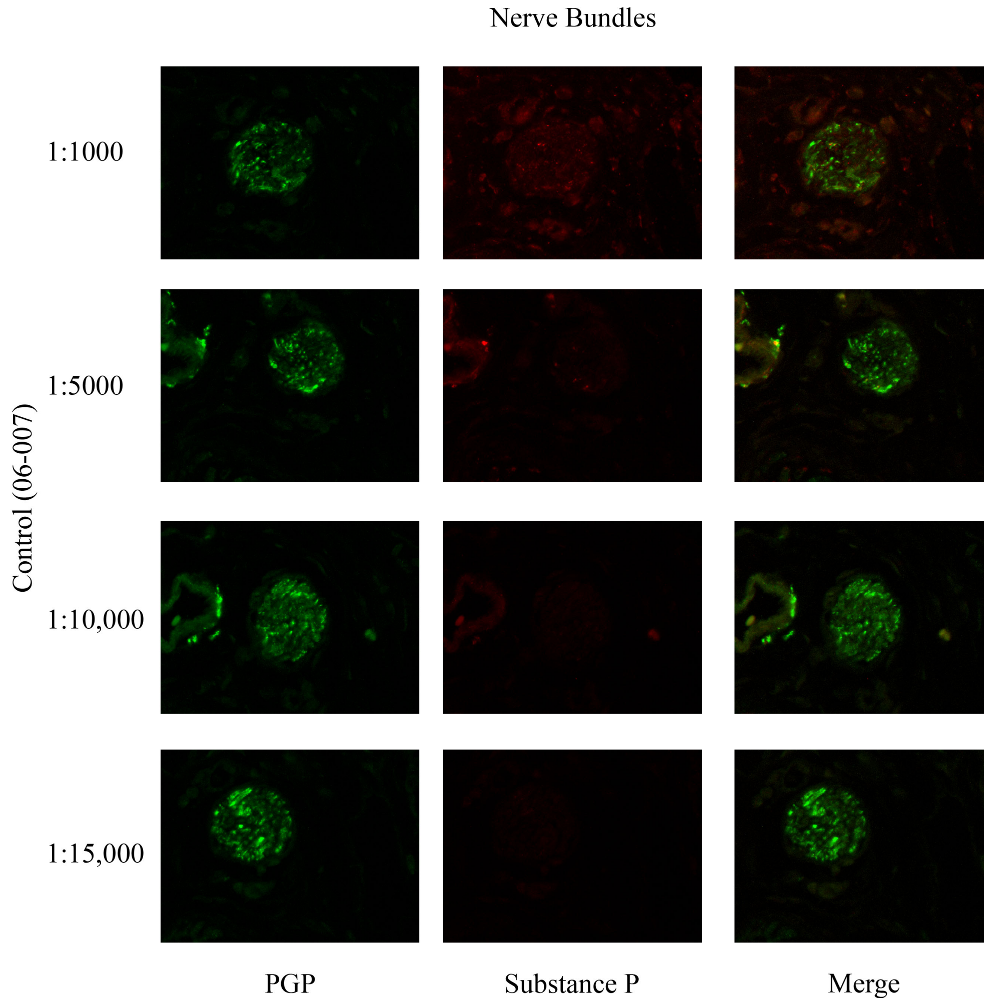


Figure 2.5. Guinea pig lung sections from a control animal embedded in paraffin were stained with antibodies to PGP9.5 (protein gene product 9.5, pan-neuronal marker, green) and substance P (red, multiple dilutions). Airway nerve bundles were photographed using filters for PGP (left panels) and substance P (middle panels) and overlayed (right panels). There was positive and specific staining with the PGP antibody, but not with the substance P antibody at any primary antibody dilution tested (listed on the left).

I then switched to frozen sections of guinea pig lung that were fixed in Zamboni's fixative. A similar protocol was followed where slides were rinsed in PBS, blocked with 3% bovine serum albumin, 15% normal goat serum, and 0.1% tween-20 for one hour at room temperature, and primary antibodies for PGP9.5 and substance P incubated overnight. Secondary antibody was added in blocking solution (goat anti-mouse Alexa 488 1:5000, and goat anti-rabbit Alexa 594 1:2000) for 2 hours at room temperature. Slides were rinsed with PBS and mounted with aqueous fluorescence media containing DAPI. This protocol did not work for the PGP9.5 antibody. Two possibilities for why this protocol failed was the wrong fixative was used and there was no antigen retrieval step. The protocol was repeated with an antigen unmasking step and still there was no positive PGP9.5 staining.

The final staining method that had positive staining for both PGP9.5 and substance P and was used in this dissertation was a protocol modified from Canning et al. (Canning *et al.*, 2002). Frozen sections fixed in 4% formaldehyde were rinsed in PBS and incubated in antigen unmasking solution as for PGP and MBP staining. Slides were blocked with 3% bovine serum albumin, 15% normal goat serum, and 0.1% Tween-20 in PBS for 1 hr at room temperature and then incubated overnight at 4°C with a rabbit polyclonal antibody to substance P and with mouse monoclonal antiserum to PGP9.5. Control slides were incubated without primary antibody. All slides were incubated with secondary antibodies conjugated to alexa fluorophores (goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 555) for two hours at room temperature.

Slides were coded and analysis performed blinded. The slides were photographed with an epifluorescence microscope equipped with appropriate filters to visualize fluorescein or rhodamine. Exposure times were fixed to allow comparison between treatment groups. There was no positive fluorescent staining in the absence of primary antibodies. For analysis, airway nerve bundles were identified and photographed based on PGP9.5 staining then the filter was switched to photograph substance P staining. Images were overlayed and nerve bundles were analyzed by three independent reviewers for presence or absence of substance P.

5. Quantification of mucus containing goblet cells in airways

Paraffin guinea pig lung sections were stained with periodic acid-Shiff stain (PAS) by the histology core (laboratory of Dr. Christopher Corless). Slides were dewaxed and hydrated as above, then incubated in 1% periodic acid for 10 minutes. Slides were rinsed in distilled water, and then incubated with Schiff's reagent for 10 minutes. They were rinsed again in warm running water for 10 minutes, and counterstained in hematoxylin for 2 minutes. Hematoxylin was rinsed off in tap water lithium carbonate solution was applied for 5 seconds and rinsed again in tap water. Slides were dehydrated as above and mounted with coverslips.

Slides were coded and analysis performed blinded. Airways were photographed and length of airway epithelium was measured using MetaMorph software. Mucus containing goblet cells in airway epithelium were counted and the data expressed as the number of

mucus containing goblet cells per length of airway epithelium. At least 4 airways were counted for each animal, with 3-6 animals per treatment group.

D. MEASUREMENT OF IL-1β IN BRONCHOALVEOLAR LAVAGE AND BONE MARROW

Supernatant from the first bronchoalveolar lavage (~10ml) was used to measure IL-1β protein levels. After the animal was killed by exsanguination, bone marrow was collected from the left femur by flushing the same 1 ml of PBS through the bone six times. Marrow lavage was centrifuged and supernatant saved. Total protein in bronchoalveolar lavage and bone marrow was measured using the bicinchoninic acid method (BCA protein assay kit; Pierce) and IL-1β was measured using a commercially available ELISA kit with standard controls (Immunotech, Marseille, France) as described by Sato et al. (Sato *et al.*, 1999). IL-1β concentrations were normalized to total protein.

E. GUINEA PIG PARASYMPATHATIC NERVE PRIMARY CULTURE

Parasympathetic nerves were isolated and grown from guinea pig tracheas. Of the nerves innervating lungs only parasympathetic nerves have their cell bodies in the trachea (Baker *et al.*, 1986; Myers, 2001).

Guinea pigs (adult females, 300-700g) were euthanized with a lethal overdose of pentobarbital (150 mg/kg, ip). Animals were placed on their back and their necks sterilized with 70% ethanol. The trachea was removed with sterile instruments and placed in PBS with penicillin and streptomycin (pen-strep). In the hood, the trachea was opened

through the cartilage and epithelium wiped away with cotton swabs soaked in PBS/pen-strep. Cartilage was trimmed off and discarded and the remaining smooth muscle chopped into fine pieces and incubated in 0.05% collagenase at 37°C for 4 hours. To quench the collagenase an equal volume of 5% fetal bovine serum (FBS) was added to the tissue and dissociated tissue was spun at 300xg for 10 minutes. The pellet was resuspended in 10% FBS with pen-strep and preplated on a 100mL culture dish overnight at 35.5°C with 5% CO_2. During the preplating step, non-neuronal cells adhere to the plastic dish, while nerves remain in suspension. Supernatant from the preplate, containing parasympathetic nerves, was spun (300xg, 10 minutes), resuspended in guinea pig serum free media containing pen-strep and nerve growth factor (NGF, 0.1ng/ml), and plated on either 4-well chamber slides or 35mm culture dishes coated with matrigel. To coat the slides or dishes, frozen matrigel was thawed on ice at 4°C, suspended in 4-6ml of cold DMEM, and added to cell culture dishes using a frozen serological pipette. Cells incubated for 24 hours, and then media was changed again to guinea pig serum free media containing pen-strep, NGF, and cytosine arabinoside, to kill dividing cells. Two days later, culture media was changed again to serum free media plus pen-strep and cells were treated and harvested between days four and eight.

F. GUINEA PIG PERITONEAL LAVAGE

Guinea pigs were anesthetized with ketamine (30mg/kg, im) and an analgesic, xylazine (5mg/kg, im). This level of anesthesia lasts about half an hour (Brown *et al.*, 1989). The peritoneum was lavaged with 50ml of warm, sterile, endotoxin-free PBS that is administered and then collected through an 18-gauge catheter (Lindor *et al.*, 1981).

Lavage fluid was centrifuged at 300xg for 10 minutes. Cells were resuspended in 20ml of cold sterile PBS and layered over 12ml of sterile percoll (density 1.090, adjusted with PIPES buffer), and centrifuged at 1400 rpm for 20 minutes with no brake. Eosinophils and red blood cells pellet at the bottom of the percoll layer (density >1.090), while macrophages and lymphocytes collect at the interface between the percoll and the PBS (density <1.090). The PBS and percoll were removed, along with the macrophages and lymphocytes. Red blood cells were lysed in a hypotonic solution by resuspending red blood cells and eosinophils in 18ml of sterile water for 30 seconds, and then adding 2ml of 10X PBS to restore isotonicity. Cells were spun again at 300xg for 10 minutes and resuspended in guinea pig serum free media. Cell viability was assessed using trypan blue exclusion, and purity using differential cell counting of a hemacolor stained cytospin slide.

G. STAINING SUBSTANCE P IN GUINEA PIG PARASYMPATHETIC NERVE PRIMARY CULTURE

Guinea pig parasympathetic nerves were grown as above (section E) on 4-well chamber slides. They were co-cultured with eosinophils for 2 days, eosinophils were then washed off with PBS and the nerves were fixed with 4% paraformaldehyde for 5 minutes. Slides were rinsed with PBS (2 times, 5 min) and blocked with a solution of 3% bovine serum albumin, 15% normal goat serum, and 0.05% Tween 20 in PBS, for one hour at room temperature. Chambers were removed, and individual wells outlined with a hydrophobic pen, and incubated with primary antibody (mouse anti-SMI 311, mouse anti-SMI 312, and rabbit anti-substance P) overnight at 4°C. SMI311 antibodies label non-

phosphorylated neurofilaments and SMI312 antibodies label pan-axonal neurofilaments and both were used to identify neurons. Control slides were incubated without primary antibody to substance P or with rabbit IgG. Slides were then rinsed in PBS (3 times, 5 min) and incubated with secondary antibodies (goat anti-mouse Alexa 488, and goat anti-rabbit Alexa 555) for 2 hours at room temperature. Slides were rinsed again in PBS and then mounted with soft set fluorescence mounting media with DAPI.

Nerves were photographed using an epifluorescence microscope equipped with appropriate filters to visualize fluorescein or rhodamine as above. Exposure times were fixed to allow comparison between treatment groups. There was no positive fluorescent staining in the absence of primary antibodies. Nerves were identified and photographed using the filter for SMI antibodies, the filter was switched to identify substance P and nerves were photographed again. Fluorescence was quantified as above (section C.3) using a technique adapted from Tuder et al. (Tuder *et al.*, 2003). Background fluorescence intensity was measured in a noncellular region and values were averaged and subtracted from each image. Using MetaMorph software, neurites were outlined using the SMI labeled images and the regions copied to substance P labeled images. Average intensity was measured and averaged over the entire picture and well (individual treatment replicate). Treatment replicates were then averaged together. Fold change relative to control untreated was calculated for each experiment, and averaged together.

H. RNA HARVEST, cDNA GENERATION, AND REAL-TIME PCR AMPLIFICATION OF mRNA

1. RNA harvest from guinea pig parasympathetic nerve primary culture

Guinea pig parasympathetic nerves in culture grow with their cell bodies in a cluster with neurites radiating outward from the central cell bodies (Figure 2.6). In addition, primary cultures are not homogenous cell populations and in order to minimize the RNA contribution of non-neuronal cells, a method was developed to harvest clusters of nerve cell bodies. Nerves were grown on 35mm cell culture dishes, as described above (section E), and following treatment, culture dishes were rinsed with ice cold PBS and then 1ml of PBS was added for harvest. Using an inverted light microscope, neurites surrounding a cluster of cell bodies were severed with a pulled glass micropipette. Neuronal cell bodies were then collected by applying suction with a syringe and then put into a microcentrifuge tube on ice. Between 5-15 clusters of cell bodies were harvested per treatment group.

FIGURE 2.6. Guinea pig parasympathetic nerves in culture.

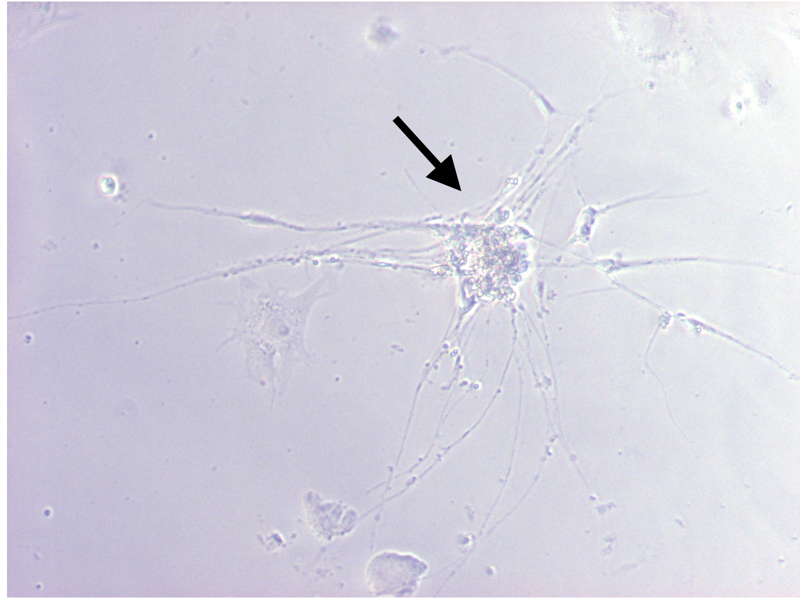


Figure 2.6. Parasympathetic nerves isolated from guinea pig tracheas, grown in culture on a matrigel coated chamber slide. Neuronal cell bodies are together in a cluster (arrow) with their neurites growing outward.

For RNA isolation, the Power SYBR Green Cells-to-Ct kit (Ambion) was used because it has been optimized for small numbers of cells, which is optimal for isolating RNA from neuronal cell body clusters. Cell bodies were centrifuged (2,000 rpm for 7 minutes at 4°C) and all but 5µl of PBS and the pellet were recovered. Cells were resuspended in lysis solution containing DNase to remove genomic DNA.

2. Reverse transcription of nerve mRNA

cDNA was made from isolated nerve mRNA using the Cells-to-Ct kit. RNA was combined with the supplied reverse transcription buffer containing dNTPs and random hexamers, and with the supplied enzyme mix. Reaction mixtures were run in the thermal cycler (Px2 Thermal Cycler, Thermo) at 37°C for 60 minutes to generate cDNA, and then 95°C for 5 minutes to inactivate the reverse transcriptase enzyme. At least one sample from each experiment was incubated without the reverse transcriptase to control for genomic contamination with real time PCR.

3. Real-time PCR of nerve cDNA

For real-time PCR reactions, 4µl of sample cDNA was added to reaction mixtures containing forward and reverse primers for the gene of interest (Table 2.2), reaction buffer containing SYBR Green, dNTPs, uracil-DNA glycosylase, and AmpliTaq Gold DNA polymerase. Reactions were run 95°C for 10 minutes to denture DNA and activate the polymerase, then 35 cycles of 95°C for 15 seconds to denature DNA, then 59°C for 1 minute for primer annealing and elongation (7500 Fast Real Time PCR System, Applied

Biosystems). Dissociation curves were obtained following completion of amplification cycles.

Primers for substance P were designed against preprotachykinin-β alternative splice variant of the preprotachykinin-A gene (Carter *et al.*, 1990). This splice variant contains all 7 exons, and substance P originates from exon three (refer to Figure 1.8), therefore the primers were designed to cross the third exon.

TABLE 2.2. Oligonucleotides for real-time polymerase chain reaction.

Gene	5' sequence	3' sequence	Product Size
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	151
PGP 9.5	CAACAACCGAGACAAACTGG	TGTCATCTACCCGACACTGG	170
IL-1β	CTTTGAAGAAGAGCCCATCG	CGAAGCTCATGGAGAACACC	194
PPTK	AAATCCTCGTCGCTTTGG	ATTGCTGAGGCTTGGGTCTC	186
NGF	CAGCGTCTGGACCCAATAAC	ATATGGTTCTGCCTGGATGC	153
NEP	CGACTTGGACTTCCTTCCAG	AGAAGCATTGGGTCATTTCG	230

Table 2.2. Oligonucleotides were designed against guinea pig genes. PPTK (preprotachykinin), NGF (nerve growth factor), NEP (neutral endopeptidase).

4. RNA harvest from guinea pig lung

Snap frozen guinea pig lung pieces were homogenized (Polytron PT10-35, Kinematica) in nuclease free tubes in RLT buffer containing β-mercaptoethanol. RNA was isolated using the RNeasy mini kit from Qiagen. 600μl of lung homogenate was combined with an equal volume of 70% ethanol. Samples were added to RNeasy mini columns and centrifuged at 10,000 rpm for 15 seconds. Samples were treated with DNase to remove genomic DNA and then rinsed and eluted from the silica-gel membrane.

5. cDNA generation from guinea pig lung/brain mRNA

Guinea pig lung and control brain mRNA was transcribed into cDNA using SuperScript III (Invitrogen). Brain mRNA was obtained from a lab stock of previously isolated brain mRNA. A master mix containing dNTP's, random hexamers, reaction buffer, magnesium chloride, dithiothreitol, and RNase inhibitors was combined with 1μg of mRNA. SuperScript III reverse transcriptase was added and tubes were incubated in a thermal cycler (Px2 Thermal Cycler) at 25°C for 10 minutes, 50°C for 50 minutes, and 70°C for 15 minutes. At least one sample from each tissue was incubated without the reverse transcriptase to control for genomic contamination during the subsequent real time PCR reactions.

6. Real-time PCR of guinea pig lung/brain cDNA

For real-time PCR reactions, cDNA was diluted in nuclease free water 1:100 for 18s primers, and 1:10 for all other genes. 2μl of cDNA was mixed with forward and reverse primers for genes of interest (Table 2.2), reaction buffer containing SYBR Green, dNTPs,

uracil-DNA glycosylase to reduce carryover contamination, and AmpliTaq Gold DNA polymerase. Reactions were run 95°C for 15 minutes, then 45 cycles of 95°C for 30 seconds, 59°C for 1 minute, then 72°C for 30 seconds (7500 Fast Real Time PCR System, Applied Biosystems). Dissociation curves were obtained following completion of amplification cycles.

I. DATA ANALYSIS AND STATISTICS

All data are expressed as means ± SE. In vivo, frequency response and dose response curves were compared using two-way ANOVA for repeated measures. Bronchoalveolar lavage, baseline data, and MBP deposition data were analyzed with a one-way ANOVA and Fisher's post hoc test. All other histology data were analyzed using one-way ANOVA and Bonferroni correction. A p value of less than 0.05 was considered significant. Analyses were made with Kaleidagraph (version 4.01, Synergy Software), StatView 4.5 (Abacus Concepts), or GraphPad Prism (version 5.0; GraphPad Software, La Jolla, CA).

TABLE 2.3. Reagents and supplies.

Pharmacological Reagents		
Drugs	**Supplier**	**Formula Wt**
acetylcholine chloride	Acros Organics	181.66
anakinra	Amgen	17.3 kD
atropine sulfate	Sigma	676.8
collagenase	Sigma	
gallamine triethiodide	Sigma	891.5
guanethidine monosulfate	Sigma	296.4
heparin sodium	American Pharmaceutical Partners	5,000 USP unts/ml
DL isoproterenol hydrochloride	Sigma	247.72
ketamine hydrochloride	Hospira	100 mg/ml
pentobarbital	Sigma	248.3
succinylcholine chloride	Sigma	397.3
urethane	Sigma	89.09
xylazine HCl	AmTech	100 mg/ml

Immunocytochemistry		
Antibodies	**Supplier**	**Dose/Concentration**
Gt x M Alexa 488	Invitrogen	1:1000
Gt x Rb Alexa 555	Invitrogen	1:1000 or 1:2000
Ab x betaNGF	R&D Systems	10 ug/kg
Rb x substance P	Chemicon	1:200 or 1:500
M x PGP9.5	Biogenesis	1:1000
Rb x MBP	gift from G. Gleich	1:1000
M x SMI 311	Covance	1:500
M X SMI 312	Covance	1:2000

Other Reagents	
antigen unmasking solution	Vector
bovine serum albumin	Fisher
CAS block	Vector
Chromotrope 2R	Sigma
DAB	Vector
Hemacolor	EMD Chemicals, Inc
normal goat serum	Vector
OCT	Sakura
Tween-20	Fisher

Commercially available reagents and kits	
BCA protein assay	Pierce
IL-1β ELISA	Immnotech
PCR Reagents	
1 Kb Plus DNA Ladder	Invitrogen
Ethidium Bromide	Fluka Biochemicals
Power SYBR Green Cells-to-Ct Kit	Ambion
QIAquick Gel Extraction Kit	Qiagen
SuperScript III	Invitrogen
Ultra Pure Agarose	Invitrogen

Guinea pig primary culture reagents		
Reagent	**Supplier**	**Formula weight (g/mol)**
Collagenase Type XI	Sigma	
Cytosine arabinoside	Sigma	
DMEM with L-glutamine	Mediatech	
Fetal bovine serum	HyClone	
Guinea pig transferrin	MP Biomedicals, LLC	
Ham's F-12 with L-glutamine	Mediatech	
HBSS with Ca^{2+} and Mg^{2+}	Mediatech	
Insulin-Transferrin-Selenite	Mediatech	
Matrigel	BD Biosciences	
2.5S Nerve growth factor	Harlan Bioprods BT	
PBS	Mediatech	
Penicillin-Streptomycin solution	Mediatech	
Pentobarbital	Sigma	248.3
Rat transferrin	Jackson ImmunoResearch	
60 mm x 15 mm cell culture dish	Corning	
35 mm x 10 mm cell culture dish	Corning	
100 mm x 20 mm cell culture dish	Corning	
25 cm^2 cell culture flask	Corning	
Cotton-tipped applicators	Fisherbrand	
SteriFlip – 50 mL disposable vacuum filtration system 0.22 mM	Millipore	

CHAPTER III.

p38 AND JNK MITOGEN ACTIVATED PROTEIN KINASES MEDIATE OZONE-INDUCED AIRWAY HYPERREACTIVITY

ABSTRACT

Ozone exposure causes airway hyperreactivity and increases hospitalizations resulting from pulmonary complications. Ozone reacts with the epithelial lining fluid and airway epithelium to produce reactive oxygen species and lipid peroxidation products, which then activate cell signaling pathways, including the mitogen activated protein kinase pathway. Both p38 and c-Jun NH_2 terminal kinase (JNK) are mitogen activated protein kinase family members that are activated by cellular stress and inflammation. To test the contribution of both p38 and JNK mitogen activated protein kinase to ozone-induced airway hyperreactivity, guinea pigs were pretreated with dual p38 and JNK mitogen activated protein kinase inhibitors (30mg/kg, ip) 30 minutes before exposure to 2ppm ozone or filtered air for 4 hours. One day later airway reactivity was measured in anesthetized animals. Ozone caused airway hyperreactivity one day after exposure, and blocking p38 and JNK mitogen activated protein kinase completely prevented ozone-induced airway hyperreactivity. Blocking p38 and JNK mitogen activated protein kinase also suppressed parasympathetic nerve activity in air exposed animals, suggesting p38 and JNK mitogen activated protein kinase contribute to acetylcholine release by airway parasympathetic nerves. Ozone inhibited neuronal M_2 muscarinic receptors and blocking both p38 and JNK prevented M_2 receptor dysfunction. Neither neutrophil influx into bronchoalveolar lavage or mucus secretion was affected by mitogen activated protein kinase inhibitors. Thus p38 and JNK mitogen activated protein kinase mediate ozone-induced airway hyperreactivity through multiple mechanisms including prevention of neuronal M_2 receptor dysfunction.

Data in this chapter have been prepared and sent to Vertex for final approval for publication in:

Verhein KC *et al*., Dual p38/JNK mitogen activated protein kinase inhibitors prevent ozone-induced airway hyperreactivity in guinea pigs. [Submitted to: *PLoS One*, February 2010].

INTRODUCTION

Ozone forms reactive oxygen species and lipid peroxides at the airway epithelial layer of humans and animals (Hamilton *et al.*, 1996; Kirichenko *et al.*, 1996). These end products activate cell signaling pathways, including mitogen activated protein kinase pathways (Kumagai *et al.*, 2002). Activation of the mitogen activated protein kinase pathway results in inflammation (Cui *et al.*, 2002; Underwood *et al.*, 2000), mucus hypersecretion (Atherton *et al.*, 2003; Fujisawa *et al.*, 2008) and airway hyperreactivity (Duan *et al.*, 2005; Nath *et al.*, 2006).

Inhibition of mitogen activated protein kinases is protective in allergen challenge models of asthma. Inhibition of p38, either pharmacologically or with antisense oligonucleotides, partially prevents airway hyperreactivity after sensitization and challenge in mice (Duan *et al.*, 2005; Nath *et al.*, 2006). A p38 inhibitor prevents eosinophil influx into bronchoalveolar lavage in guinea pigs and mice (Underwood *et al.*, 2000). Blocking p38 also prevents IL-13 induced mucus metaplasia in human and mouse airway epithelial cells (Atherton *et al.*, 2003; Fujisawa *et al.*, 2008).

What is not known is if the mitogen activated protein kinase pathway is involved in ozone-induced airway hyperreactivity through inhibition of neuronal M_2 muscarinic receptors. These studies were carried out to test whether dual inhibition of both p38 and JNK mitogen activated protein kinase pathways prevents ozone-induced inflammation and subsequent airway hyperreactivity in guinea pigs.

EXPERIMENTAL DESIGN

Ozone exposure

Guinea pigs were exposed to 2ppm ozone or filtered air for 4 hours as described in the methods section (Chapter II).

Treatments

Guinea pigs were given 30 mg/kg ip of the dual p38 and JNK mitogen activated protein kinase inhibitors V-05-013, V-05-014, or V-05-015 (Vertex Pharmaceuticals, Cambridge, MA) one hour before ozone exposure. Inhibitors were dissolved in 25% DMSO in phosphate buffered saline (PBS). Control animals were given 25% DMSO in PBS one hour before ozone exposure. All three drugs have similar affinity for the p38 and JNK (see table 3.1, Vertex unpublished data).

Measurements

One day after exposure to ozone or filtered air, pulmonary inflation pressure in response to both vagal stimulation and intravenous acetylcholine was measured as described in the methods (Chapter II). M_2 receptor function was assessed with intravenous gallamine administration. Inflammatory cells in bronchoalveolar lavage were counted and lung tissue sections were evaluated for number of mucus containing goblet cells as described in the methods.

Statistics

All data are expressed as means ± SE. In vivo frequency response and dose response curves were compared using two-way ANOVA for repeated measures. Histology and baseline data were analyzed by one-way ANOVA and Bonferroni's correction. A p value of less than 0.05 was considered significant. Analyses were made with GraphPad Prism (version 5.0).

RESULTS

One day after ozone exposure, baseline pulmonary inflation pressure was significantly increased compared to air-exposed controls (Table 3.2). All the dual p38 and JNK inhibitors partially attenuated the ozone induced increase in baseline airway inflation pressure, although the attenuation only reached statistical significance in the group treated with V-05-013. None of the mitogen activated protein kinase inhibitors affected baseline inflation pressure in air-exposed controls. Neither ozone nor the mitogen activated protein kinase inhibitors affected baseline heart rate or blood pressure.

Ozone significantly potentiated bronchoconstriction in response to electrical stimulation of the vagus nerves compared to air-exposed controls as previously reported (Figure 3.1). Treatment with each of the dual mitogen activated protein kinase inhibitors prevented ozone induced airway hyperreactivity (Figure 3.1A-C). M_2 muscarinic receptors were dysfunctional in ozone treated animals as gallamine, an M_2 selective inhibitor, potentiated bronchoconstriction in response to vagal stimulation in air-exposed animals but not in ozone-exposed animals (Figure 3.2); an effect that is consistent with decreased function of neuronal M_2 muscarinic receptors (Fryer *et al.*, 1991b). Ozone induced M_2 receptor dysfunction was prevented by treatment with V-05-014 and V-05-015 (Figure 3.2B-C), and attenuated by treatment withV-05-013 (Figure 3.2A). Airway smooth muscle responses to intravenous acetylcholine were potentiated by ozone (Figure 3.3). This was not prevented by any of the mitogen activated protein kinase inhibitors, but was partially attenuated by V-05-015 (Figure 3.3C). V-05-015 also produced a paradoxical increase in airway response to intravenous acetylcholine in air exposed animals.

Ozone exposure potentiated falls in heart rate in response to vagal stimulation compared to air-exposed controls (Figure 3.4A-C). Ozone and air-exposed controls are the same in Figure 3.4A-C. Separate pretreatment with all three dual mitogen activated protein kinase inhibitors prevented the ozone-induced potentiation in falls in heart rate and had no effect in air-exposed animals (Figure 3.4A-C). Falls in heart rate in response to intravenous acetylcholine were not affected by either ozone or the mitogen activated protein kinase inhibitor (Figure 3.4D-F). Ozone and air exposed controls are the same in Figure 3.4D-F.

One day after ozone exposure neutrophils were increased in bronchoalveolar lavage (Figure 3.5D). All the mitogen activated protein kinase inhibitors slightly attenuated the ozone induced increase in neutrophils (Figure 3.5D). None of the other inflammatory cell types were affected by either ozone or the mitogen activated protein kinase inhibitors (Figure 3.5).

There were no significant differences between inflammatory cells in peripheral blood either after ozone exposure, or treatment with the dual mitogen activated protein kinase inhibitors (Figure 3.6).

Ozone exposure significantly reduced the numbers of mucus containing goblet cells in the airways compared to air exposed controls demonstrating mucus secretion had occurred in the ozone-exposed guinea pigs (Figure 3.7). Treatment with the dual mitogen

activated protein kinase inhibitors did not affect goblet cell numbers in either air or ozone exposed guinea pigs.

TABLE 3.1. Ki values for dual p38 and JNK mitogen activated protein kinase inhibitors.

	Ki (nM)		
Compound	p38α	JNK1	JNK2
V-05-013	13	10	8
V-05-014	-	-	-
V-05-015	8	2	2

Table 3.1. All compounds have a Ki greater than 1μM for all other kinases tested (data generated by Vertex Pharmaceuticals). Data for V-05-014 is pending from co-investigator at Vertex Pharmaceuticals.

TABLE 3.2. Baseline pulmonary and cardiovascular parameters.

Group	*n*	Heart Rate (beats/min)	Blood Pressure (mmHg)		Pulmonary Inflation Pressure (mmH_2O)
			Systolic	Diastolic	
Air	7	306±6	40±4	20±3	110±4
Ozone	5	309±10	48±4	23±4	248±21 *
Ozone + V-05-013	5	301±7	40±3	20±2	190±15 * ‡
Ozone + V-05-014	4	286±16	38±4	21±1	225±5 *
Ozone + V-05-015	3	353±7	40±1	18±1	213±7 *
Air + V-05-013	4	291±7	32±2	17±1	102±3
Air + V-05-014	5	306±9	38±2	19±1	102±2
Air + V-05-015	5	327±14	40±3	22±3	94±5

Table 3.2. Values are means ± SEM. Baseline pulmonary inflation pressure significantly increased after ozone exposure. Treatment with dual p38/JNK mitogen activated protein kinase inhibitor V-05-013 significantly reduced the ozone-induced increase in baseline pulmonary inflation pressure. *$p<0.05$ Significantly different from air exposure. ‡$p<0.05$ Significantly different from ozone exposure.

FIGURE 3.1. Inhibiting both p38 and JNK mitogen activated protein kinase prevented ozone-induced airway hyperreactivity.

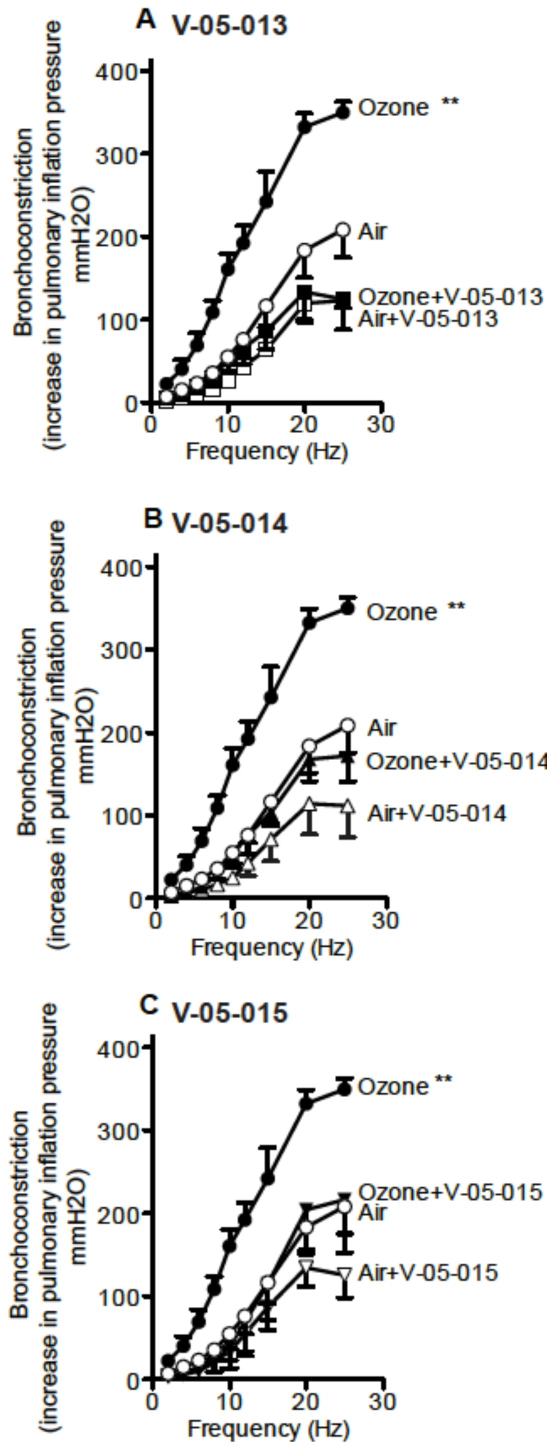


Figure 3.1. In anesthetized and vagotomized guinea pigs, stimulation of the vagus nerves caused frequency dependent bronchoconstriction (open circles) that is significantly potentiated one day after ozone exposure (closed circles). Pretreatment with dual mitogen activated protein kinase inhibitors V-05-013 (A closed squares), V-05-014 (B closed triangles), or V-05-015 (C closed inverted triangles) completely prevented ozone-induced airway hyperreactivity. All three dual mitogen activated protein kinase inhibitors suppressed parasympathetic nerve activity (A open squares, B open triangles, C open inverted triangles). Ozone and air exposed control data are the same in A-C. *$p<0.05$, **$p<0.01$ Significantly different from air exposed controls. Data are mean ± SEM. n=4-7

FIGURE 3.2. Inhibiting both p38 and JNK mitogen activated protein kinase prevented M_2 receptor dysfunction.

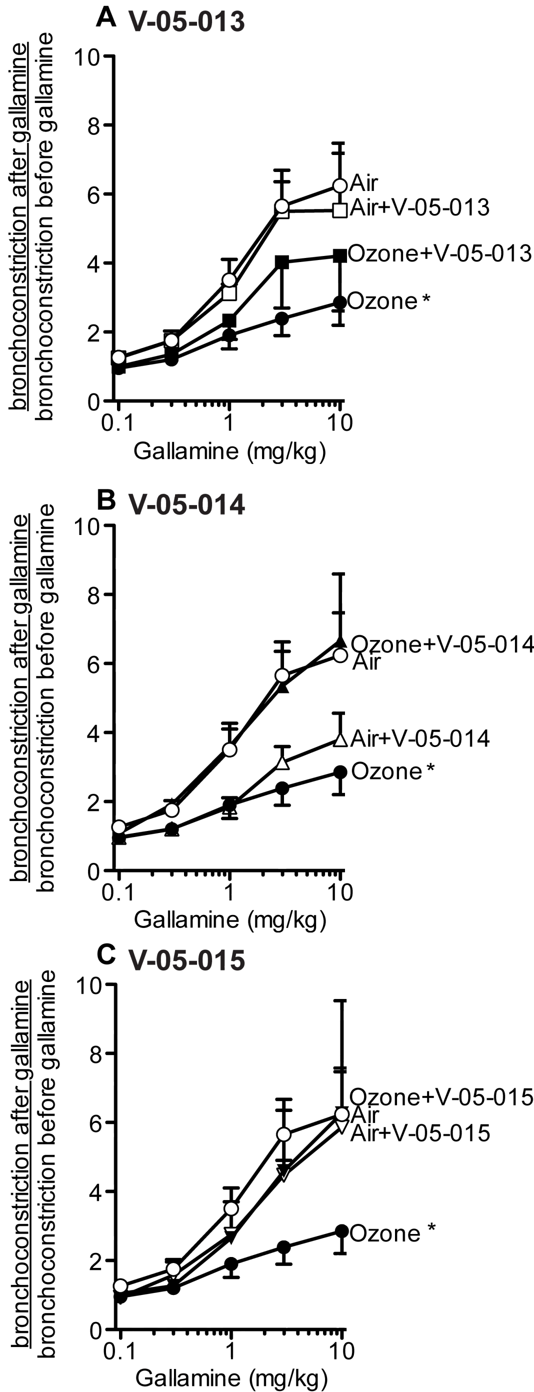


Figure 3.2. An M_2 receptor antagonist, gallamine, potentiated bronchoconstriction in response to vagal stimulation in air exposed animals (open circles) demonstrating a functional M_2 receptor. M_2 receptors were dysfunctional after ozone exposure (closed circles) and V-05-013 partially prevented M_2 receptor dysfunction (C closed squares), while V-05-014 (B closed triangles) and V-05-015 (C closed inverted triangles) completely prevented M_2 receptor dysfunction. Ozone and air exposed controls are the same in A-C. *$p<0.05$, **$p<0.01$ Significantly different from air exposed controls. Data are mean ± SEM. n=4-7

FIGURE 3.3. Inhibiting both p38 and JNK mitogen activated protein kinase did not prevent ozone-induced smooth muscle hyperresponsiveness.

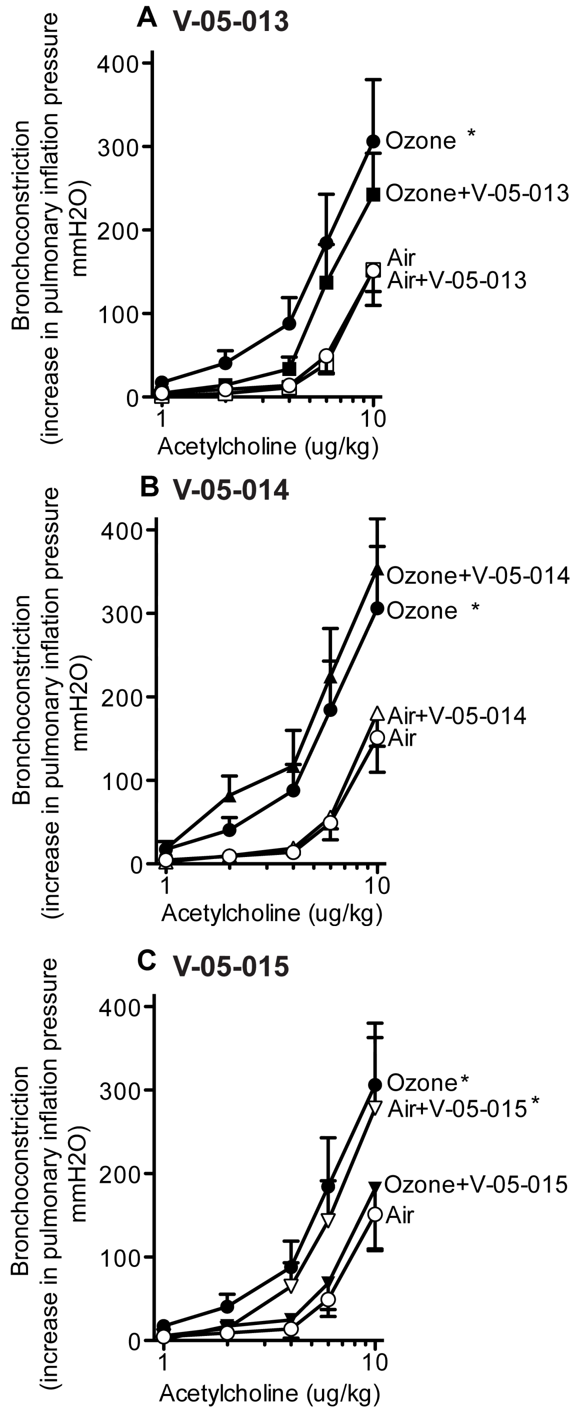


Figure 3.3. Bronchoconstriction in response to intravenous acetylcholine was significantly potentiated one day after ozone (closed circles) compared to air exposed controls (open circles), and was not blocked by V-05-013 (A closed squares), or V-05-014 (B closed triangles). V-05-015 (C closed inverted triangles) attenuated ozone-induced smooth muscle hyperreactivity while air exposed animals pretreated with V-05-015 were hyperreactive to intravenous acetylcholine (C open inverted triangles). Ozone and air exposed controls are the same in A-C. *p<0.05, **p<0.01 Significantly different from air exposed controls. Data are mean ± SEM. n=3-7

FIGURE 3.4. Inhibiting both p38 and JNK mitogen activated protein kinase prevented ozone-induced potentiation of falls in heart rate.

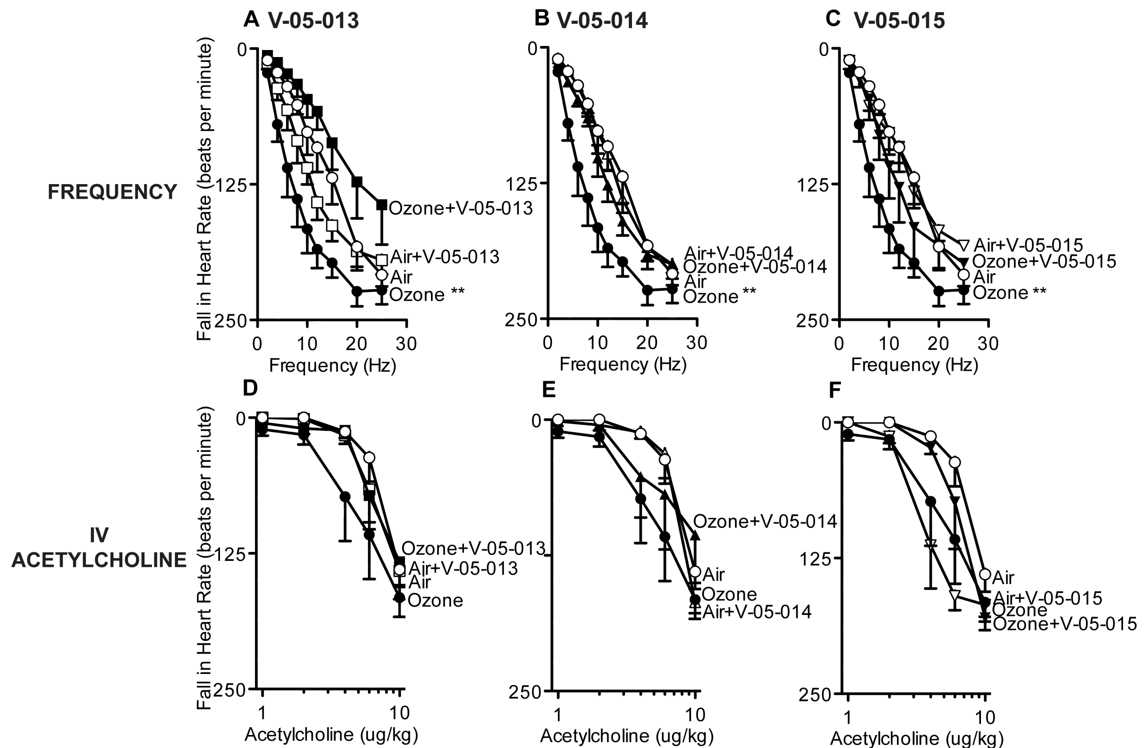


Figure 3.4. Ozone potentiated vagally mediated falls in heart rate (A-C closed circles) compared to air exposed animals (A-C open circles). Separate pretreatment with all three dual mitogen activated protein kinase inhibitors (V-05-013: A closed squares; V-05-014: B closed squares; V-05-015: C closed squares) prevented the ozone-induced potentiation of frequency induced falls in heart rate. Fall in heart rate following intravenous acetylcholine administration was not changed by either ozone, or mitogen activated protein kinase inhibitors (D-E). Ozone and air exposed controls are the same for A-C, and are the same for D-F. **$p<0.01$ Significantly different from air exposed controls. Data are mean ± SEM. n=3-7

FIGURE 3.5. Blocking both p38 and JNK mitogen activated protein kinase did not prevent ozone-induced increased in neutrophils in bronchoalveolar lavage.

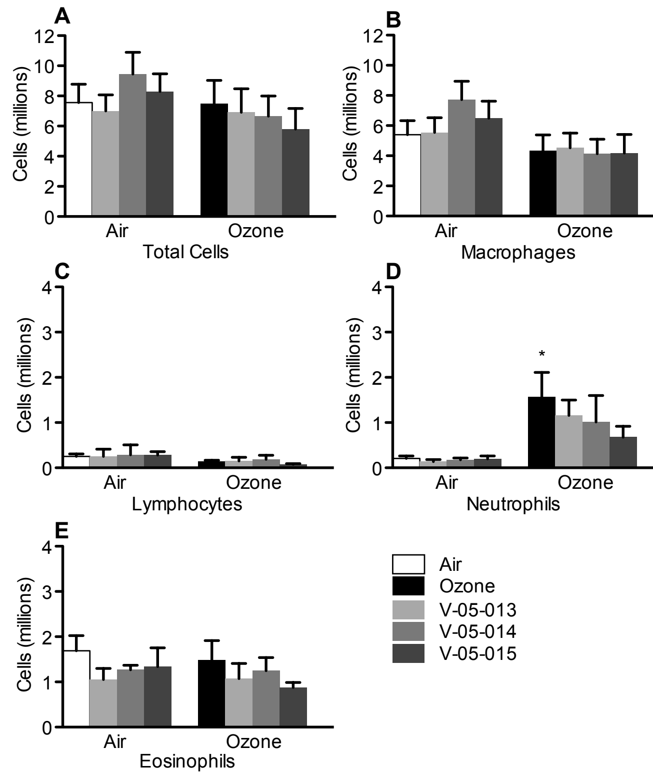


Figure 3.5. Ozone exposure increased neutrophils in bronchoalveolar lavage (D closed bar). No other inflammatory cell type number was affected by either ozone or the dual p38/JNK mitogen activated protein kinase inhibitors. *p<0.05 Significantly different from air exposed controls. Data are mean ± SEM. n=3-6

FIGURE 3.6. Neither ozone nor the dual mitogen activated protein kinase inhibitors affected inflammatory cell numbers in peripheral blood.

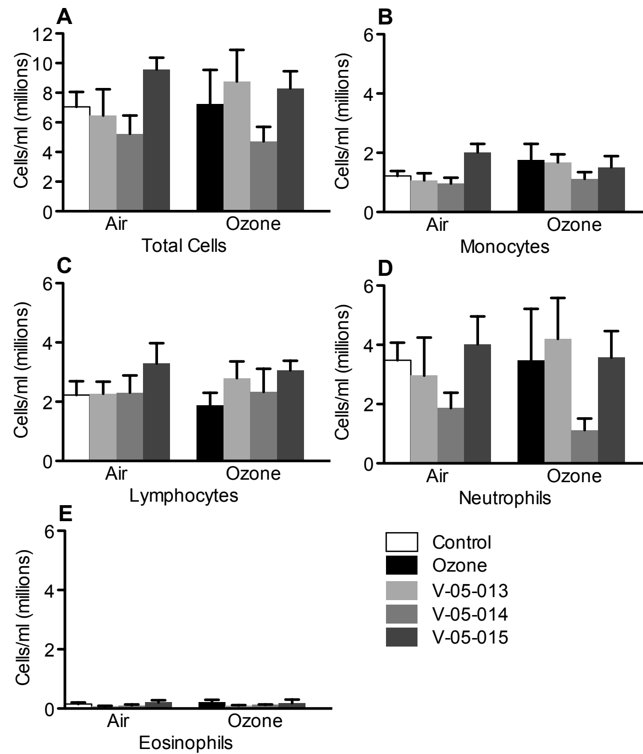


Figure 3.6. Ozone exposure did not change inflammatory cell numbers in peripheral blood. Treatment with the dual p38 and JNK mitogen activated protein kinase inhibitors also had no effect on inflammatory cell numbers in peripheral blood. Data are mean ± SEM. n=3-6

FIGURE 3.7. Blocking both p38 and JNK mitogen activated protein kinase did not prevent ozone-induced decrease in airway mucus containing goblet cells.

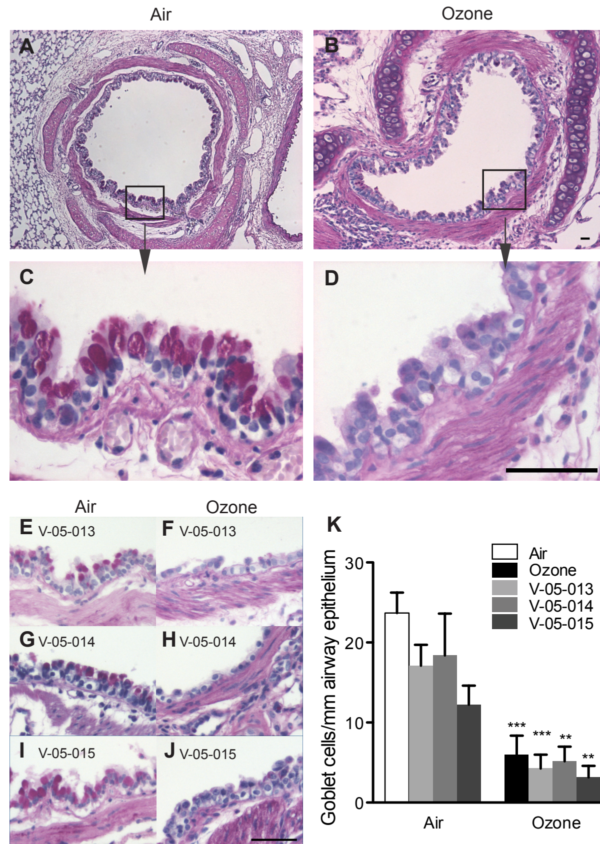


Figure 3.7. Ozone (B, D) significantly decreased mucus containing goblet cells compared to air exposed controls (A, C). Guinea pig lung sections were stained with periodic acid-Schiff and the number of mucus containing goblet cells per length of airway epithelium were counted. Treatment with dual p38/JNK mitogen activated protein kinase inhibitors did not affect goblet cell numbers (E-J). Goblet cells were counted as number of mucus containing goblet cells per mm of airway epithelium (K). **$p<0.01$, ***$p<0.001$ Significantly different from air exposed controls. Data are mean ± SEM. n=3-6

DISCUSSION

Ozone induces airway hyperreactivity, measured as potentiation of vagally induced bronchoconstriction, in guinea pigs one day after exposure confirming previous studies (Schultheis *et al.*, 1994b; Yost *et al.*, 2005). Ozone also significantly potentiated bronchoconstriction in response to intravenous acetylcholine, an effect that has also been previously reported (Schultheis *et al.*, 1994b). Inhibiting p38 prevents ozone-induced airway hyperreactivity in mice while inhibiting JNK is partially protective (Williams *et al.*, 2008; Williams *et al.*, 2007a). Ozone-induced increases in inflammatory cells in bronchoalveolar lavage are significantly inhibited in *Jnk1* knockout mice (Cho *et al.*, 2007). These studies suggest a potential mechanism for how p38 and JNK mitogen activated protein kinases contribute to ozone-induced airway hyperreactivity; inhibition of both p38 and JNK prevents neuronal M_2 muscarinic receptor dysfunction.

Blocking both p38 and JNK mitogen activated protein kinases with three different, but related, inhibitors prevented vagally mediated hyperreactivity in ozone-exposed animals but had no effect on ozone induced mucus secretion or inflammatory cell numbers in bronchoalveolar lavage. The prevention of vagally mediated hyperreactivity was associated with prevention of ozone-induced M_2 receptor dysfunction that was complete in animals treated with V-05-014 and V-05-015, and partial in animals treated with V-05-013. Ozone induced hyperreactivity to intravenous acetylcholine was partially attenuated by treatment with the mitogen activated protein kinase inhibitors.

Treatment of air exposed guinea pigs with any of the three mitogen activated protein kinase inhibitors decreased the airway response to vagal stimulation slightly, but not significantly. This effect was most pronounced at high frequency stimulation, but could not be explained by changes in M_2 receptor function, as the effects of gallamine were not potentiated by the mitogen activated protein kinase inhibitors in air exposed animals. This effect was also not due to decreased smooth muscle responsiveness, as the effects of intravenous acetylcholine were not decreased by the mitogen activated protein kinase inhibitors. Thus, in air exposed guinea pigs, p38 and JNK mitogen activated protein kinase inhibitors likely inhibit vagally induced bronchoconstriction by suppressing release of acetylcholine from airway parasympathetic nerves, a novel observation.

The mechanism for this decreased acetylcholine release is unknown. p38 and JNK are involved in nerve regeneration and development (Agthong *et al.*, 2009; Hirai *et al.*, 2005) but whether they inhibit ganglionic transmission, action potentials or transmitter release (by a mechanism separate from M_2 receptors, since there was no change in the response to gallamine) is not well studied. In *Aplysia*, activation of p38 by the peptide neurotransmitter FMRFa leads to long-term depression in sensory neurons in the pleural ganglia (Guan *et al.*, 2003), although the mechanism is not known. In *Drosophila* motor neurons, expression of constitutively active JNK decreases neurotransmitter release (Etter *et al.*, 2005) while in primary cultures of rat cortical neurons, IL-1β signaling activates p38, decreasing synaptophysin, a protein involved in synaptic transmission (Li *et al.*, 2003). These varied and sometimes contradictory effects of mitogen activated protein kinases on neural function and transmitter release may be involved in the effects I

observed. In neutrophils, activation of p38 mitogen activated protein kinase is required for granule exocytosis after stimulation by CXCR1/2 ligands (Rittner *et al.*, 2007); if neurotransmitter exocytosis were similarly mediated by mitogen activated protein kinases, kinase inhibitors would block secretion. Thus, the role of mitogen activated protein kinases are cell type dependent and additionally may differ between central neurons where kinases inhibit neurotransmission and peripheral neurons, where they have not been well studied. The novel results in this chapter suggest p38 or JNK mitogen activated protein kinase may additionally play a previously unrecognized role in release of acetylcholine from lung parasympathetic nerves.

Ozone exposure induced mucus secretion but this effect was not blocked by the p38 and JNK mitogen activated protein kinase inhibitors. Ozone also significantly increased the numbers of neutrophils in bronchoalveolar lavage compared to air exposed controls confirming previously published data (Verhein *et al.*, 2008; Yost *et al.*, 2005). However, blocking both p38 and JNK mitogen activated protein kinase did not prevent the neutrophil influx. No other inflammatory cell numbers in the lavage were affected by ozone or by the p38 and JNK mitogen activated protein kinase inhibitors. Thus, prevention of ozone-induced airway hyperreactivity did not occur via a decrease in airway inflammatory cells.

Major basic protein, released from eosinophils, inhibits neuronal M_2 muscarinic receptor function (Jacoby *et al.*, 1993), thereby increasing acetylcholine release and subsequently leading to increased bronchoconstriction and airway hyperreactivity (Evans *et al.*, 1997;

Fryer *et al.*, 1992). Depletion of eosinophils with an antibody to IL-5, or by blocking major basic protein, prevents M_2 receptor dysfunction and ozone-induced airway hyperreactivity one day after ozone exposure (Yost *et al.*, 1999). Thus, although neutrophils are the cells that increase in the bronchoalveolar lavage early after ozone, it is tissue eosinophils that mediate ozone-induced hyperreactivity (Yost *et al.*, 2005). In eosinophils, eotaxin and IL-5 signal through both ERK and p38 mitogen activated protein kinase activation (Adachi *et al.*, 2000; Kampen *et al.*, 2000). Inhibition of p38 reduces eosinophil degranulation as measured by decreased eosinophil cationic protein release (Kampen *et al.*, 2000). Major basic protein has also been shown to alter smooth muscle contractility (White *et al.*, 1990), thus blocking eosinophil degranulation could also contribute to preventing the smooth muscle hyperreactivity.

These data demonstrate blocking both p38 and JNK mitogen activated protein kinases prevents airway hyperreactivity twenty-four hours after ozone exposure (Figure 3.8). Inhibition of both p38 and JNK mitogen activated protein kinase prevents enhancement of acetylcholine release both by preventing M_2 receptor dysfunction, and possibly via a novel mechanism directly involving parasympathetic nerves (Figure 3.1). These studies do not show what mediators induced by mitogen activated protein kinases are responsible for ozone-induced airway hyperreactivity and this will be examined in Chapter IV and V with whether IL-1 or NGF regulate ozone-induced airway hyperreactivity.

FIGURE 3.8. p38 and JNK mitogen activated protein kinase mediate ozone-induced airway hyperreactivity one day after exposure.

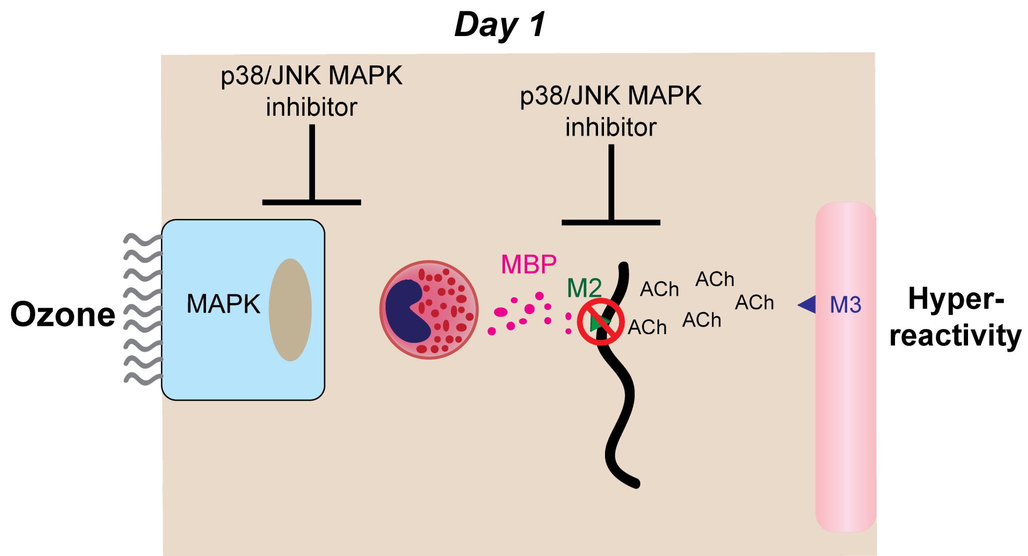


Figure 3.8. One day after ozone exposure airway hyperreactivity is mediated by eosinophil major basic protein (MBP) inhibiting neuronal M_2 muscarinic receptors thereby increasing acetylcholine (ACh) release onto M_3 muscarinic receptors on airway smooth muscle (pink band). It is known that ozone increases mitogen activated protein kinase activation in airway epithelial cells (see chapter I for references), therefore I tested the role of p38 and JNK mitogen activated protein kinase in ozone-induced airway hyperreactivity. Blocking both p38 and JNK mitogen activated protein kinase completely prevented ozone-induced airway hyperreactivity by preventing M_2 receptor dysfunction and preventing acetylcholine release from airway nerves.

CHAPTER IV.

IL-1 MEDIATES PERSISTENT, BUT NOT ACUTE, OZONE-INDUCED AIRWAY HYPERREACTIVITY

ABSTRACT

Ozone exposure in the lab and environment causes airway hyperreactivity in humans and animals lasting at least three days. In guinea pigs one day after ozone, airway hyperreactivity is mediated by eosinophils that block neuronal M_2 muscarinic receptor function, thus increasing acetylcholine release from airway parasympathetic nerves. Inhibition of both p38 and JNK mitogen activated protein kinases also prevents ozone-induced airway hyperreactivity one day after exposure (Chapter III). However, mechanisms of ozone-induced airway hyperreactivity change over time so that depleting eosinophils three days after ozone makes airway hyperreactivity worse rather than better. IL-1β is a potent mediator of airway inflammation. To test whether IL-1β mediates ozone-induced airway hyperreactivity one and three days after ozone, guinea pigs were pretreated with an IL-1 receptor antagonist (anakinra, 30mg/kg ip) thirty minutes before exposure to filtered air or to ozone (2ppm, 4 hours). Ozone exposure increased IL-1β in bone marrow three days after exposure. One or three days after exposure, airway reactivity was measured in anesthetized guinea pigs. The IL-1 receptor antagonist prevented ozone-induced airway hyperreactivity three days, but not one day, after ozone. Ozone-induced airway hyperreactivity was vagally mediated since bronchoconstriction induced by intravenous acetylcholine was not changed by ozone. The IL-1 receptor antagonist selectively prevented ozone-induced reduction of eosinophils around nerves and prevented ozone-induced deposition of extracellular eosinophil major basic protein in airways. These data demonstrate that IL-1 mediates ozone-induced airway hyperreactivity at three days, but not one day, after ozone. Furthermore, preventing

hyperreactivity was accompanied by decreased eosinophil major basic protein deposition within the lung suggesting that IL-1 affects eosinophil activation three days after ozone.

Data in this chapter have been published in:

Verhein KC *et al*., Persistent, but not immediate, airway hyperreactivity is mediated through IL-1 receptors in guinea pigs. *Am J Respir Cell Mol Biol* 39:730-8, 2008.

INTRODUCTION

Interleukin 1β (IL-1β) is a key mediator of inflammation that increases granulocyte hematopoiesis (Hestdal *et al.*, 1994), induces chemokines and adhesion molecules (Birdsall *et al.*, 1992; Dinarello, 1996; Jedrzkiewicz *et al.*, 2000), and activates eosinophils (Gounni *et al.*, 2000; Okada *et al.*, 1995). IL-1β activates the mitogen activated protein kinase signaling pathway, and stimulates release of eotaxin from human airway smooth muscles cells by activating both p38 and JNK mitogen activated protein kinases (Freshney *et al.*, 1994; Oltmanns *et al.*, 2003; Wuyts *et al.*, 2003a). Additionally, activation of the mitogen activated protein kinase pathway by other inflammatory mediators increases synthesis and release of IL-1β (Lee *et al.*, 1994). Inhibiting IL-1β or blocking IL-1 receptors prevents virus-induced, toluene diisocyanate-induced, and antigen challenge-induced hyperreactivity in animals (Hakonarson *et al.*, 1999; Johnson *et al.*, 2005; Selig *et al.*, 1992; Watson *et al.*, 1993). In asthmatic humans, IL-1β is present in bronchoalveolar lavage, epithelial cells, and alveolar macrophages (Arsalane *et al.*, 1995; Borish *et al.*, 1992; Jarjour *et al.*, 1995; Sousa *et al.*, 1996).

Ozone interacts with airway epithelium and alveolar macrophages to produce inflammation via increasing inflammatory cytokines including IL-1β (Arsalane *et al.*, 1995; Cohen *et al.*, 2001; Fakhrzadeh *et al.*, 2004; Pendino *et al.*, 1994; Polosa *et al.*, 2004). The role of IL-1β in ozone induced airway hyperreactivity early after ozone exposure has been suggested since an IL-1 receptor antagonist, anakinra, prevents airway hyperreactivity and inflammation in ozone-exposed mice (Johnston *et al.*, 2007; Park *et al.*, 2004b). Anakinra, a recombinant human form of the IL-1 receptor antagonist, is a

very potent, highly selective inhibitor of IL-1 type 1 receptors with no biological activity of its own (Dripps *et al.*, 1991). These studies were carried out to test in guinea pigs whether IL-1β affects eosinophil activation and mediates airway hyperreactivity one day after ozone where the role of eosinophils has been firmly established (Yost *et al.*, 1999; Yost *et al.*, 2005), and during the lag phase, where the role of IL-1 is unknown, three days after ozone exposure where eosinophils and M_2 receptors do not cause hyperreactivity.

EXPERIMENTAL DESIGN

Ozone exposure

Guinea pigs were exposed to 2 ppm ozone or filtered air for 4 hours as described in the methods section (Chapter II).

Treatments

10-30 mg/kg recombinant human IL-1 receptor antagonist (anakinra) or vehicle (6.5 mM sodium citrate, 140 mM NaCl, 48 mM EDTA, 1 mg/ml polysorbate 80) was diluted in phosphate buffered saline (PBS), and given intraperitoneally 30 minutes before ozone or air exposure, and once daily thereafter.

Measurements

One or three days after exposure to filtered air or ozone, pulmonary inflation pressure was measured as described in the methods. Vagal and smooth muscle reactivity, and bronchoalveolar lavage were measured as described in the methods. Lung tissue sections fixed in zinc buffered formalin were evaluated for eosinophil influx into the airways and major basic protein deposition was measured as described in the methods. Total protein and IL-1β were measured in bronchoalveolar lavage and bone marrow supernatant as described in the methods.

Statistics

All data are expressed as means ± SE. Frequency and dose response curves were compared using two-way ANOVA for repeated measures. Lavage and MBP deposition

data were analyzed by a multiple one-way ANOVA with Fisher's post hoc test. Baseline and histology data were analyzed by one-way ANOVAs with Bonferroni correction. A *P* value of less than 0.05 was considered significant. In all figures, symbols designate comparisons that were statistically significant; comparisons with no statistical significance have no symbols.

RESULTS

IL-1β mRNA levels in guinea pig lungs did not increase either one or three days after ozone exposure (Figure 4.1). One day after ozone exposure total protein in bronchoalveolar lavage was significantly increased compared to air exposed controls (Figure 4.2). Three days after ozone, IL-1β concentration in bone marrow was almost doubled (Figure 4.3), while one day after ozone IL-1β was increased slightly. In contrast, IL-1β was below the limit of detection of the ELISA assay (2 pg/ml) in bronchoalveolar lavage from both ozone and air exposed guinea pigs.

Ozone exposure significantly increased baseline pulmonary inflation pressure one day after ozone (Table 4.1). Pretreatment with the IL-1 receptor antagonist did not affect baseline pulmonary inflation pressure one day after ozone. Neither ozone nor the IL-1 receptor antagonist affected resting heart rate one or three days after ozone. Resting blood pressure was not affected by ozone or by the IL-1 receptor antagonist. Vehicle treatment did not affect baseline parameters compared to controls.

Bronchoconstriction in response to electrical stimulation of the vagus nerves was significantly potentiated above control one and three days after ozone (Figure 4.4A-B). The IL-1 receptor antagonist (30mg/kg, ip) had no effect on ozone-induced airway hyperreactivity at day one (Figure 4.4A). In contrast, the IL-1 receptor antagonist completely blocked ozone-induced hyperreactivity to electrical stimulation of the vagus nerves three days after ozone (Figure 4.4C). The effect was dose related since 10 mg/kg partially prevented ozone-induced airway hyperreactivity at three days (280±73 mmH_2O

at 25Hz). Vehicle administration did not inhibit ozone-induced airway hyperreactivity (Figure 4.4B).

Ozone slightly, but not significantly, increased bronchoconstriction in response to intravenous acetylcholine at one and three days (Figure 4.5A-B). This increase probably does not contribute to ozone-induced airway hyperreactivity since it was small compared to ozone's potentiation of vagally induced bronchoconstriction. The IL-1 receptor antagonist increased smooth muscle responsiveness to acetylcholine and occurred regardless of whether animals were exposed to air or ozone (Figure 4.5C). Vehicle treatment did not affect acetylcholine-mediated bronchoconstriction in either air or ozone exposed animals (Figure 4.5B).

In the heart, ozone exposure increased acetylcholine-induced bradycardia, although not significantly, compared to air exposed controls one day after ozone (Figure 4.6C). Ozone did not affect either vagally induced or intravenous acetylcholine-induced bradycardia after three days (Figure 4.6B,D). Pretreatment with the IL-1 receptor antagonist also did not affect bradycardia in air or ozone exposed animals. Vehicle treatment did not affect vagally or acetylcholine-induced bradycardia in either air or ozone exposed guinea pigs (Figure 4.6B,D).

Only neutrophils were increased in bronchoalveolar lavage one day after ozone exposure (Figure 4.7A). In contrast, all inflammatory cells were increased three days after ozone (Figure 4.7B). The protective effect of the IL-1 receptor antagonist three days after ozone

(Figure 4.4B) was not associated with a change in inflammatory cells in the bronchoalveolar lavage at this time point (Figure 4.7B). Neither was airway hyperreactivity nor the protective effect of the antagonist due to changing inflammatory cell numbers in the blood since circulating numbers of white cells were not changed by ozone, although the IL-1 receptor antagonist decreased circulating lymphocytes three days after ozone (Figure 4.8). Vehicle treatment did not change inflammatory cell numbers in bronchoalveolar lavage or blood in either air or ozone exposed guinea pigs (data not shown).

Since the IL-1 receptor antagonist only blocked airway hyperreactivity three days after ozone, I counted eosinophils around the nerves only at this time point. Ozone alone decreased the number of eosinophils in the lungs and around the nerves (Figure 4.9). While the antagonist did not inhibit ozone-induced depletion of eosinophils in the whole lung, it did prevent loss of eosinophils around airway nerves (Figure 4.9E-F). The selective protection of eosinophils around nerves by the IL-1 receptor antagonist was accompanied by decreased degranulation as assessed by major basic protein deposition (Figure 4.10). Air exposed animals have a basal level of eosinophil degranulation that was not enhanced by ozone exposure (Figure 4.9A,C).

TABLE 4.1. Baseline cardiovascular and pulmonary parameters.

Treatment	Group	*n*	Heart Rate (beats/min)	Blood Pressure (mmHg) Systolic	Blood Pressure (mmHg) Diastolic	Pulmonary Inflation Pressure (mmH_2O)
1 day post O3	Air	5	311±24	40±2	18±2	104±12
	Ozone	6	295±13	43±4	21±1	253±30 *
	Ozone + IL-1Ra	4	301±6	38±3	17±2	240±20 *
3 days post O3	Air	7	298±14	43±5	21±4	104±4
	Air + IL-1Ra	6	283±7	40±4	22±2	98±4
	Ozone	5	321±7 *	49±3	26±3	121±10 *
	Ozone + IL-1Ra	4	283±8 ‡	40±3	24±4	95±5 ‡
	Air + Vehicle	4	300±8	38±4	18±2	105±3
	Ozone + Vehicle	3	297±20	54±7	31±1	103±3

Table 4.1. Values are means ± SE. Baseline pulmonary inflation pressure increased one and three days after ozone. Resting heart rate increased three days after ozone and was prevented by the IL-1 receptor antagonist (IL-1Ra). *Significantly different from air exposed controls. ‡Significantly different from ozone exposed animals.

FIGURE 4.1. Ozone did not increase IL-1β mRNA levels in guinea pig lung.

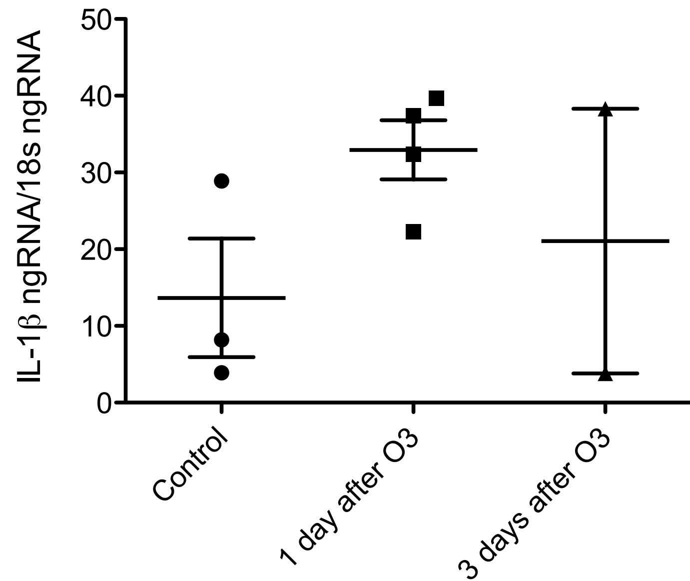


Figure 4.1. RNA from guinea pig lungs was reverse transcribed and cDNA was screened for IL-1β. IL-1β was normalized to 18s ribosomal RNA. There was no difference in IL-1β levels one or three days after ozone exposure (O3) compared to air exposed controls. Data are means ± SE, *n*=2-4.

FIGURE 4.2. Ozone significantly increased total protein levels in bronchoalveolar lavage one day after exposure.

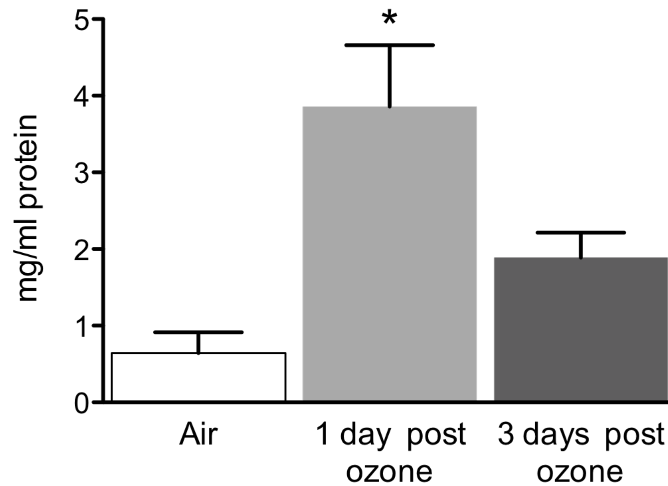


Figure 4.2. Total protein was measured in bronchoalveolar lavage fluid and bone marrow supernatant. Total protein was significantly increased one day after ozone exposure (light gray bar) compared to air exposed animals (open bar) and recovered to control levels three days after ozone (dark grey bar). There was no change in total protein concentration in bone marrow one or three days after ozone exposure (data not shown). *Significantly different from air exposed controls. Data are means ± SE, *n*=3.

FIGURE 4.3. Ozone increased IL-1β in bone marrow three days after exposure.

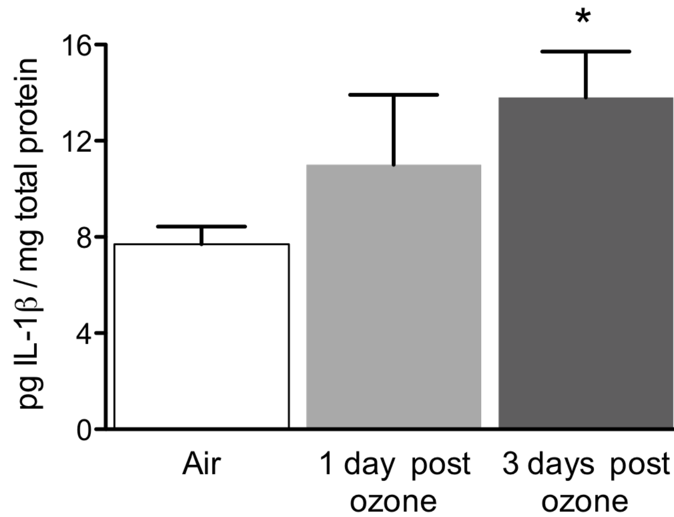


Figure 4.3. IL-1β protein levels were measured in bronchoalveolar lavage fluid and bone marrow supernatants one and three days after ozone exposure. IL-1β was below the limit of detection in bronchoalveolar lavage (data not shown). IL-1β was present in bone marrow of controls (open bar) and was slightly increased one day after ozone (light gray bar). At three days after ozone (dark gray bar), IL-1β levels in bone marrow were increased compared to air exposed controls. IL-1β concentrations were normalized to total protein. *p=0.057 from control. Data are means ± SE, *n*=5.

FIGURE 4.4. Blocking IL-1 receptors did not prevent airway hyperreactivity one day after ozone exposure, but does prevent airway hyperreactivity three days after ozone exposure.

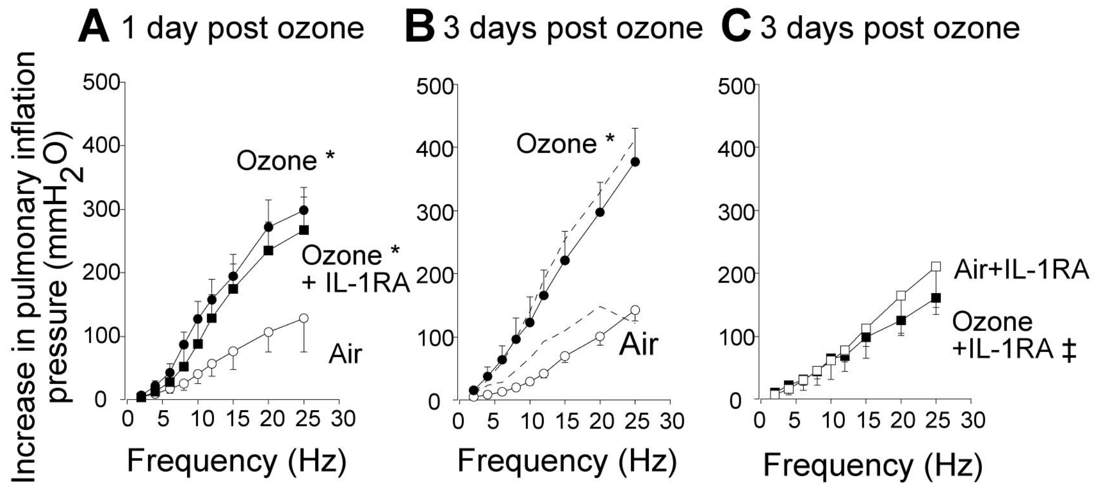


Figure 4.4. In anesthetized guinea pigs, electrical stimulation of both vagus nerves causes frequency-dependent bronchoconstriction (measured as an increase in pulmonary inflation pressure) in air-exposed guinea pigs (open circles) that was significantly potentiated one (A) and three days (B) after ozone exposure (solid circles). Pretreatment with the IL-1 receptor antagonist (IL-1RA) prevented airway hyperreactivity three days after ozone (C solid squares) but not one day after ozone (A solid squares). The IL-1 receptor antagonist did not affect air-exposed controls (C open squares). Vehicle treatment did not change vagally mediated bronchoconstriction in either ozone or air exposed animals (B dashed lines next to respective controls). *Ozone is significantly different from air exposed guinea pigs. ‡The entire frequency response in the presence of IL-1 receptor antagonist is significantly different from ozone exposed guinea pigs. Data are expressed as means ± SE, n = 3-7.

FIGURE 4.5. Blocking IL-1 receptors increased airway smooth muscle contraction to intravenous acetylcholine.

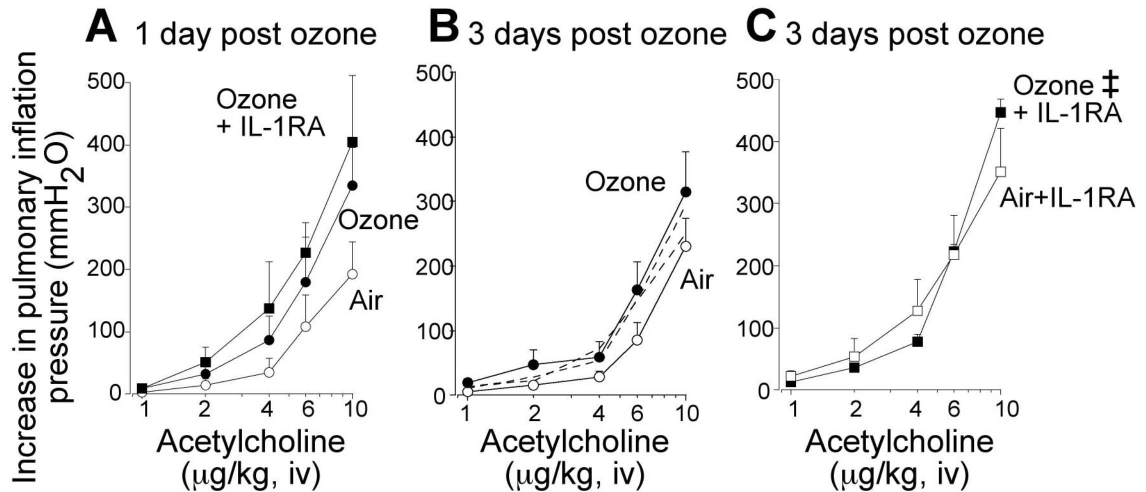


Figure 4.5. Acetylcholine induced bronchoconstriction was measured in vagotomized guinea pigs as an increase in pulmonary inflation pressure. One (A) and three days (B) after ozone exposure smooth muscle contraction slightly increased (solid circles) compared to air exposed animals (open circles). The IL-1 receptor antagonist (IL-1RA) slightly increased smooth reactivity in all groups (C). Vehicle treatment did not change acetylcholine-induced bronchoconstriction in either air or ozone exposed animals (B. dashed lines next to respective controls). ‡The entire frequency response in the presence of IL-1 receptor antagonist (C solid squares) is significantly different from ozone exposed guinea pigs (B solid circles). Data are expressed as means ± SE, *n* = 4-7.

FIGURE 4.6. Neither ozone nor the IL-1 receptor antagonist affected vagally or acetylcholine induced bradycardia.

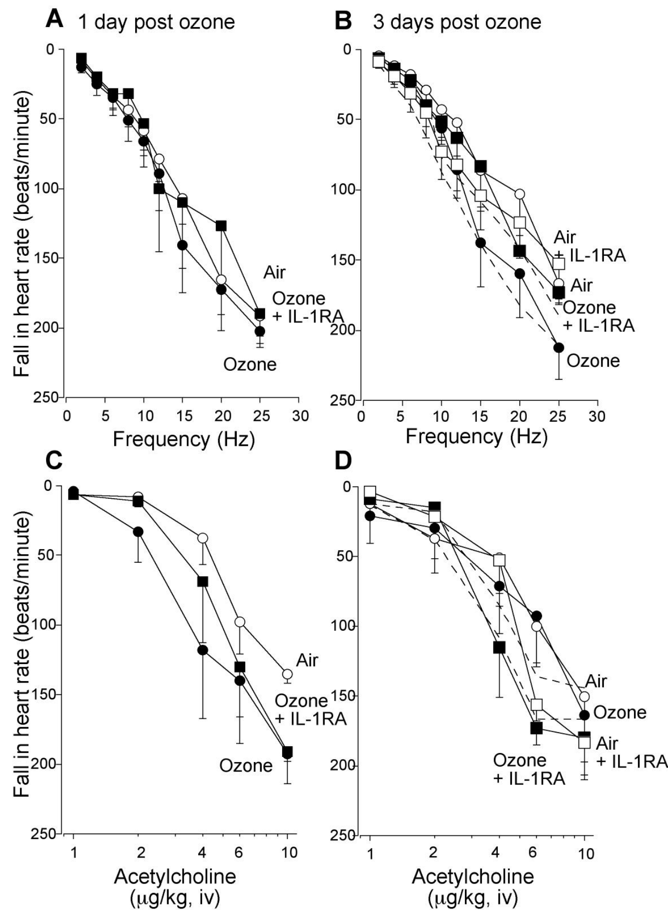


Figure 4.6. Bradycardia in response to electrical stimulation of both vagus nerves (A and B) or to intravenous acetylcholine in vagotomized guinea pigs (C and D) was measured as a fall in heart rate in beats per minute. Neither ozone (A-D solid circles) nor the IL-1 receptor antagonist (IL-1RA) (A-D solid squares) affected vagally or acetylcholine induced bradycardia. Vehicle treatment did not change bradycardia in response to vagal stimulation or acetylcholine in either air or ozone exposed animals (B, D dashed lines next to respective controls). Data are expressed as means ± SE, n = 4-6.

FIGURE 4.7. Ozone increased inflammatory cells in bronchoalveolar lavage three days after exposure.

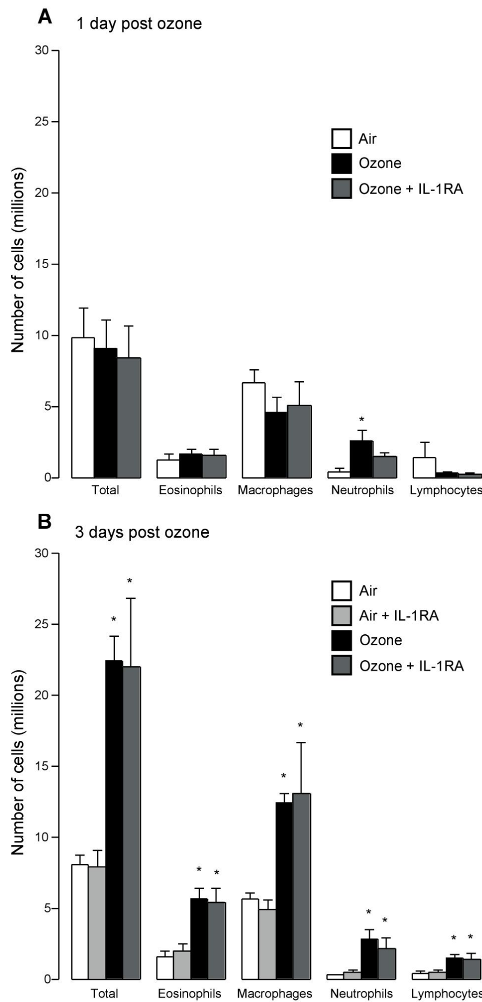


Figure 4.7. Inflammatory cells in bronchoalveolar lavage were not increased one day after ozone, with the exception of neutrophils (A). However, three days after ozone all inflammatory cells were significantly increased in bronchoalveolar lavage (B). Treatment with IL-1 receptor antagonist (IL-1RA) did not change inflammatory cells in bronchoalveolar lavage in controls or at either time point after ozone. *Significantly different from air exposed controls. Data are expressed as means ± SE, n = 4-7.

FIGURE 4.8. Circulating peripheral blood inflammatory cells were not changed by ozone.

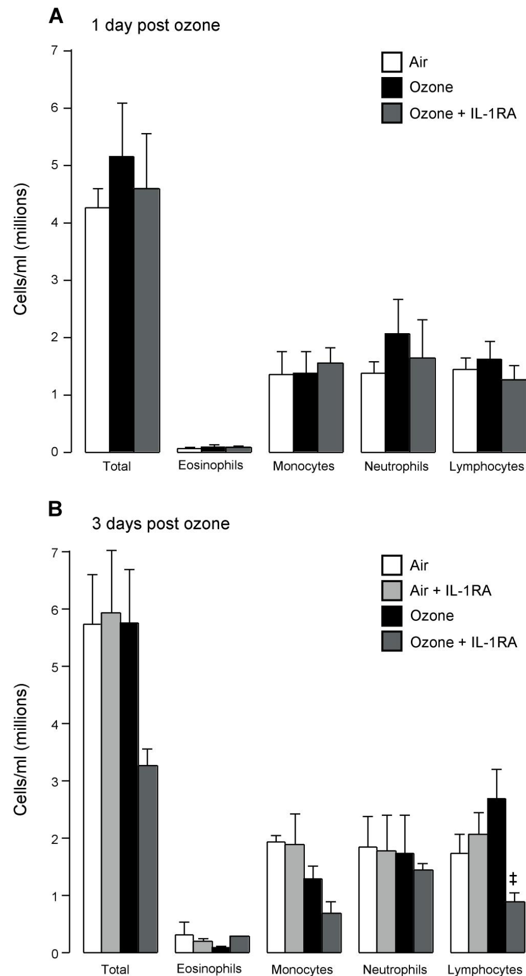


Figure 4.8. Circulating inflammatory cells in peripheral blood were not changed by ozone or by the IL-1 receptor antagonist (IL-1RA) at one (A) or three (B) days after ozone with the exception of lymphocytes, which were significantly inhibited by the IL-1 receptor antagonist three days after ozone (B). Data are expressed as means ± SE, n = 3-6.

FIGURE 4.9. Blocking IL-1 prevented ozone-induced loss of eosinophils around airway nerves three days after exposure.

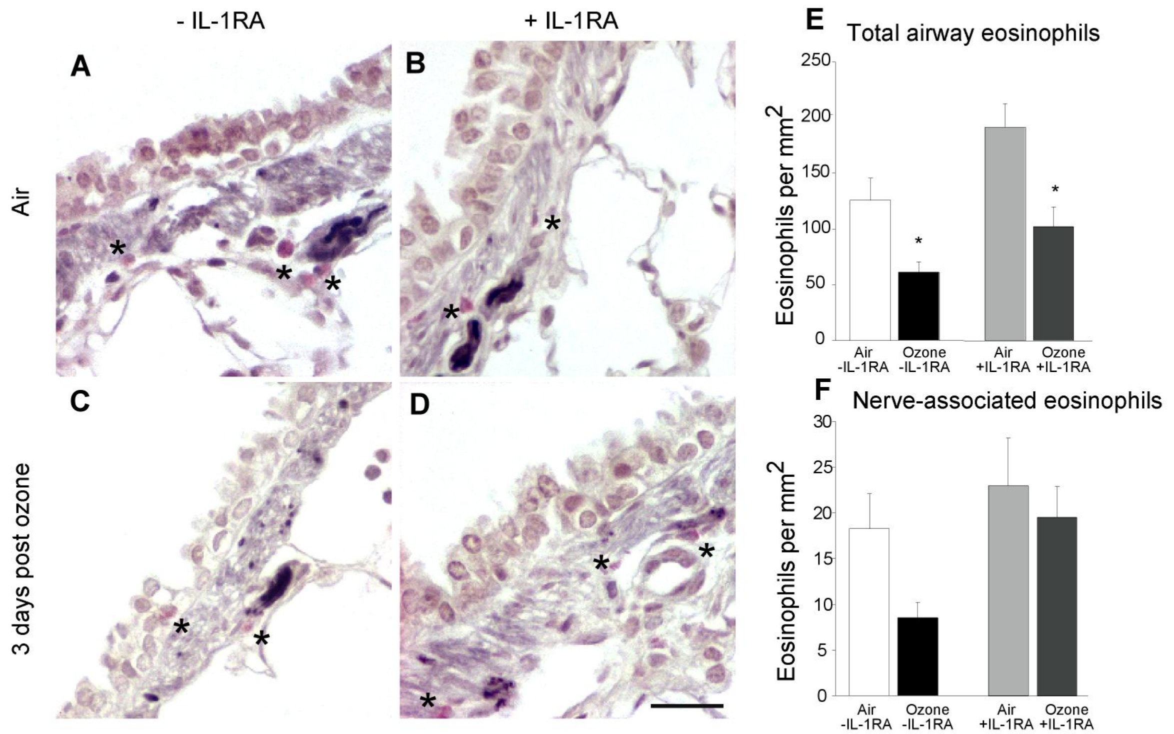


Figure 4.9. Eosinophils are present in airways and around nerves in control and ozone exposed animals. Nerves in guinea pig lungs were stained black with antibody to PGP9.5 and eosinophils were stained red with chromotrope 2R (asterisks) (A-D) and quantified in 6-8 different airways per guinea pig (E-F). Even in air exposed control animals there are some eosinophils around airway nerves (A; open bar E and F), which are decreased by ozone (C; solid bar in E and F). The ozone-induced decrease in total lung eosinophils was not affected by the IL-1 receptor antagonist (IL-1RA) (E), but the IL-1 receptor antagonist did prevent ozone induced eosinophil loss around nerves (F). Data in E and F are expressed as means ± SE, *n* = 4-6. *Significantly different from air exposed animals treated with the IL-1 receptor antagonist.

FIGURE 4.10. Blocking IL-1 receptors prevented extracellular deposition of eosinophil major basic protein within airways three days after ozone.

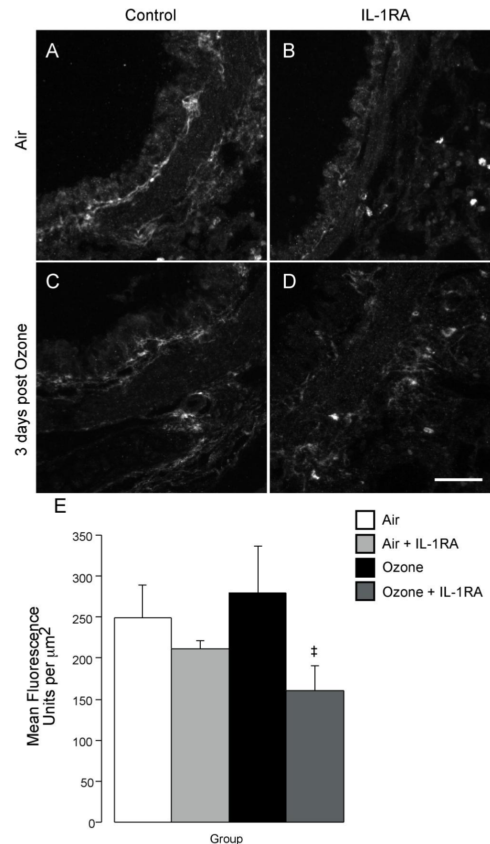


Figure 4.10. Following staining with an antibody to major basic protein (MBP), slides were coded and analysis performed blinded and intact eosinophils as identified by solid spheres of MBP staining in excess of 8 μm in diameter were excluded. Airways of air exposed control guinea pigs do contain some extracellular MBP (A and E open bar), which was not significantly changed three days after ozone exposure (C and E solid bar). IL-1 receptor antagonist (IL-1RA) significantly decreased deposition of extracellular MBP (D and E dark gray bar). Data (E) are expressed as mean fluorescence intensity per area of the airway in μm^2. Data are means ± SE, *n* = 4-6. ‡Significantly different from ozone exposed animals. Scale bar, 50 μm. (A-D).

DISCUSSION

Ozone transiently increases IL-1β in airway epithelium (Dokic *et al.*, 2006) and lungs (Johnston *et al.*, 2007; Park *et al.*, 2004b), and this declines over 24 hours. My data at 24 hours are in agreement with this since I did not see measurable levels of IL-1β in bronchoalveolar lavage at that time point. Eosinophils are known to increase in lungs after ozone (Yost *et al.*, 2005), therefore I measured IL-1β in guinea pig bone marrow since it is involved in granulocyte hematopoiesis (Hestdal *et al.*, 1994). IL-1β increased in bone marrow three days after ozone exposure and may be the systemic link between the lungs and increased eosinophil production. IL-1β may be stimulating an increase in a "beneficial" phenotype of eosinophil.

In guinea pigs ozone induces airway hyperreactivity that lasts at least three days confirming previous findings (Yost *et al.*, 2005). This is similar to what is seen in human populations after environmental exposure to ozone (Ko *et al.*, 2007; Lewis *et al.*, 2005). In contrast to my original hypothesis, IL-1β had no effect on ozone-induced airway hyperreactivity one day after ozone. However, it completely prevented hyperreactivity three days after ozone, demonstrating that the mechanisms of hyperreactivity have changed from IL-1 independent to IL-1 mediated over these three days. Thus, an IL-1 receptor antagonist given at the time of ozone exposure has no effect on airway hyperreactivity one day after ozone, but completely prevents hyperreactivity three days after ozone. Similarly, ozone-induced inflammation was not prevented one day after ozone in IL-1 receptor type I knockout mice, but was prevented three days after ozone (Johnston *et al.*, 2007). These data show that hyperreactivity and inflammation after

ozone change between one and three days post exposure. Protection against ozone-induced airway hyperreactivity occurs at the level of the nerves and not airway smooth muscle since the IL-1 receptor antagonist does not reduce acetylcholine-induced bronchoconstriction in air or ozone exposed animals.

Ozone-induced airway hyperreactivity is linked to inflammation (Gambone *et al.*, 1994; Yost *et al.*, 2005). Therefore, if mechanisms of hyperreactivity change, it might be expected that inflammatory cell populations also change over time. Although ozone exposure is typically associated with increased neutrophils, neutrophil populations in the lung do not change between one and three days after ozone (Yost *et al.*, 2005) (Figure 4.7). Ozone also induces eosinophilia in animals (Fabbri *et al.*, 1984; Hyde *et al.*, 1992; Ishii *et al.*, 1998; Park *et al.*, 2004a) and humans (Hiltermann *et al.*, 1997; Peden *et al.*, 1997; Peden *et al.*, 1995), and eosinophil populations in the lung do change between one and three days post ozone (Yost *et al.*, 2005) (Figure 4.7). Depleting eosinophils prior to ozone exposure completely prevents airway hyperreactivity one day later demonstrating a role for eosinophils in ozone-induced hyperreactivity in guinea pigs (Yost *et al.*, 2005). Preventing eosinophil migration into the lungs is not protective at this time point, suggesting that ozone is activating eosinophils that are resident in the lungs (Yost *et al.*, 2005). Conversely, depleting eosinophils or preventing eosinophils from entering the lungs three days after ozone makes hyperreactivity significantly worse (Yost *et al.*, 2005) suggesting a beneficial role for late eosinophils that enter the lung three days after ozone.

In asthmatic humans, antigen challenged, virus infected, and ozone or organophosphate exposed animals, airway hyperreactivity is mediated in part by loss of neuronal M_2 receptor function on the vagus nerves, leading to increased acetylcholine release and increased bronchoconstriction (Evans *et al.*, 1997; Fryer *et al.*, 1991a; Lein *et al.*, 2005; Minette *et al.*, 1989; Yost *et al.*, 1999). This mechanism is established in mouse, rat, guinea pig, and horse models of airway hyperreactivity and in some humans with asthma (Belmonte *et al.*, 1998; Elbon *et al.*, 1995; Evans *et al.*, 1997; Larsen *et al.*, 2000; Minette *et al.*, 1989; Yost *et al.*, 1999; Zhang *et al.*, 1999). In antigen challenged guinea pigs, loss of neuronal M_2 muscarinic receptor function is mediated by degranulation of eosinophils at the nerves in the lungs, resulting in release of the M_2 antagonist major basic protein (Elbon *et al.*, 1995; Fryer *et al.*, 2006). A similar mechanism of hyperreactivity has been established one day after ozone (Yost *et al.*, 1999). However, mechanisms of hyperreactivity three days after ozone are not understood. Although IL-1β decreases M_2 receptor expression (Haddad *et al.*, 1996), this is unlikely to explain the ability of the IL-1 receptor antagonist to prevent airway hyperreactivity three days after ozone since my laboratory has previously shown that restoring M_2 receptor function at this time point does not prevent airway hyperreactivity (Yost *et al.*, 2005).

Airway hyperreactivity does not correlate with eosinophils in bronchoalveolar lavage or in airway tissues (Yost *et al.*, 2005), but does correlate with the presence of activated eosinophils, as measured by major basic protein deposition in airways and especially around airway nerves (Costello *et al.*, 1997; Verbout *et al.*, 2007). Similarly, it is the presence of eosinophils in airway tissues, but not bronchoalveolar lavage or blood, that

accompanies airway hyperreactivity in human asthma (Flood-Page *et al.*, 2003) and that may be important after ozone exposure. However, there are more eosinophils in bronchoalveolar lavage three days after ozone than one day after ozone, suggesting that eosinophils are moving through the lungs differently at these time points. A new, beneficial population of eosinophils has been suggested since eosinophil depletion makes airway hyperreactivity significantly worse (Yost *et al.*, 2005) and these beneficial eosinophils may arrive at the later time point. Here I show that eosinophils within the lungs are significantly decreased three days after ozone while they are increased in bronchoalveolar lavage. Thus, ozone is either moving eosinophils from the lungs to the bronchoalveolar lavage, or they are disappearing via activation and degranulation (Verbout *et al.*, 2007).

IL-1β is known to activate eosinophils directly to stimulate cytokine release (Gounni *et al.*, 2000). IL-1 may also affect eosinophil reactivity since IL-1 receptor antagonists decrease hypodense eosinophils in bronchoalveolar lavage of antigen challenged animals (Okada *et al.*, 1995) suggesting that IL-1 contributes to eosinophil activation. The same mechanism is suggested here since the IL-1 receptor antagonist prevented eosinophil degranulation in ozone-exposed animals, measured as a decrease in extracellular major basic protein. Additionally, the IL-1 receptor antagonist also prevented ozone-induced loss of eosinophils around airway nerves. Since hyperreactivity depends upon an interaction between eosinophils and nerves (Fryer *et al.*, 2006) it may be significant that only eosinophils around nerves were protected by the antagonist. Thus, the IL-1 receptor

antagonist protects eosinophil populations around nerves and prevents eosinophil activation in ozone-exposed animals.

Depletion of eosinophils does not prevent hyperreactivity three days after ozone but actually makes airway hyperreactivity significantly worse, suggesting that eosinophils three days after ozone are beneficial (Yost *et al.*, 2005). The IL-1 receptor antagonist may inhibit hyperreactivity three days after ozone not by preventing eosinophil degranulation, but by protecting beneficial eosinophils (Yost *et al.*, 2005). Eosinophils are known to release neurotrophins, including nerve growth factor (Noga *et al.*, 2003), leukemia inhibitory factor (Zheng *et al.*, 1999), and bone derived neurotrophic factor (Noga *et al.*, 2003), which may all be involved in neural repair mechanisms. It has been suggested that there are multiple populations of eosinophils (Giembycz *et al.*, 1999), thus while the IL-1 receptor antagonist may have no effect on resident eosinophils that mediate hyperreactivity one day after ozone, it may preserve the integrity of secondary, beneficial eosinophils, preventing airway hyperreactivity three days after ozone.

Substance P containing airway nerves are increased in asthmatics compared to nonasthmatics (Ollerenshaw *et al.*, 1991) and ozone-induced airway hyperreactivity is associated with increased substance P in bronchoalveolar lavage in humans and in ferret lung (Hazbun *et al.*, 1993; Wu *et al.*, 2003). Substance P is a peptide neurotransmitter usually expressed by sensory nerves. Although not usually present in efferent parasympathetic nerves of humans or guinea pigs (Canning *et al.*, 2002; Lundberg *et al.*, 1984), ozone induces substance P expression in guinea pig parasympathetic nerves

(Hazari *et al.*, 2003) and increases substance P in ferret parasympathetic nerves (Wu *et al.*, 2003). Ozone-induced airway hyperreactivity is blocked by a neurokinin receptor (NK_1) antagonist in guinea pigs three days after ozone (Hazari *et al.*, 2003) and in ferret tracheas *in vitro* (Wu *et al.*, 2003) demonstrating a role for tachykinins in ozone induced hyperreactivity. IL-1β can stimulate substance P expression directly (Hurst *et al.*, 1993; Wu *et al.*, 2002), and also indirectly via induction of nerve growth factor (Frossard *et al.*, 2005), which also increases substance P (de Vries *et al.*, 2006). One potential source of nerve growth factor is eosinophils (Noga *et al.*, 2003). Therefore, the IL-1 receptor antagonist may prevent hyperreactivity by decreasing eosinophil activation, resulting in decreased nerve growth factor mediated induction of substance P. The role of NGF in ozone-induced airway hyperreactivity will be addressed in Chapter V.

The data presented here support the hypothesis that the mechanisms of ozone-induced airway hyperreactivity have switched from eosinophil mediated to dependent on IL-1 (Figure 4.11). It is unclear from these data what other mediators may be involved in ozone-induced airway hyperreactivity and whether NGF or eosinophils directly increase substance P expression in airway nerves will be examined in Chapter V.

FIGURE 4.11. Blocking IL-1 prevents ozone-induced airway hyperreactivity three days, but not one day, after ozone exposure.

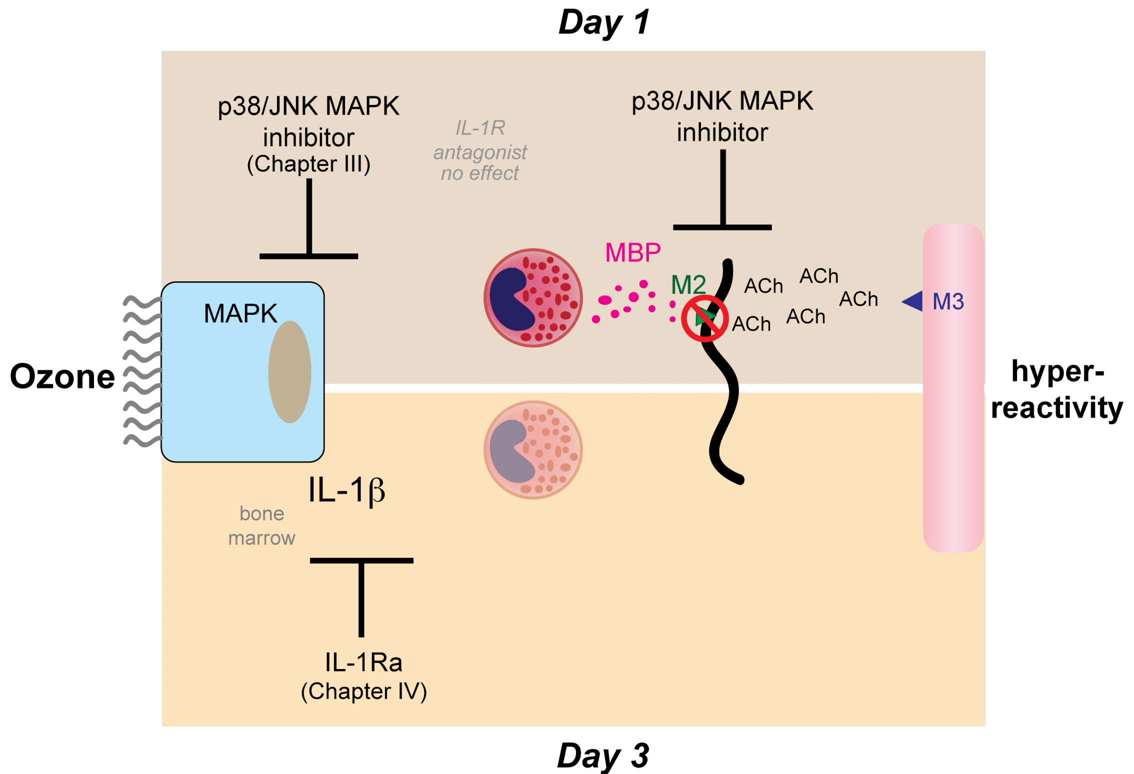


Figure 4.11. The mechanisms of ozone-induced airway hyperreactivity change over time from MAPK (Chapter III) and eosinophil mediated, to IL-1 mediated. Blocking IL-1 completely prevents ozone-induced airway hyperreactivity three days, but not one day, after ozone exposure.

CHAPTER V.

OZONE-INDUCED AIRWAY HYPERREACTIVITY IS DEPENDENT ON SUBSTANCE P AND NGF THREE DAYS AFTER EXPOSURE

ABSTRACT

Ozone causes persistent airway hyperreactivity in humans and animals. One day after ozone exposure airway hyperreactivity is mediated by eosinophil major basic protein and inhibition of neuronal M_2 muscarinic receptors, leading to increased acetylcholine release and smooth muscle contraction in guinea pigs. Three days after ozone IL-1β and tachykinins, but not eosinophils, mediate ozone-induced airway hyperreactivity, but the mechanism at this later time-point is largely unknown. IL-1β increases nerve growth factor (NGF) and substance P, both of which play a role in neural plasticity. Since eosinophils make NGF, I tested whether NGF or substance P play a role in ozone-induced airway hyperreactivity one to three days after exposure and whether NGF or eosinophils induce substance P in parasympathetic nerves. Guinea pigs were pretreated with an antibody to NGF or IgG, exposed to filtered air or ozone (2ppm, 4 hours) and vagally mediated and acetylcholine mediated bronchoconstriction was measured one or three days later in anesthetized and vagotomized animals. To test changes in neural plasticity, parasympathetic nerves were cultured from guinea pig tracheas and treated with isolated guinea pig peritoneal lavage eosinophils, or NGF for 2 days. Ozone caused vagally mediated airway hyperreactivity lasting over three days and airway smooth muscle was hyperreactive three days after ozone. The antibody to NGF completely prevented airway hyperreactivity three days, but not one day after ozone, and significantly reduced the number of substance P positive airway nerve bundles. NGF did not increase substance P expression in isolated parasympathetic nerves. Co-culture with eosinophils for 2 days significantly increased substance P expression measured by immunostaining. Thus, mechanisms of ozone-induced airway hyperreactivity change

over time so that three days after ozone hyperreactivity is mediated by an increased role for substance P orchestrated by NGF.

Data in this chapter have been submitted for publication in:

Verhein KC *et al*., Three days after a single exposure to ozone the mechanism of airway hyperreactivity is dependent upon substance P and nerve growth factor. [Submitted: *Am J Physiol Lung Cell Mol Physiol*, February 2010].

**Some experiments in this chapter (Figure 5.2-5.4) were in collaboration with Mehdi Hazari and we share first authorship on this paper.

INTRODUCTION

The mechanisms of hyperreactivity three days after ozone exposure are unknown, but have changed to involve increased smooth muscle contraction in addition to increased vagally mediated bronchoconstriction (Schultheis *et al.*, 1994b; Yost *et al.*, 2005). The systemic nature of the secondary response suggests a role for inflammatory mediators including IL-1β, which is increased in the bone marrow three days post ozone (Verhein *et al.*, 2008). Blockade of IL-1 inhibits hyperreactivity three days post ozone, but has no effect on hyperreactivity one day post ozone demonstrating that it is important specifically to the secondary hyperreactivity (Chapter IV). In addition, inhibition of substance P receptors prevents ozone-induced airway hyperreactivity after three days (Hazari, 2005). Since IL-1β is capable of stimulating nerve growth factor (NGF) (Frossard *et al.*, 2005) and substance P (Frossard *et al.*, 2005; Hurst *et al.*, 1993; Wu *et al.*, 2002), both of which can mediate neural plasticity and hyperreactivity (Hazari *et al.*, 2007), I tested whether NGF contributes to ozone induced hyperreactivity one or three days after ozone exposure, and whether NGF or eosinophils contribute to neural plasticity by increasing substance P in airway nerves.

EXPERIMENTAL DESIGN

Ozone exposure

Guinea pigs were exposed to 2ppm ozone or filtered air for 4 hours as described in the methods section (Chapter II).

Treatments

Guinea pigs were treated with either antibody to nerve growth factor (AbNGF; 10μg/kg ip) or goat IgG (10μg/kg ip), one hour prior to ozone exposure.

Measurements

One and three days after exposure to ozone or filtered air, pulmonary inflation pressure in response to both vagal stimulation and intravenous acetylcholine was measured as described in the methods (Chapter II). Inflammatory cells in bronchoalveolar lavage were counted, and substance P was detected with immunostaining in airway tissue sections fixed in formaldehyde as described in the methods.

Parasympathetic nerve culture

Guinea pig parasympathetic nerves were isolated as described in the methods (Chapter II) and treated with NGF (0.1-0.8μg/ml), antibody to NGF (2μg/ml), or eosinophils (250,000 eosinophils per well) for 2-4 days. Some cultures were stained with an antibody to substance P and immunofluorescense was measured as described in the methods (Chapter II), and from other cultures substance P was measured with RT-PCR using RNA isolated from neuronal cell bodies as described in the methods.

Statistics

All data are expressed as means ± SE. In vivo frequency response and dose response curves were compared using two-way ANOVA for repeated measures. Histology of guinea pig lung sections and baseline physiology data were analyzed using one-way ANOVA and Bonferroni's correction. Substance P immunofluorescence in cultured parasympathetic nerves was analyzed using one-way ANOVA and Bonferroni's correction by using the antibody to NGF treated wells as a control for NGF treatment and dead eosinophils as the control for eosinophil treatment. A *p* value of less than 0.05 was considered significant. Analyses were made with GraphPad Prism (version 5.0).

RESULTS

Ozone significantly increased baseline pulmonary inflation pressure one and three days after exposure compared to air exposed controls (Table 5.1). Treatment with an antibody to NGF did not prevent the ozone-induced increase in pulmonary inflation pressure one day after ozone. However, antibody to NGF, but not IgG, significantly reduced the baseline rise in pulmonary inflation pressure three days after ozone. Exposure to ozone did not have any consistent effect on resting heart rate. Similarly, resting blood pressure in the control groups ranged from 46±2 mmHg systolic/22±2 mmHg diastolic to 51±2 mmHg systolic/26±3 mmHg diastolic and exposure to ozone did not effect resting blood pressure either. Furthermore, none of the treatments altered resting heart rate or blood pressure in ozone or control animals.

Ozone did not increase either NGF or preprotachykinin mRNA in guinea pig lungs either one or three days after exposure (Figure 5.1).

Simultaneous electrical stimulation of both vagus nerves (1-25 Hz) caused frequency-dependent bronchoconstriction in all animals that was significantly potentiated (by 300%) one day and (by 100%) three days following a single exposure to ozone (Figure 5.2) and was completely blocked by 1mg/kg atropine (data not shown). Pretreatment with antibody to NGF had no effect on vagally mediated bronchoconstriction in air exposed controls. While pretreatment with antibody to NGF did not prevent ozone-induced airway hyperreactivity one day after exposure (Figure 5.2A), it completely prevented ozone-induced airway hyperreactivity three days after exposure (Figure 5.2B). The control IgG

had no effect on vagally induced bronchoconstriction in ozone-exposed animals (Figure 5.2B).

Intravenous acetylcholine in vagotomized animals bypasses the nervous system and directly induces bronchoconstriction via an effect at airway smooth muscle. Acetylcholine induced bronchoconstriction was not changed one day after ozone but was slightly though significantly potentiated (by 33%) three days after ozone compared to air exposed controls (Figure 5.3). This potentiation at three days was also inhibited by antibody to NGF (Figure 5.3B). The control IgG had no effect on acetylcholine-induced bronchoconstriction in ozone-exposed animals (Figure 5.3B).

One day after ozone, the only cell type that significantly increases in bronchoalveolar lavage is neutrophils and the antibody to NGF had no effect on this increase (Figure 5.4A). Three days post ozone only macrophages and eosinophils were significantly increased (Figure 5.4B). The increase in macrophages was not blocked by the antibody to NGF. Eosinophils were significantly inhibited by the antibody to NGF three days after ozone (Figure 5.4B).

There was no difference in the number of substance P positive nerve bundles in the lungs of ozone and air exposed controls three days after exposure (Figure 5.5A, C, E). However, the antibody to NGF significantly reduced the number of substance P positive nerve bundles regardless of whether they were air or ozone exposed (Figure 5.5B, D, E).

NGF did not increase expression of substance P by cultured guinea pig parasympathetic nerves measured by immunofluorescence (Figure 5.6). Co-culture with eosinophils for two days slightly, but not significantly, increased substance P staining in parasympathetic nerves (Figure 5.7). The slight increase in substance P staining required live eosinophils and contact with nerves since neither dead eosinophils nor eosinophil supernatant increased substance P staining (Figure 5.7). Preprotachykinin mRNA, the precursor for substance P, was undetected in parasympathetic nerve cultures (Figure 5.8) but was present in guinea pig peritoneal eosinophils (Figure 5.9).

TABLE 5.1. Baseline pulmonary parameters.

Treatment	Group	*n*	Pulmonary Inflation Pressure (mmH_2O)
1 day	Air	5	106±2
	Ozone	5	242±17 *
	Ozone + AbNGF	5	208±8 *
3 days	Air	5	92±6
	Ozone	5	155±14 *
	Ozone + AbNGF	6	118±19
	Ozone + IgG	5	160±12 *

Table 5.1. Values are means ± SE. Baseline pulmonary inflation pressure increased one and three days after ozone. The antibody to NGF (AbNGF) prevented the ozone-induced increase in baseline pulmonary inflation pressure three days after exposure.
*Significantly different from air exposed controls.

FIGURE 5.1. Ozone exposure did not appear to increase NGF or preprotachykinin expression in guinea pig lungs.

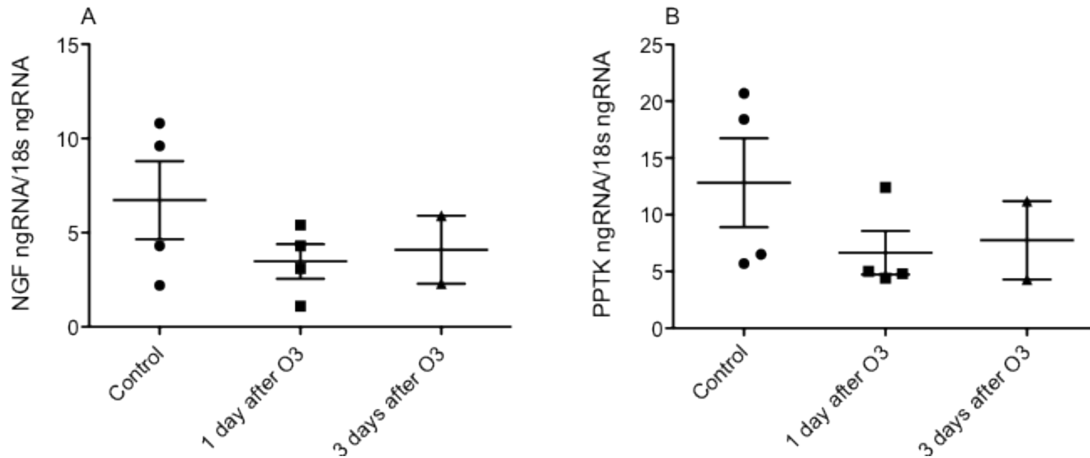


Figure 5.1. RNA isolated from guinea pig lungs was reverse transcribed and cDNA was screened by RT-PCR using primers for guinea NGF (A) and preprotachykinin (B). Neither NGF nor preprotachykinin, normalized to 18s ribosomal RNA, increased one or three days after ozone exposure compared to air exposed controls. Data are expressed as means ± SE, *n* = 2-4.

FIGURE 5.2. Blocking NGF prevented ozone-induced airway hyperreactivity three days but not one day after ozone.

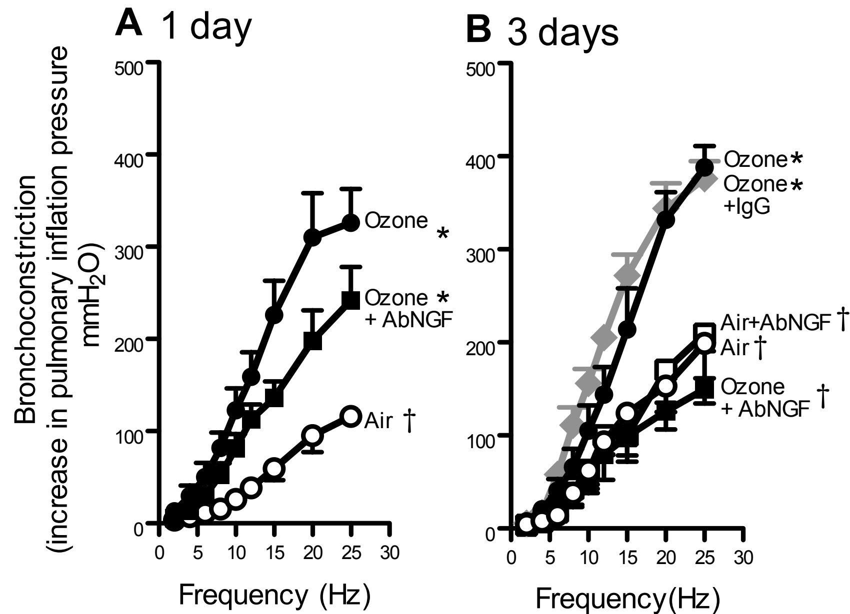


Figure 5.2. Electrical stimulation of both vagus nerves in anesthetized guinea pigs caused frequency dependent bronchoconstriction, measured as an increase in pulmonary inflation pressure, in air-exposed controls (open circles in A and B) that was significantly potentiated both one and three days after ozone exposure (filled circles in A and B). Pretreatment with an antibody to NGF (AbNGF) did not prevent airway hyperreactivity one day after ozone (A filled squares) but was completely protective three days after ozone (B filled squares). Blocking NGF had no effect on vagally mediated bronchoconstriction in air-exposed controls (B open squares). An isotype control (IgG) had no effect in ozone-exposed animals (B gray diamonds). *Significantly different from air exposed guinea pigs. †Significantly different from ozone-exposed guinea pigs. Data are expressed as means ± SE, $n = 5$.

FIGURE 5.3. Blocking NGF prevented ozone-induced smooth muscle hyperresponsiveness three days after ozone.

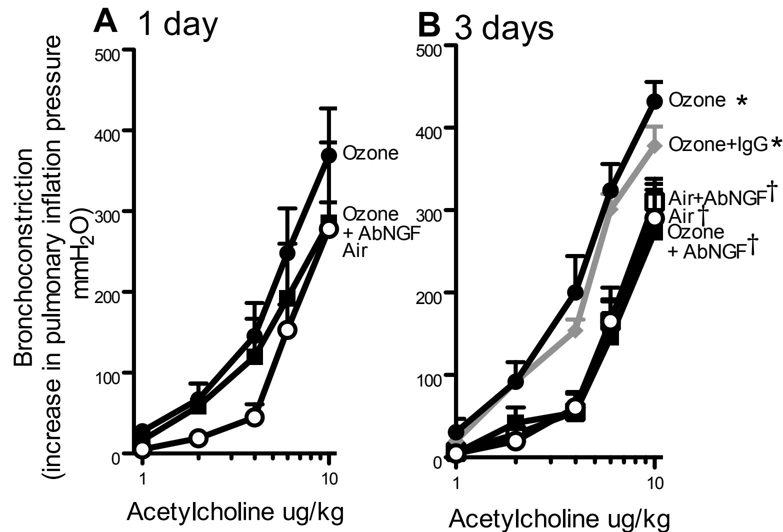


Figure 5.3. In vagotomized guinea pigs acetylcholine caused dose related bronchoconstriction in air exposed animals (open circles A and B). Acetylcholine-induced bronchoconstriction was not altered one day after ozone but was significantly potentiated three days after ozone compared to air exposed controls (filled circles A and B). The antibody to NGF did not affect acetylcholine-induced bronchoconstriction one day after ozone (A filled squares) but did inhibit acetylcholine-induced bronchoconstriction three days after ozone (B filled squares). Neither IgG (B gray diamonds) in ozone exposed animals nor antibody to NGF (B open squares) in air exposed animals had any effect on acetylcholine induced bronchoconstriction. *Significantly different from air exposed guinea pigs. †Significantly different from ozone-exposed guinea pigs. Data are expressed as means ± SE, n = 4-6.

FIGURE 5.4. Blocking NGF prevented eosinophil influx into bronchoalveolar lavage three days after ozone exposure.

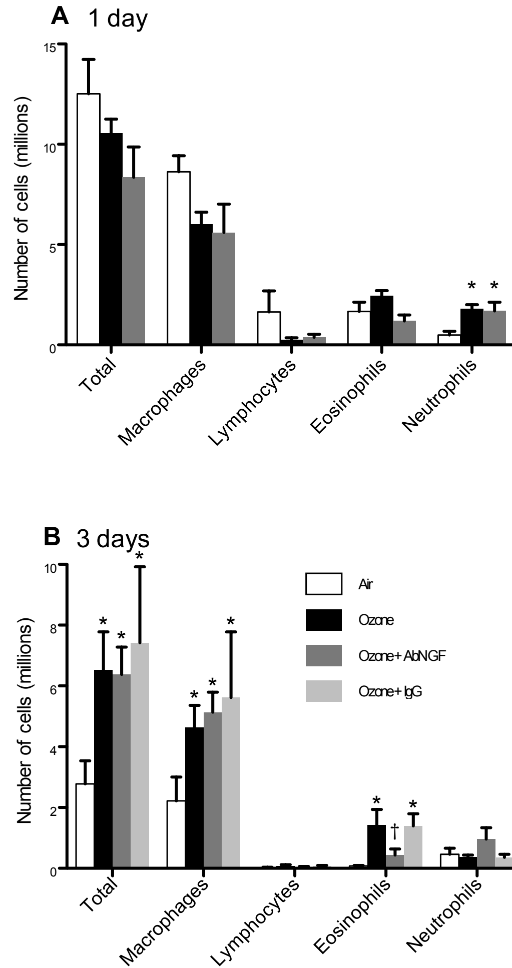


Figure 5.4. Neutrophils are the only inflammatory cells that increase one day after ozone exposure (A closed bars) and blocking NGF does not prevent the increase (A gray bar). Eosinophils, macrophages, and total cells in the bronchoalveolar lavage are significantly increased three days after ozone (closed bars) compared with air exposed controls (open bars). The antibody to NGF reduced eosinophils in ozone exposed animals (A dark gray bars). *Significantly different from air exposed controls. †Significantly different from ozone exposed animals. Data are expressed as mean ± SE, *n* = 5.

FIGURE 5.5. Blocking NGF significantly decreased the percentage of substance P positive nerve bundles in guinea pig airways.

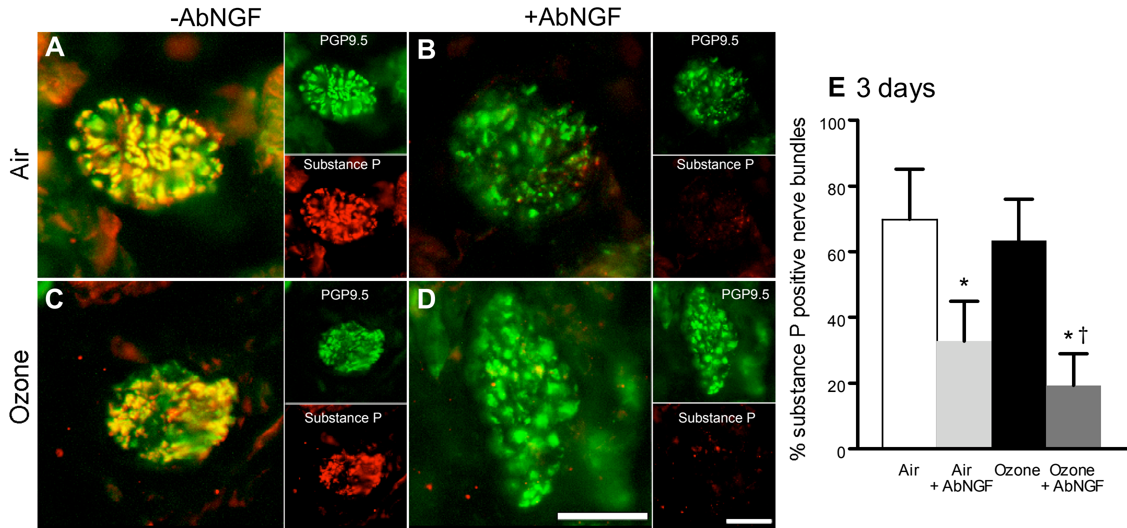


Figure 5.5. Representative images of airway nerve bundles in guinea pig lung stained with antibodies to pan neuronal marker PGP9.5 (green) and substance P (red) are shown with their merged overlay (A-D). Quantification of substance P positive bundles is shown in E. Three days after ozone (C, E filled bar) there is no change in the percentage of substance P positive nerve bundles compared to air exposed controls (A, E open bar). Pretreatment with an antibody to NGF significantly reduced the number of substance P positive nerve bundles in both air (B, E light gray bar) and ozone (D, E dark gray bar) exposed animals. *Significantly different from air exposed controls. †Significantly different from ozone exposed animals. Scale bar is 50μm. Data are expressed as mean ± SE, n = 3-5.

FIGURE 5.6. NGF did not increase substance P in parasympathetic nerves in culture.

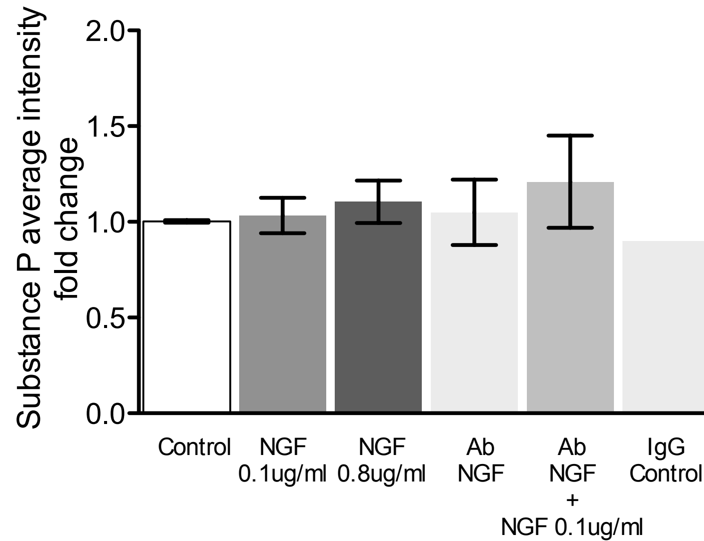


Figure 5.6. Parasympathetic nerves were cultured from guinea pig tracheas and treated with varying concentrations of NGF (0.1μg/ml or 0.8μg/ml, for 4 days) or an antibody to NGF (2μg/ml). Cultures were fixed and stained with an antibody to substance P. Substance P fluorescence was measured and treatments were compared to untreated control wells. NGF (dark gray bars) did not increase substance P staining above untreated controls (open bar) or background IgG staining (light gray bar) neither did the antibody to NGF (AbNGF medium gray bars) have any effect on substance P expression. To verify NGF was functional, the same aliquots of NGF and the antibody to NGF were used in sensory nerve cultures by another lab member at the same time these experiments were performed. Data are expressed as mean ± SE, n = 3-4.

FIGURE 5.7. Eosinophils slightly increased substance P in parasympathetic nerves in culture.

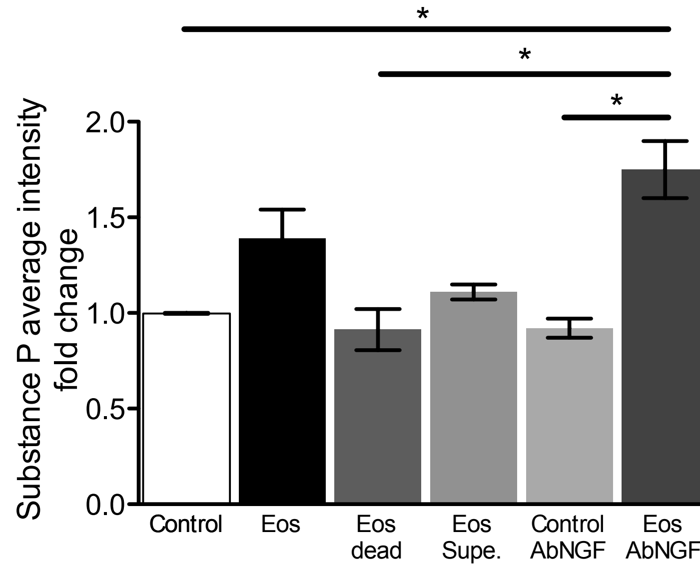


Figure 5.7. Parasympathetic nerves were cultured from guinea pig tracheas and co-cultured with eosinophils (250,000 eosinophils) or an antibody to NGF (AbNGF, 2μg/ml). Cultures were fixed and stained with an antibody to substance P. Substance P fluorescence was measured and treatments were compared to untreated control wells. Eosinophils (filled bar, Eos) slightly, but not significantly, increased substance P staining above untreated controls (open bar). The antibody to NGF (AbNGF medium gray bars) had no effect on substance P staining. Neither dead eosinophils nor eosinophil supernatant (Eos supe.) increased substance P staining. To verify the antibody to NGF was functional, another lab member used the antibody to NGF in sensory nerve cultures at the same time these experiments were performed. ANOVA $p = 0.0048$ Data are expressed as mean ± SE, n = 3-8.

FIGURE 5.8. Preprotachykinin mRNA was not detected by PCR in parasympathetic nerve cultures.

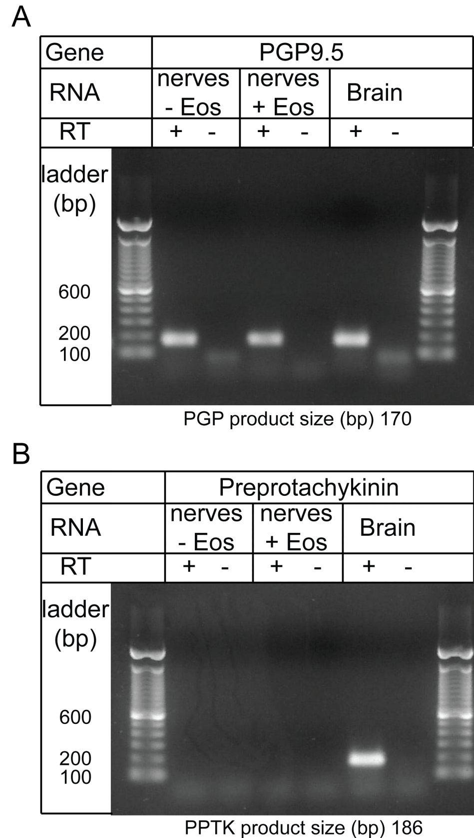


Figure 5.8. RNA isolated from guinea pig parasympathetic nerve cell bodies cultured with (nerves +Eos) or without eosinophils (nerves –Eos) and reverse transcribed in the presence (+) or absence (-) of reverse transcriptase (RT) was screened by RT-PCR using primers for (A) the pan neuronal marker protein gene product 9.5 (PGP9.5) and (B) the precursor for substance P, preprotachykinin (PPTK). Guinea pig brain cDNA was used as a positive control for both gene products. All samples contained PGP9.5 and only the brain contained PPTK. PCR products were sequenced to confirm correct gene products. Data are representative from one experiment of n=3-6.

FIGURE 5.9. Guinea pig peritoneal eosinophils express preprotachykinin mRNA.

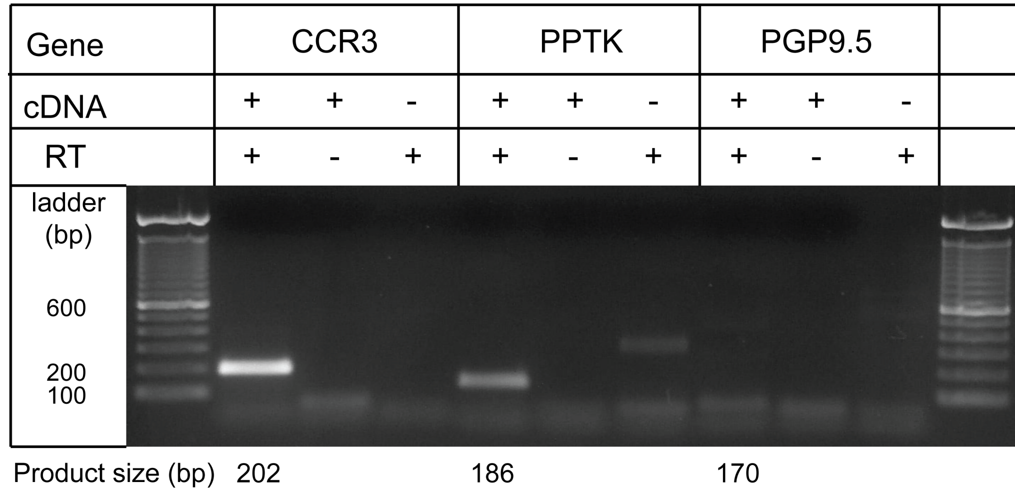


Figure 5.9. RNA isolated from guinea pig peritoneal eosinophils and reverse transcribed in the presence (+) or absence (-) of reverse transcriptase (RT) was screened by RT-PCR using primers for the chemokine receptor CCR3 (positive control), the precursor for substance P, preprotachykinin (PPTK), and the pan neuronal marker protein gene product 9.5 (PGP9.5) (as a negative control). Guinea pig eosinophils express mRNA for CCR3 receptors and preprotachykinin and do not have mRNA for the pan neuronal marker PGP. PCR products were sequenced to confirm correct gene products. Data are from one set of isolated guinea pig eosinophils.

DISCUSSION

Data presented here demonstrate that persistent, but not acute, ozone-induced airway hyperreactivity is mediated by NGF, possibly through an interaction with tachykinins, confirming that different mechanisms mediate acute versus persistent airway hyperreactivity after ozone (Figure 5.10).

Ozone increases substance P in bronchoalveolar lavage of humans (Hazbun *et al.*, 1993), and in ferrets, ozone increases the number of substance P positive parasympathetic nerve fibers in the trachea (Wu *et al.*, 2001; Wu *et al.*, 2003). In guinea pigs, airway hyperreactivity three days after ozone exposure is blocked by neurokinin receptor antagonists (Hazari, 2005) suggesting ozone increases substance P in guinea pigs as well. However, in these experiments, ozone did not increase the number of substance P positive airway nerve bundles three days after exposure. This was unexpected and suggests another mechanism for an increased role for substance P beside an increase in expression. It is possible concentrations of substance P were increased in airway nerves after ozone exposure and this would not have been reflected in my measurements since I did not measure the amount of substance P within each airway nerve in tissue sections.

Another possible mechanism for an increased role for substance P is neurokinin receptors increase or are unmasked three days after ozone exposure. NK_1 receptors are located on airway parasympathetic nerves (Mapp *et al.*, 2000) although pharmacological data show that NK_1 receptors are not normally involved in release of acetylcholine (Canning *et al.*, 2002; Grumann-Junior *et al.*, 2000; Hall *et al.*, 1989; Tramontana *et al.*, 1998). Receptor

unmasking has been described for NK_2 receptors on sensory nerves in antigen challenged guinea pigs where receptor activation occurs within minutes of antigen challenge (Weinreich *et al.*, 1997). The mechanisms for receptor unmasking are unclear, but may occur through post-translational modification, or trafficking of receptors to the cell membrane, rather than new receptor production. Thus, enhanced substance P, or NK_1 receptor activation on parasympathetic nerves could increase acetylcholine release resulting in ozone-induced airway hyperreactivity.

A third possible explanation is neutral endopeptidase, the enzyme that breaks down substance P, is inhibited three days after ozone exposure resulting in increased substance P due to decreased break down rather than increased production. Ozone does inhibit neutral endopeptidase activity in guinea pigs (Yeadon *et al.*, 1992), thus, an increased role for substance P after ozone exposure may be due to increased production, an increase in tachykinin receptors on parasympathetic nerves, and/or decreased break down of substance P.

NGF increases Substance P expression in the lungs of rabbits (Larsen *et al.*, 2004) and mice (Hoyle *et al.*, 1998). In ferret trachea, NGF induces hyperreactivity to electrical stimulation of airway nerves that is blocked by a NK_1 receptor antagonist, suggesting NGF induces airway hyperreactivity by increasing substance P (Wu *et al.*, 2006). NGF also increases neurokinin A and substance P expression in dorsal root ganglia sensory nerves (Vedder *et al.*, 1993). In these experiments, an antibody to NGF prevented ozone-induced airway hyperreactivity three days after exposure by decreasing substance P at a

time when the role of substance P is enhanced. Even though the antibody to NGF reduced substance P in air exposed control animals as well, there was no effect on vagally mediated bronchoconstriction. It is possible NGF is permissive for substance P expression and without it, substance P decreases. Substance P only contributes to vagally mediated bronchoconstriction, or airway hyperreactivity, three days after ozone exposure.

What is unclear from these experiments is the source of NGF and substance P. Eosinophils are a source of NGF (Noga *et al.*, 2003; Solomon *et al.*, 1998) and since NGF increases substance P expression in sensory nerves (Hoyle *et al.*, 1998; Larsen *et al.*, 2004; Vedder *et al.*, 1993) it is possible that it also increases substance P in parasympathetic nerves. However, in cultured guinea pig parasympathetic nerves, NGF did not increase substance P expression. Co-culture with eosinophils slightly increased substance P fluorescence intensity with an antibody to substance P. Although the antibody to NGF blocked ozone-induced airway hyperreactivity in vivo and decreased substance P staining in lung tissue sections, NGF did not mediate the increased substance P staining in isolated nerves since an antibody to NGF did not block increased substance P. Though small the eosinophil-induced increase in substance P staining appears to be real. It required eosinophils to be alive and in contact with parasympathetic nerves (Figure 5.7). Although eosinophils slightly increased substance P immunostaining of cultured parasympathetic nerves, no preprotachykinin mRNA was detected in mRNA from cultured parasympathetic nerves. It is possible that the increase in immunostaining was due to substance P released by another cell in the culture that was sticking to the nerves. Eosinophils also release substance P (Figure 5.9) (Aliakbari *et al.*, 1987;

Weinstock *et al.*, 1988) and that may be the source of the increase in substance P staining on nerves in culture. An alternative explanation is the antibody to substance P was not selective, or parasympathetic nerves are making substance P in response to eosinophils but the levels of mRNA are below the limit of detection.

An additional explanation for the lack of measurable substance P in parasympathetic nerves is perhaps substance P levels are changing in vivo in sensory nerves. The decreased expression of substance P in airway nerve bundles after administration of the antibody to NGF may have been in sensory nerves, since approximately 80% of nerves in the vagus are sensory.

The novel data presented here suggest a role for substance P in ozone-induced airway hyperreactivity three days after exposure. Substance P may contribute to ozone-induced airway hyperreactivity by multiple mechanisms including increased substance P expression, increased or unmasked tachykinin receptors, or inhibition of neutral endopeptidase.

FIGURE 5.10. Role of NGF and tachykinins in ozone-induced airway hyperreactivity.

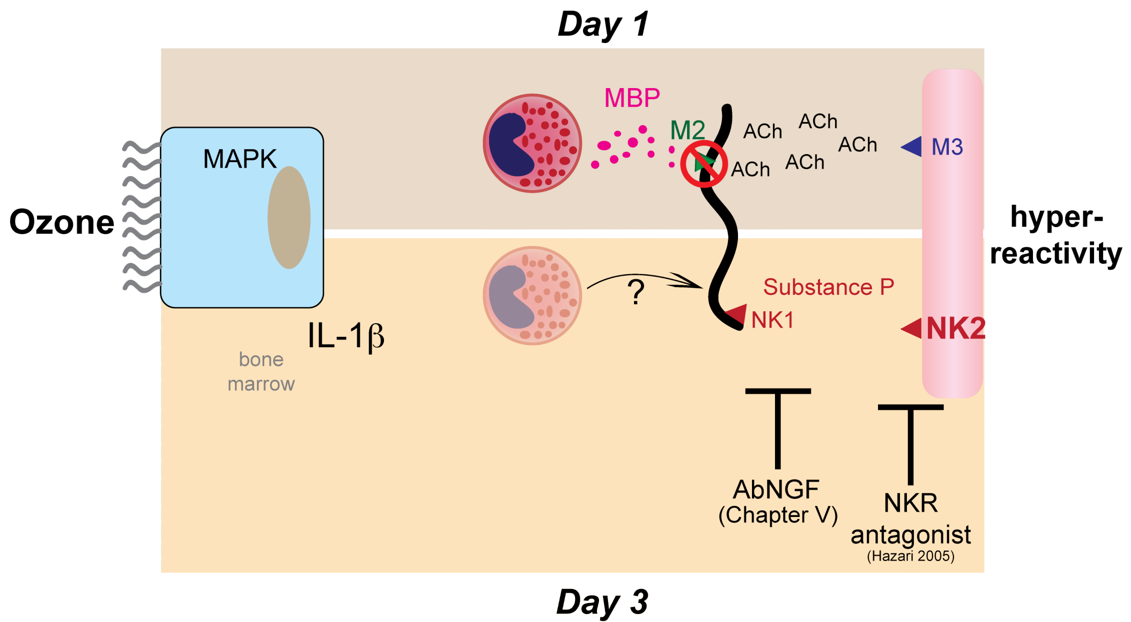


Figure 5.10. One day after ozone exposure airway hyperreactivity is mediated by eosinophil major basic protein (MBP) inhibiting neuronal M_2 muscarinic receptors thereby increasing acetylcholine (ACh) release onto M_3 muscarinic receptors on airway smooth muscle (pink band). It is known that IL-1β and substance P contribute to airway hyperreactivity three days after ozone exposure (Chapter IV) (Hazari, 2005). Since IL-1β increases both NGF and substance P expression (see text for references) I tested whether NGF mediates ozone-induced airway hyperreactivity through increasing substance P. Blocking NGF prevented ozone-induced airway hyperreactivity three days but not one day after ozone exposure. Eosinophils slightly increased substance P staining on parasympathetic nerves in culture that was not mediated by NGF.

CHAPTER VI.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Previous work demonstrated the initial hyperreactivity to ozone was mediated by degranulation of eosinophils, release of major basic protein, blockade of inhibitory M_2 muscarinic receptors on parasympathetic nerves, increased acetylcholine release and increased vagally mediated bronchoconstriction (Chapter I, see figure 1.17). The data included in this thesis add that p38 and JNK activation precede these events (Chapter III). Furthermore, the mechanisms of hyperreactivity change over time so that blocking IL-1 (Chapter IV) or blocking tachykinin receptors or depleting substance P (Chapter V) each prevents vagally-mediated hyperreactivity three days, but not one day, after ozone. These data significantly advance the field in that they definitively show that mechanisms of ozone-induced hyperreactivity originate with mitogen activated protein kinase activation and change over three days to include tachykinins. I have adapted figure 1.17 to include my findings here and included it here as figure 6.1. One of the first changes I made was to move the epithelial cells only to the one day compartment only since ozone is not present three days later, although it appears to induce a delayed, systemic effect three days after ozone.

It is known the interaction of ozone with airway epithelial cells produces reactive oxygen species that activate the mitogen activated protein kinase signaling pathway (Hamilton *et al.*, 1996; Kirichenko *et al.*, 1996; Kumagai *et al.*, 2002). I have shown inhibition of both p38 and JNK mitogen activated kinases prevents M_2 receptor dysfunction and airway hyperreactivity one day after ozone (Chapter III) suggesting that kinase activation precedes M_2 receptor dysfunction. Inhibition of both p38 and JNK may protect M_2

receptor function and prevent airway hyperreactivity at multiple and intersecting levels. Activation of mitogen activated protein kinase signaling increases inflammatory cytokines (Cui *et al.*, 2002; Kalesnikoff *et al.*, 2001; Peng *et al.*, 2004) that may lead to activation of eosinophils and release of eosinophil major basic protein. Inhibition of p38 and JNK may also directly inhibit eosinophil activation by preventing major basic protein release since inhibiting p38 activation in eosinophils prevents IL-5 induced cytokine production (Adachi *et al.*, 2000). Since it is known that acute airway hyperreactivity after ozone is mediated by eosinophils and M_2 receptor dysfunction, p38 and JNK mitogen activated protein kinases contribute to ozone-induced airway hyperreactivity possibly by initiating, recruiting, activating or otherwise enhancing eosinophil degranulation. Blocking them would prevent neuronal M_2 muscarinic receptor dysfunction by decreasing release of eosinophil major basic protein. The exact mechanism for how the mitogen activated protein kinase pathway contributes to airway hyperreactivity is unclear because every cell type expresses these kinases and systemic inhibition of the pathways does not allow for elucidation of particular cell types that may be involved. Additionally, these inhibitors are not specific and other kinases may also contribute to ozone-induced inflammation and airway hyperreactivity. What is definitive is that p38 and JNK mitogen activated kinases play an early role in ozone-induced hyperreactivity.

Three days after ozone exposure I have shown that the mechanisms of airway hyperreactivity have completely changed and are no longer mediated by eosinophil major basic protein or by inhibition of neuronal M_2 muscarinic receptors, confirming what was suggested by Yost et al in 2005. Since IL-1β increases in lungs following ozone exposure

(Arsalane *et al.*, 1995; Cohen *et al.*, 2001; Fakhrzadeh *et al.*, 2004; Pendino *et al.*, 1994; Polosa *et al.*, 2004) I blocked this cytokine and have shown that an IL-1 antagonist prevents ozone-induced airway hyperreactivity three days, but not one day after ozone (Chapter IV) (Verhein *et al.*, 2008). I have shown that IL-1β is increased in bone marrow three days post ozone, again suggesting that ozone induces a systemic response that takes several days to mature. IL-1 may enter systemic circulation from the lungs and increase synthesis of IL-1 in the bone marrow. This is included in Figure 6.1 as an initial increase early after ozone exposure, followed by an as yet unknown systemic signal increasing IL-1β in bone marrow. There is no literature regarding the presence of inflammatory markers in blood in the days following ozone, but such measurements could identify whether there were circulating systemic signals following ozone. The IL-1 receptor antagonist may be blocking one, or all three potential actions of IL-1 three days after ozone exposure.

IL-1β induces airway hyperreactivity to substance P that is blocked by an antibody to NGF (Frossard *et al.*, 2005), suggesting that NGF may be an intermediary between IL-1β and substance P. I have included data suggesting a similar pathway mediates airway hyperreactivity three days after ozone in guinea pigs. In Figure 6.1 IL-1 is early in the pathway since blocking it prevents hyperreactivity. Following that is the antibody to NGF because that prevents airway hyperreactivity three days after ozone, probably by blocking substance P. Thus, NGF may be an intermediate in the pathway between IL-1 and substance P three days after ozone.

What is also still unclear from these data is the source of both NGF and substance P. NGF is produced by many cells including mast cells, macrophages, eosinophils, smooth muscle, and epithelial cells (Braun *et al.*, 1998; Fox *et al.*, 2001; Freund *et al.*, 2002; Hahn *et al.*, 2006; Leon *et al.*, 1994; Noga *et al.*, 2003; Solomon *et al.*, 1998). Regardless of the source of NGF, the absence of NGF either decreases substance P production or increases metabolism so there is less substance P. Similar to NGF, substance P is produced by a number of airway cells besides nerves including epithelial cells, macrophages, and eosinophils (Bost *et al.*, 1992; Killingsworth *et al.*, 1997; Weinstock *et al.*, 1988). Substance P content in the lungs is only important three days after ozone exposure where decreased substance P, via an antibody to NGF, prevents airway hyperreactivity. The contribution of NGF to airway hyperreactivity three days after ozone exposure has been included in figure 6.1 as a depletion of substance P with the antibody to NGF. The source is unmarked since it is as yet unknown.

Although eosinophils may have increased substance P immunostaining on parasympathetic nerves in culture, because substance P was not detected in nerves cultured with eosinophils the results were inconclusive as to whether substance P was being synthesized by nerves or not. It may be nerves in cell culture and in vivo behave differently, thus measuring the presence of preprotachykinins in ganglia in vivo may yield different results than measuring in cell culture.
Another possibility is the as yet unexplored interaction between eosinophils and sensory nerves. The original observation that eosinophils co-localize with airway nerves in lungs were made using nerves labeled with a nonspecific pan-neuronal marker, so it is possible

that eosinophils are associated with airway sensory nerves in addition to parasympathetic nerves (Costello *et al.*, 1997). The role of eosinophils in neural plasticity of sensory nerves could be tested by co-culturing eosinophils with sensory nerves and measuring substance P expression and release in these cell bodies. Although not tested, figure 6.1 depicts probable airway sources of substance P as eosinophils and sensory nerves, based upon the literature, and lack of substance P mRNA in parasympathetic ganglia. Substance P, or other tachykinins, from these sources might both directly constrict airway smooth muscle, and increase acetylcholine release from airway parasympathetic nerves.

Eosinophils and nerves are not the only cells found in lungs that may contribute to airway hyperreactivity following ozone exposure. Airway macrophages respond to ozone exposure by producing inflammatory cytokines, including IL-1 and TNF, and via IL-1 alone, could contribute to both recruitment and activation of inflammatory cells after ozone exposure. Neutrophils are the first inflammatory cells recruited out of the airways after ozone exposure however, their role in hyperreactivity is completely unknown. They may be intermediary between epithelial cells and/or macrophages leading to eosinophil activation downstream; or may be dying cells exiting the lungs in the lumen. Neither neutrophils nor macrophages have been included in figure 6.1 in order to keep the figure relevant to results described in this thesis.

In conclusion, I have shown that ozone activates signaling kinases, likely in epithelial cells, that triggers a systemic response mediated by IL-1 that over two to three days increases substance P in either nerves or eosinophils. Depletion of substance P with an

antibody to NGF or neurokinin receptor antagonists prevents airway hyperreactivity three days but not one day after ozone exposure (Figure 6.1).

The data included in this thesis and the conclusions I have drawn are important for human health since over half of the population of the United States lives in areas with unhealthy levels of ozone (Association, 2009). The observation that ozone increases asthma exacerbations and increases hospitalizations and mortality not only the same day ozone levels are high, but also two to three days after a high exposure as well has not been well characterized (Chan *et al.*, 2005; Lewis *et al.*, 2005; Romieu *et al.*, 1995). Currently there are no specific therapies available to treat ozone-induced lung complications and the only options are standard asthma therapies (i.e. bronchodilators and steroids). Controlled human exposure studies measuring airway inflammation and decrements in lung function over time will be necessary to fully understand the long term effects of ozone on the lungs in humans. The findings presented in this thesis demonstrate different treatments may be required depending on when symptoms occur following ozone exposure since the mechanisms of ozone-induced airway hyperreactivity change over time.

In summary, I have identified important new mediators that contribute to ozone-induced airway hyperreactivity. These include mitogen activated protein kinases one day after ozone, IL-1, substance P, and nerve growth factor three days after ozone. In addition, I have definitively shown that mechanisms of hyperreactivity are different one and three days after ozone in that neither IL-1 nor NGF contributes to hyperreactivity one day after

ozone. These results provide additional drug targets for future therapies and suggest that specific therapies may need to be tailored to inhibit the specific and different mechanisms of hyperreactivity in the days following ozone exposure.

FIGURE 6.1. Mechanisms of ozone-induced airway hyperreactivity change over time.

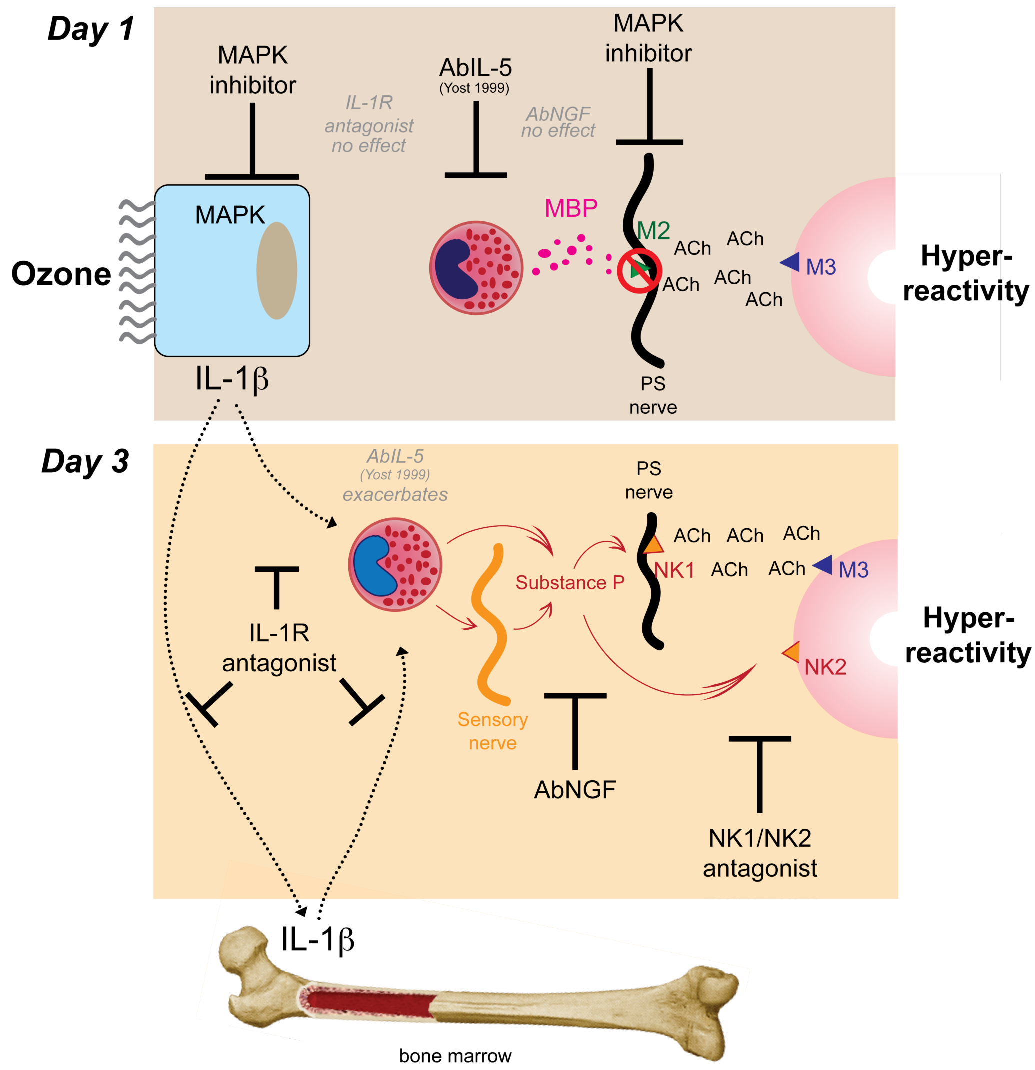


Figure 6.1. Using a guinea pig model, I have demonstrated the mechanisms of ozone-induced airway hyperreactivity change over time. One day after ozone, hyperreactivity is mediated by p38 and JNK mitogen activated protein kinases (MAPK) (Chapter III) and inhibition of neuronal M_2 muscarinic receptors. Three days after ozone, airway hyperreactivity is mediated by IL-1 (Chapter IV), nerve growth factor (NGF) and substance P (Chapter V).

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APPENDIX

A. Review: Neural control of airway inflammation

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Abstract

Abnormal neural function contributes to the pathogenesis of airway disease. In addition to effects on airway physiology, the nerves produce and release inflammatory mediators, contributing to the recruitment and activation of leukocytes. Activated inflammatory cells, in turn, affect the function of airway nerves, changing the production and release of neurotransmitters. Crosstalk between airway nerves and leukocytes participates both in maintaining chronic inflammation and in causing accentuated neural control of the airways.

Introduction

Airway inflammation is a characteristic of many airway diseases, including asthma and chronic obstructive pulmonary disease (COPD). Inflammation results from a number of different stimuli including allergen exposure, viral infection, and environmental pollutants. Inflammatory cells found in asthmatic and antigen, ozone or virus exposed lungs include eosinophils, neutrophils, basophils, mast cells, macrophages, and T lymphocytes. These various cell types contribute to airway remodeling and airway hyperreactivity. Inflammation-induced changes in airway nerves are a major mechanism of airway hyperreactivity [1**]. However, communication between inflammatory cells and airway nerves is a two way street: nerves produce chemotactic factors that recruit

inflammatory cells [2**], and inflammatory cells produce neuromodulatory signals [3, 4]. This review describes recent advances in our understanding of interactions between airway nerves and airway inflammation.

Airway innervation

The lungs are innervated by parasympathetic, sympathetic, and sensory nerves, which control gland secretion, baseline airway smooth muscle tone, and bronchoconstriction. The majority of sensory and all of the parasympathetic nerves travel to airways via the vagus nerves, while sympathetic nerves arrive via the spinal cord. The vagus nerves innervate chains of parasympathetic ganglia in the trachea and main bronchi. Parasympathetic nerve targets include airway smooth muscle, mucus glands, and blood vessels. Sensory nerve cell bodies are located in the nodose and jugular ganglia at the base of the skull and innervate airway epithelium, submucosal layers, and smooth muscle. Some sensory nerves originate in dorsal root ganglia and arrive in the lungs with spinal sympathetic nerves. In humans, sympathetic nerves target mucus glands, blood vessels and parasympathetic ganglia, but do not innervate smooth muscle directly. In animals, innervation differs between species, with guinea pigs and mice having more extensive sympathetic innervation than humans.

Neuron-inflammatory cell communication

Muscarinic Acetylcholine Receptors

Parasympathetic nerves release the neurotransmitter acetylcholine, which binds to nicotinic and muscarinic receptors throughout the airways. Although principally a

neuronal product, non-neuronal release of acetylcholine has been described in the airways. Many cells have machinery necessary for acetylcholine synthesis, including epithelial cells and immune cells [5]. In the lungs, parasympathetic nerves provide the dominant autonomic control over airway smooth muscle tone through release of acetylcholine onto M3 muscarinic receptors. Acetylcholine release from parasympathetic nerves is normally limited by inhibitory M2 muscarinic receptors on the nerves [6], and dysfunction of neuronal M2 muscarinic receptors increases acetylcholine release and bronchoconstriction. This leads to airway hyperreactivity in humans with asthma [7] and animal models of asthma [1].

There are 5 subtypes of G-protein-coupled muscarinic receptors (M1-M5), all of which are found in the lung. Airway smooth muscle contains both M2 and M3 receptors and acetylcholine-induced contraction is primarily mediated through activation of M3. M3 receptor activation also increases mucus and water secretion from glands and increases ciliary beat frequency on airway epithelium [8].

Muscarinic receptors are also expressed on inflammatory cells (Table 1). Macrophages have M3 receptors through which acetylcholine stimulates release of leukotriene B4 [9]. Mast cell release of histamine is inhibited by acetylcholine [10*]. In isolated bronchi from COPD patients, histamine release induced by calcium ionophore is enhanced compared to bronchi from non-COPD patients [11]. Oxotremorine, a muscarinic agonist, did not significantly inhibit the enhanced histamine release suggesting inhibitory acetylcholine receptors on mast cells are not functional in patients with COPD [11]. M3,

M4 and M5 receptor mRNA has been found in human neutrophils [12] although the function of these receptors on neutrophils is unknown.

Both human and guinea pig eosinophils express M3 and M4 receptors, while human eosinophils also express M5 receptors [13]. These muscarinic receptors inhibit eosinophil activation, since administering atropine, a muscarinic antagonist, prior to antigen challenge significantly potentiates antigen-induced eosinophil activation, measured by an increase in major basic protein deposition in the airways [14**]. As a result, animals treated with atropine before antigen challenge are substantially more hyperreactive than animals antigen challenged without atropine. In vitro studies demonstrate that acetylcholine blocks platelet activating factor-induced increase in intracellular calcium in eosinophils in the presence of a nicotinic receptor antagonist confirming eosinophil muscarinic receptors are inhibitory [13]. Inhibition of eosinophil activation by acetylcholine likely occurs through M4 receptors since an M4 selective antagonist, MT3, prevents acetylcholine-induced inhibition of eosinophil activation [13] (Figure A.1). This proinflammatory effect of anticholinergic treatment may limit the effectiveness of anticholinergics in asthma. Increased activation of inflammatory cells, especially eosinophils, in the presence of anticholinergics may additionally increase release of mediators that adversely affect nerves [13].

Nicotinic Acetylcholine Receptors

Nicotinic receptors are ion channels and are found in the airways on parasympathetic nerves, macrophages, epithelial cells, eosinophils, neutrophils, and mast cells [15-19]

(Table A.1). Recently, Gu and colleagues discovered functional nicotinic receptors expressed on vagal pulmonary sensory nerves in the rat [20]. The authors speculate nicotinic receptors on sensory nerves may be involved in smoking-induced cough.

Vagal cholinergic pathways have an anti-inflammatory role, termed the 'cholinergic anti-inflammatory pathway' mediated by nicotinic acetylcholine receptors. Stimulation of the vagus nerves inhibits TNFα synthesis during endotoxemia and prevents development of shock in rats. In isolated macrophages, acetylcholine inhibits lipopolysaccharide-induced production of inflammatory cytokines TNFα, IL-1β, and IL-6 [21*]. Acetylcholine's anti-inflammatory properties in peripheral tissues results from activation of the α7 nicotinic receptor on macrophages [15]. In addition to anti-inflammatory effects on macrophages, activation of α7 nicotinic receptors on endothelial cells inhibits TNFα induced expression of the adhesion molecule ICAM-1 and chemokines IL-8, RANTES, and MCP-1 [22] preventing migration of inflammatory cells from the blood to the tissues. Systemic administration of nicotine, or a selective α7 agonist, attenuates acid-induced lung injury by reducing TNFα concentrations and neutrophil influx into bronchoalveolar lavage in rats [16]. Thus, nicotinic agonists, including acetylcholine that can be released by endothelial cells themselves [23], can limit cytokine release and tissue inflammation.

Anti-inflammatory effects of nicotinic receptor activation may not always be beneficial. Nicotine increases growth of Streptococcus pneumoniae and increases TNFα and IFNγ concentrations 24 hours after infection in mice [24]. In addition, chronic nicotine administration promotes influenza infection by inhibiting inflammatory cell influx into

the lungs [25], suggesting that, in some infectious models, inhibiting inflammatory processes via nicotinic acetylcholine receptors prevents clearance of pathogens.

Adrenergic Receptors

Sympathetic nerves release the catecholamines norepinephrine and epinephrine onto adrenergic receptors. Adrenergic receptors are G-protein coupled and are classified into two subclasses, α and β. In the lungs, α, β1 and β2 receptors are present on many airway cells. Activating β1 receptors on parasympathetic nerves inhibits acetylcholine release and relaxes airway smooth muscle. However, catecholamine-induced relaxation of airways is primarily mediated by β2 receptors on smooth muscle.

Adrenergic receptors are also located on inflammatory cells although the exact role of these receptors during airway inflammation is still unclear (Table A.1). Rat alveolar macrophages and blood neutrophils express α2-adrenoreceptors and make norepinephrine and epinephrine upon stimulation with LPS [26]. In addition, human and guinea pig peripheral blood eosinophils express β2 receptors [27]. Functional evidence also demonstrates mast cells may express inhibitory β2 receptors [28].

Activation of adrenergic receptors on inflammatory cells can be both pro- and anti-inflammatory. Norepinephrine and epinephrine stimulate release of inflammatory cytokines in macrophages including TNFα, IL-1β, and IL-6 in vitro [29]. Conversely, activation of β receptors on human blood eosinophils prevents degranulation in response

to platelet activating factor, and on mast cells inhibits release of histamine after IgE activation [28].

Tachykinin Receptors and Neurogenic Inflammation

Activation of sensory nerves by antigen or environmental stimuli initiates a central reflex mediated through the parasympathetic nerves and also local reflexes. The local reflexes were described in the early 20th century, when it was observed that neurogenic vasodilation in the skin in response to noxious stimuli was independent of central connections of sensory nerves. Likewise in the lungs, sensory nerves produce peptide neurotransmitters that are released directly from the nerve terminals in the airways through a local axon reflex. Neurogenic inflammation refers to the process of neuropeptide release from nerves, leading to inflammatory effects including edema, neural plasticity and recruitment of inflammatory cells.

Substance P is one of the primary peptide neurotransmitters released by sensory nerves in the airways, and release of substance P causes smooth muscle contraction, submucosal gland secretion, vasodilation, stimulation of cholinergic nerves, and both stimulation and chemoattraction of inflammatory cells. As with acetylcholine, substance P is not exclusively a neuronal product, and is made by epithelial cells in which expression is mediated by viral infection [30] and inflammatory cells, including macrophages and eosinophils. Substance P exerts its effects through G-protein coupled tachykinin receptors, and has the greatest affinity for neurokinin receptor 1 (NK1). Other tachykinin receptors include NK2 and NK3 receptors, which preferentially bind neurokinin A and

neurokinin B, respectively. This review will focus on the effects of substance P although all tachykinins can signal through all three tachykinin receptors.

In asthma, substance P increases in bronchoalveolar lavage fluid [31] and there is immunohistochemical evidence for an increase in NK1 receptor expression in airway epithelium compared to non-asthmatic controls [32]. In ovalbumin-sensitized and challenged guinea pigs, substance P protein increases in airways, and antigen challenge also increases production of substance P mRNA in sensory nerves [33]. Antigen challenge increases vagally mediated reflex bronchoconstriction by decreasing the function of inhibitory M2 muscarinic receptors. Tachykinin receptor antagonists prevent antigen-challenge induced airway hyperreactivity by protecting the function of neuronal M2 receptors, thus limiting acetylcholine release from parasympathetic nerves and preventing development of airway hyperreactivity [34]. Thus, substance P is important in airway hyperreactivity.

Substance P may increase with asthma and airway inflammation by either increased protein production by nerves or non-neuronal sources, or by inhibition of substance P degradation. Metabolism of substance P occurs mainly through cleavage by neutral endopeptidase. Neutral endopeptidase activity has been found on neutrophils, but not on lymphocytes, monocytes, eosinophils, or basophils. Neutral endopeptidase activity is reduced during viral infections and following ozone exposure [35]. In asthma, levels of epithelial neutral endopeptidase are inversely proportional to bronchial hyperreactivity and asthma symptoms. Inhaled corticosteroids increase neutral endopeptidase activity,

suggesting that regulation of substance P levels by epithelial neutral endopeptidase is related to airway hyperreactivity [36].

Tachykinin receptors are located throughout the airways, including on inflammatory cells (Table A.1). Human and mouse alveolar macrophages, neutrophils, and mast cells all express NK1 receptors [37-42]. Activation of NK1 receptors on isolated macrophages induces metalloelastase (MMP-12) production [39]. In human connective tissue type mast cells activation of NK1 receptors by substance P stimulates release of RANTES and IL-8. Furthermore, activation of mast cells through crosslinking of IgE receptors upregulates NK2 and NK3 receptor expression, suggesting that exposure to allergens may prime mast cells to be more responsive to neuropeptides, including substance P [42]. In rats, an NK1 receptor antagonist administered prior to ozone exposure prevented ozone-induced epithelial cell death and attenuated epithelial cell proliferation suggesting a role for both injury and repair upon activation of NK1 receptors [43]. In another inhalation injury model, an NK1 antagonist prevented ICAM-1 upregulation following oil smoke exposure [44].

NK2 receptors are also present on mast cells and macrophages [37, 45]. NK3 receptors are found in airway parasympathetic ganglia, and activation of these receptors depolarizes postganglionic parasympathetic nerves, increasing acetylcholine release [46], that can interact with muscarinic receptors on inflammatory cells as described above.

There is functional evidence for tachykinin receptor expression on eosinophils. Substance P induces peripheral blood eosinophil chemotaxis that is blocked by an NK1 receptor antagonist in vitro [47], and functional NK2 receptors have been described on eosinophils in the intestine [48]. However, confirmation of tachykinin receptor subtype with PCR and or antibody binding has not been described in either peripheral blood or tissue-resident eosinophils.

Communication between inflammatory cells and nerves

Increased release of acetylcholine from parasympathetic nerves and increased substance P in sensory nerves both contribute to airway hyperreactivity. The cellular interactions underlying neural plasticity of the sensory and parasympathetic nervous systems almost certainly involve inflammatory cells. Depleting granulocytes with cyclophosphamide prevents M2 receptor dysfunction and airway hyperreactivity induced by ozone inhalation, antigen challenge, and viral infection [49, 50]. Mast cell deficient mice do not develop airway hyperreactivity after antigen challenge [51]. Depleting macrophages prevents virus-induced M2 receptor dysfunction and hyperreactivity [52]. Thus, in addition to inflammatory cells being affected by the neurotransmitters acetylcholine, norepinephrine, and substance P, inflammatory cells are capable of changing nerve function, life span, transmitter release, and neurotransmitter content through release of cytokines and neurotrophins [53, 54].

One well-studied pathway of nerve-inflammatory cell interactions is the interaction of eosinophils with parasympathetic nerves. In fatal asthma and in animal models of airway

hyperreactivity, eosinophils cluster around airway nerves [55**]. Eosinophil recruitment to nerves is not limited to asthma or the airways, as there are also increased eosinophils around nerves in the intestine following parasitic infections [56], and they are found along nerves in inflamed skin [57].

Inflammatory cells are recruited to airways by production of chemokines. Blocking CCR3 receptors inhibits recruitment of eosinophils to airway nerves, and prevents neuronal M2 receptor dysfunction and airway hyperreactivity in antigen challenged guinea pigs [2]. The CCR3 agonist eotaxin, is made by airway parasympathetic nerves exposed to inflammatory cytokines, thus a source for eosinophil attractant cytokines is the airway nerves. Eotaxin is upregulated in airway lavage fluid of humans with asthma and also in antigen challenged animals suggesting a role in asthma.

Activated eosinophils release eosinophil major basic protein, an endogenous antagonist of neuronal M2 muscarinic receptors [3]. Therefore, eosinophils in close proximity to airway nerves release major basic protein, that inhibits neuronal M2 receptor function resulting in increased acetylcholine release, increased bronchoconstriction, and vagally mediated airway hyperreactivity [1] (Figure 1). Eosinophil major basic protein and other eosinophil derived cationic proteins also increase the sensitivity of sensory neurons to capsaicin and to electrical stimulation [58]. Pretreatment of sensory nerves (isolated from rat nodose and jugular ganglia) with major basic protein significantly increased capsaicin-evoked inward currents and number of action potentials, which lasted for over an hour after treatment. Although the mechanism of eosinophil sensitization of sensory

nerves is unclear, the authors ruled out membrane injury as the cause of increased sensitivity and conclude that sensitization is based on charge interactions between the cationic proteins and the nerves.

Recruitment of mast cells to airway nerves has also been described. Antigen-induced depolarization of airway parasympathetic nerves is mediated by histamine release from mast cells located near the nerves (Myers *et al.*, 1995; Weigand *et al.*, 2009).

Other proteins released from inflammatory cells are capable of interacting with nerves, including nerve growth factor (NGF). NGF is a neurotrophin that is involved in neuronal outgrowth and differentiation during development. In the adult, NGF is important in inflammation and neural plasticity. For example, NGF promotes nerve cell growth, increases eosinophil survival (Solomon *et al.*, 1998), and upregulates tachykinins in airway nerves (Mingomataj *et al.*, 2008). Patients with asthma have significantly increased levels of NGF in bronchoalveolar lavage compared to non-asthmatics (Olgart Hoglund *et al.*, 2002), and nasal allergen provocation in humans with allergic rhinitis increases NGF levels in nasal mucosa and serum 24 hours after challenge (Raap *et al.*, 2008b).

In vitro evidence suggests sources of NGF in inflamed airways include eosinophils, mast cells, macrophages, epithelial cells, and smooth muscle cells (Caroleo *et al.*, 2001; Xiang *et al.*, 2000). Inflammatory conditions increase production of NGF, as eosinophils from allergic humans make and release more NGF than eosinophils from non-allergic

individuals (Noga *et al.*, 2003). Peripheral blood eosinophils from patients with atopic dermatitis and allergic rhinitis differentially express receptors for neurotrophins and respond differently to NGF stimulation (Raap *et al.*, 2008a). NGF inhibits eosinophil apoptosis in atopic dermatitis and allergic rhinitis, but not in non-allergic individuals. Thus, inflammatory cells can affect parasympathetic and sensory nerve function by release of inflammatory mediators such as NGF that can enhance production or release of substance P.

Clinical implications of muscarinic receptors on inflammatory

The finding that airway hyperreactivity is potentiated by an anticholinergic at the time of antigen challenge, along with the finding that this is accompanied by increased eosinophil activation, suggests a mechanism limiting the effectiveness of anticholinergic treatment for chronic asthma. While anticholinergics are beneficial in treating acute asthma attacks [68], their clinical efficacy in chronic stable asthma is less impressive. Possible proinflammatory effects of anticholinergics in this setting have not been adequately examined, but a study of the effects of nasal ipratropium in patients with allergic rhinitis found a five fold increase in eosinophils in the nasal lavage [69]. Further defining the mechanism of eosinophil activation by anticholinergics may suggest alternative approaches to anticholinergic treatment of chronic asthma. For example, depending on the specific muscarinic receptor subtypes involved, it may be possible to design anticholinergics that bronchodilate without activating eosinophils. Alternatively, it may be possible circumvent this problem by combining anticholinergic therapy with either more intensive general anti-inflammatory treatments or specifically targeted anti-

eosinophil treatments. It is also likely to be important to define the presence and function of muscarinic receptor subtypes on other inflammatory cells, thereby determining the optimal muscarinic receptor selectivity of an antiasthma drug.

Conclusion

Chronic airway inflammation is a hallmark of both asthma and COPD. It is clear that inflammation affects airway nerve function, just as airway nerves influence inflammation. Release of neurotransmitters in lungs affects not only smooth muscle contractility, but also inflammatory responses as well, and can be both pro- and anti-inflammatory. Crosstalk between nerves and immune cells in chronic inflammatory airway diseases is underappreciated and needs further scientific exploration.

FIGURE A.1. Crosstalk between eosinophils and parasympathetic nerves.

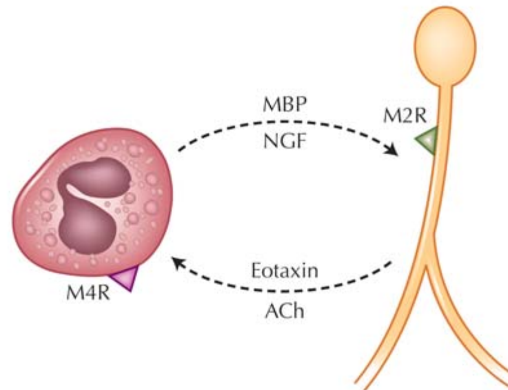

Figure A.1. An example of crosstalk between airway parasympathetic nerves and eosinophils. Parasympathetic nerves release the chemokine eotaxin, which recruits and activates eosinophils. Eosinophils release major basic protein (MBP) that inhibits neuronal M2 muscarinic receptors (M2R) and increases acetylcholine release. Eosinophils may also release nerve growth factor (NGF), which induces neural plasticity and increases substance P release from airway nerves. Acetylcholine inhibits eosinophil activation through M4 muscarinic receptors (M4R).

TABLE A.1. Neurotransmitter receptor expression on airway inflammatory cells.

Cell	Receptors											
	Muscarinic			Nicotinic			Adrenergic			Neurokinin		
Eosinophil	M3-M5	PCR	[13]	a3, a4,	PCR	[17]	b2	binding	[27]	NK1	function	[47]
		immuno.		a7	function			function		NK2	immuno.	[48]
					immuno.							
Macrophage	M3	function	[9]	a7	immuno.	[15]	a2	PCR	[26]	NK1	function	[38]
					PCR			western			western	
					western						immuno.	
										NK2	immuno.	[45]
Mast Cell	M1	function	[10]	a7	PCR	[19]	b2	function	[44]	NK1	function	[42]
											flow-cytometry	
										NK2	immuno.	[45]
Neutrophil	M3-M5	PCR	[12]	yes	function	[18]	a2	PCR	[26]	NK1	function	[41]
								western		NK1-3	western	[40]

Table A.1. Included in the table are methods used to detect neurotransmitter receptors (PCR, functional studies, western blot, or immunohistochemistry/immunocytochemistry) and corresponding references.

References

*of importance

**of major importance

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B. Raw data for MAPK paper (Chapter III)

INCREASE IN Ppi - Frequency

Treatment Date	Hz Code	2	4	6	8	10	12	15	20	25
Control										
11/6/06	06-059	13	26	38	54	80	110	135	190	210
12/20/06	06-094	3	6	7	14	25	42	75	115	120
2/15/07	07-044 dmso	15	37	58	95	135	185	230	320	260
2/23/07	07-050 dmso	4	6	9	10	21	39	76	160	160
10/26/07	07-213 dmso	2	4	5	6	8	15	27	56	100
11/5/07	07-234 dmso	3	8	9	14	31	54	105	195	340
9/25/08	08-102	11	20	38	58	86	90	170	250	270
1 day post O3										
10/16/06	06-045 dmso	20	32	68	108	156	190	250	310	320
11/9/06	06-063 dmso	32	70	100	130	200	240	330	380	380
11/16/06	06-068 dmso	30	42	80	130	180	200	240	310	360
9/19/08	08-095	8	18	30	68	108	140	150	330	340
1 day O3 + V-05-013										
10/14/06	06-044	4	6	12	18	26	42	58	80	70
10/27/06	06-053	0	1	7	5	9	18	35	56	55
11/9/06	06-064	12	18	34	60	104	110	160	230	210
12/16/06	06-090	4	10	14	26	50	44	68	108	70
9/19/08	08-096	6	15	27	40	72	115	110	195	220
1 day O3 + V-05-014										
1/5/07	07-003	0	4	10	30	54	68	88	90	80
1/24/07	07-019	5	8	14	26	58	92	120	190	140
1/24/07	07-020	6	12	24	54	90	108	160	220	230
9/26/08	08-104	2	8	9	16	31	60	76	220	250
10/17/08	08-122	2	5	12	19	33	56	74	120	160
1 day O3 + V-05-015										
12/16/06	06-091	2	4	7	8	14	20	54	144	110
12/21/06	06-095	4	8	10	10	14	38	92	170	210
12/21/06	06-096	6	6	14	44	92	150	200	300	330
Control + V-05-013										
10/26/07	07-214	3	6	10	15	26	46	75	160	140
11/5/07	07-235	4	6	7	16	26	40	56	80	120
11/9/07	07-239	2	6	12	17	28	44	60	120	110
Control + V-05-014										
1/22/07	07-016	2	8	12	22	29	50	85	175	190
1/22/07	07-017	5	12	20	34	54	100	170	230	220
2/15/07	07-045	4	5	6	11	14	18	31	60	60
2/23/07	07-051	4	6	9	11	16	24	31	60	30
11/15/07	07-243	2	4	5	8	12	22	40	46	60
Control + V-05-015										
11/15/07	07-244	1	1	4	4	8	10	24	70	75
12/19/07	07-259	2	4	6	9	15	25	60	130	65
12/19/07	07-260	8	16	25	38	62	120	170	200	200
12/20/07	07-261	6	15	23	40	58	84	130	170	180
9/25/08	08-103	4	8	12	20	25	34	52	105	110

INCREASE IN Ppi -
Acetylcholine

Treatment Date	(ug/kg) Code	1	2	4	6	10
Control						
11/6/06	06-059	2	6	10	50	190
12/20/06	06-094	0	5	11	46	160
2/23/07	07-050 dmso	3	2	4	15	66
10/26/07	07-213 dmso	0	4	5	11	66
2/16/07	07-052	0	5	9	28	95
9/25/08	08-102	22	33	46	145	330
1 day post O3						
10/16/06	06-045 dmso	5	12	18	70	165
11/9/06	06-063 dmso	18	36	160	320	340
11/16/06	06-068 dmso	32	84	130	240	390
1/5/07	07-002	6	8	8	22	116
9/19/08	08-095	26	64	124	270	520
1 day O3 + V-05-013						
10/14/06	06-044	0	16	74	260	380
11/9/06	06-064	2	5	11	50	160
12/16/06	06-090	10	10	18	88	180
9/19/08	08-096	6	26	32	150	250
1 day O3 + V-05-014						
1/5/07	07-003	2	14	44	88	200
1/24/07	07-019	20	108	170	340	460
1/24/07	07-020	10	50	68	140	270
9/26/08	08-104	50	148	260	380	520
10/17/08	08-122	9	90	45	175	320
1 day O3 + V-05-015						
12/16/06	06-091	0	0	0	26	96
12/21/06	06-095	0	0	6	50	120
12/21/06	06-096	4	52	68	130	330
Control + V-05-013						
10/26/07	07-214	1	8	23	72	200
11/5/07	07-235	0	0	0	17	90
11/7/07	07-236	2	3	9	28	130
11/9/07	07-239	2	6	13	42	190
Control + V-05-014						
1/22/07	07-016	2	12	19	64	215
1/22/07	07-017	4	20	32	80	240
2/15/07	07-045	0	1	8	16	63
11/15/07	07-243	4	7	17	64	205
Control + V-05-015						
11/15/07	07-244	3	4	3	8	28
12/19/07	07-259	2	9	62	190	360
12/19/07	07-260	7	23	140	230	400
12/20/07	07-261	10	24	54	145	325

INCREASE IN Ppi-Gallamine

Treatment Date	(mg/kg) Code	B1	0.1	0.3	1	3	10
Control							
12/20/06	06-094	17.2	1	1.32	2.25	3.45	3.78
1/19/07	07-014	18.2	1.35	1.83	4.18	5.57	4.53
1/19/07	07-015	16.4	0.95	1.21	1.94	3.75	3.89
8/18/06	06-007	16.6	1.2	1.8	3.8	5.3	4.9
12/8/06	06-086	17.5	1.4	1.5	3.2	7	5.9
11/28/06	06-072	15.7	2	3.3	6.6	8.9	12.9
9/25/08	08-102	19	0.94	1.32	2.58	5.58	7.98
1 day post O3							
10/14/06	06-043 dmso	20.4	1.27	1.37	1.27	1.27	1.53
10/16/06	06-045 dmso	32	1.22	1.58	3.06	3.75	4.79
11/16/06	06-068 dmso	22	0.97	1.18	2.09	2.97	4
1/5/07	07-002	16	0.67	1.13	2.25	2.71	2.25
9/26/08	08-105	20	0.64	0.77	0.85	1.25	1.7
1 day O3 + V-05-013							
10/14/06	06-044	21.6	0.87	0.83	0.77	0.8	0.68
11/9/06	06-064	16	1.21	1.25	2.42	3.58	3.75
12/16/06	06-090	19.2	0.94	1.11	1.53	1.81	2.4
9/19/08	08-096	17	0.86	1.81	3.22	8.1	10.1
10/17/08	08-123	20	1.09	1.77	3.7	5.83	4.09
1 day O3 + V-05-014							
1/5/07	07-003	17.6	1.14	1.66	2.65	2.57	3.03
1/24/07	07-019	18	1.07	1.52	3	4.93	6.15
1/24/07	07-020	15.6	1.24	2.48	4.19	6.67	8.42
9/26/08	08-104	19.5	0.82	1.09	2.26	2.97	2.62
10/17/08	08-122	12	1.11	2.06	5.94	9.58	13.1
1 day O3 + V-05-015							
12/16/06	06-091	9.2	0.95	1.09	2.53	3.99	4.93
12/21/06	06-095	12.8	1.3	1.62	3.02	4.74	5
12/21/06	06-096	14.4	0.88	1.11	2.41	5.05	8.89
Control + V-05-013							
10/26/07	07-214	19.4	1.2	1.5	2.4	3.3	2
11/5/07	07-235	11.6	1.3	1.9	3.4	4.7	5
11/7/07	07-236	10	1.4	1.9	3.1	5.1	5.1
11/9/07	07-239	13.8	1.1	1.7	3.6	8.9	10
Control + V-05-014							
1/22/07	07-016	15.4	1.27	1.62	2.62	4.25	5.77
1/22/07	07-017	16	0.83	1.06	1.71	2.5	2.56
2/15/07	07-045	14.8	0.99	1.03	1.64	2.25	2.7
11/15/07	07-243	12	0.79	1.14	1.4	3.54	4.2
Control + V-05-015							
11/15/07	07-244	13.8	0.82	1.09	1.33	1.16	0.92
12/19/07	07-259	12	0.86	2.38	4.94	10.4	16.4
12/20/07	07-261	18.8	1.29	2.07	3.76	5.23	5.32
9/25/08	08-103	9	0.78	0.78	0.97	1.08	0.81

FALL IN HEART RATE - Frequency

Treatment Date	Hz Code	2	4	6	8	10	12	15	20	25
Control										
11/6/06	06-059	10	30	35	35	35	40	55	60	165
12/20/06	06-094	5	10	15	25	30	45	75	210	245
2/15/07	07-044 dmso	7	20	35	45	65	95	125	225	225
2/23/07	07-050 dmso	20	30	40	65	90	105	180	200	220
10/26/07	07-213 dmso	10	25	55	85	110	130	155	185	200
11/5/07	07-234 dmso	5	15	35	75	180	195	205	225	225
9/25/08	08-102	20	25	30	35	30	30	40	175	180
1 day post O3										
10/16/06	06-045 dmso	25	100	140	175	210	220	235	250	250
11/9/06	06-063 dmso	25	95	170	190	200	210	200	200	200
11/16/06	06-068 dmso	25	45	75	105	130	155	175	200	200
9/19/08	08-095	15	40	55	85	125	155	180	245	240
1 day O3 + V-05-013										
10/14/06	06-044	0	0	2	5	5	10	10	15	25
10/27/06	06-053	3	5	10	15	20	35	50	85	95
11/9/06	06-064	5	10	20	30	50	70	140	160	175
12/16/06	06-090	10	20	35	45	60	65	105	145	215
9/19/08	08-096	15	30	50	70	100	110	130	210	210
1 day O3 + V-05-014										
1/5/07	07-003	10	40	60	75	165	185	190	210	225
1/24/07	07-019	10	15	25	35	60	95	115	185	190
1/24/07	07-020	15	35	50	70	100	125	150	175	180
9/26/08	08-104	20	40	60	80	100	120	160	185	195
10/17/08	08-122	15	30	45	65	85	110	185	200	210
1 day O3 + V-05-015										
12/16/06	06-091	5	15	25	35	50	65	90	140	190
12/21/06	06-095	10	20	55	110	150	170	200	200	200
12/21/06	06-096	25	45	60	95	125	150	205	205	205
Control + V-05-013										
10/26/07	07-214	5	15	20	45	80	110	135	160	170
11/5/07	07-235	25	50	80	115	130	150	175	210	215
11/9/07	07-239	10	45	70	95	120	165	180	190	200
Control + V-05-014										
1/22/07	07-016	10	25	40	80	105	125	135	165	185
1/22/07	07-017	10	25	35	55	70	90	120	180	225
2/15/07	07-045	5	10	20	25	30	45	180	205	215
2/23/07	07-051	15	25	45	55	80	100	110	150	155
11/15/07	07-243	10	20	40	60	100	130	155	220	220
Control + V-05-015										
11/15/07	07-244	10	20	25	35	40	50	80	130	180
12/19/07	07-259	15	35	85	105	115	125	150	175	180
12/19/07	07-260	15	30	35	45	60	75	165	185	185
12/20/07	07-261	15	35	85	105	115	125	150	175	180
9/25/08	08-103	3	15	35	45	60	80	115	175	185

FALL IN HEART RATE - Acetylcholine

Treatment Date	(ug.kg) Code	1	2	4	6	10
Control						
11/6/06	06-059	0	0	10	15	140
12/20/06	06-094	0	0	10	10	185
2/23/07	07-050 dmso	0	0	10	125	140
10/26/07	07-213 dmso	0	0	20	15	150
9/25/08	08-102	0	0	15	20	85
1 day post O3						
10/16/06	06-045 dmso	5	5	10	150	160
11/9/06	06-063 dmso	25	35	90	100	145
11/16/06	06-068 dmso	0	0	35	40	170
1/5/07	07-002	0	0	5	10	125
9/19/08	08-095	25	40	225	240	230
1 day O3 + V-05-013						
10/14/06	06-044	10	25	35	50	190
11/9/06	06-064	15	20	20	130	125
12/16/06	06-090	0	0	0	160	205
10/27/06	06-053	0	5	5	15	135
1 day O3 + V-05-014						
1/5/07	07-003	5	10	25	30	170
1/24/07	07-019	0	10	165	220	195
1/24/07	07-020	0	0	0	0	25
9/26/08	08-104	0	0	20	25	35
1 day O3 + V-05-015						
12/16/06	06-091	0	0	35	35	160
12/21/06	06-095	0	0	20	25	180
12/21/06	06-096	0	0	15	160	200
Control + V-05-013						
10/12/06	06-039	0	0	40	70	105
10/26/07	07-214	0	5	10	150	165
11/5/07	07-235	0	0	10	30	155
11/9/07	07-239	0	0	5	15	140
Control + V-05-014						
1/22/07	07-016	0	0	20	75	185
2/15/07	07-045	0	0	0	0	150
11/15/07	07-243	5	15	15	20	170
Control + V-05-015						
12/19/07	07-259	0	10	165	180	190
12/19/07	07-260	0	25	140	165	160
12/20/07	07-261	0	5	35	135	155

FALL IN HEART RATE-Gallamine

Treatment Date	(mg/kg) Code	B1	0.1	0.3	1	3	10
Control							
12/20/06	06-094	20	0.75	0.5	0.25	0.15	0
1/19/07	07-014	115	0.17	0.11	0.04	0.03	0.02
1/19/07	07-015	72	0.69	0.47	0.14	0.07	0.04
8/18/06	06-007	100	0.65	0.35	0.15	0.05	0.05
10/12/06	06-040	5	1	1	1	1	0
1 day post O3							
11/9/06	06-063 dmso	204	0.89	0.68	0.43	0.1	0.02
10/16/06	06-045 dmso	250	0.98	0.82	0.6	0.28	0.08
11/16/06	06-068 dmso	52	1.06	0.67	0.25	0.1	0
1/5/07	07-002	183	0.75	0.51	0.3	0.12	0.04
9/26/08	08-105	137	0.16	0.04	0	0	0
1 day O3 + V-05-013							
10/14/06	06-044	5	0	0	0	0	0
11/9/06	06-064	140	0.14	0.14	0.07	0.04	0
12/16/06	06-090	43	0.98	0.47	0.23	0.12	0
9/19/08	08-096	16	0.5	0.41	0	0	0
1 day O3 + V-05-014							
1/5/07	07-003	199	0.93	0.29	0.08	0.07	0.04
1/24/07	07-019	79	0.7	0.25	0.11	0.06	0.03
1/24/07	07-020	47	0.91	0.53	0.32	0.21	0.11
9/26/08	08-104	120	0.5	0.34	0.29	0.13	0.04
1 day O3 + V-05-015							
12/16/06	06-091	12	0.42	0.42	0	0	0
12/21/06	06-095	178	0.66	0.4	0.08	0.06	0.03
12/21/06	06-096	51	0.69	0.49	0.29	0.1	0
Control + V-05-013							
10/26/07	07-214	26	1.15	1.04	0.96	0.65	0.27
11/5/07	07-235	235	0.67	0.52	0.26	0.09	0.02
11/9/07	07-239	167	0.64	0.42	0.2	0.09	0.03
Control + V-05-014							
1/22/07	07-016	105	0.93	0.74	0.2	0.06	0.05
1/22/07	07-017	41	1.12	0.85	0.49	0.24	0.1
2/15/07	07-045	24	0.83	0.75	0.63	0.42	0.21
11/15/07	07-243	22	1.09	1	0.82	0.45	0.22
Control + V-05-015							
11/15/07	07-244	11	0.73	0.73	0.45	0.18	0
12/19/07	07-259	10	1.17	2	0.7	0.5	0.2
12/20/07	07-261	55	0.6	0.36	0.18	0.09	0
9/25/08	08-103	52	0.63	0.33	0.1	0.06	0

BAL Cell Numbers millions

Treatment Date	Code	Total	Macrophages	Lymphocytes	Neutrophils	Eosinophils
Control						
1/17/06		8.8	6.3	0.4	0.4	1.67
8/8/06	06-007	7.5	4.7	0.3	0.15	2.25
10/12/06	06-040	5.8	3.89	0.17	0.17	1.62
11/6/06	06-059	9.7	6.31	0.29	0.29	2.81
12/20/06	06-094	2.7	2.27	0.03	0.03	0.38
9/25/08	08-102	10.9	8.9	0.33	0.22	1.4
1 day post O3						
10/16/06	06-045 dmso	6.9	4	0.24	0.9	1.86
11/9/06	06-063 dmso	4.2	2.52	0.21	0.34	1.13
11/16/06	06-068 dmso	8.8	3.08	0.09	2.2	3.43
1/5/07	07-002	6.9	4.62	0.07	1.1	1.1
9/19/08	08-095	4	2.5	0.08	0.84	0.56
9/26/08	08-105	14.2	9.3	0.14	4	0.85
1 day O3 + V-05-013						
10/14/06	06-044	4.4	2.6	0.04	1.32	0.44
10/27/06	06-053	8	5.76	0.16	0.96	1.12
11/9/06	06-064	5.3	3.45	0.11	1.11	0.64
12/16/06	06-090	2.3	1.68	0.05	0.44	0.14
9/19/08	08-096	8.2	5.6	0.03	0.41	2.1
10/17/08	08-123	13.3	8.1	0.53	2.7	2
1 day O3 + V-05-014						
1/5/07	07-003	4.10	2.95	0.12	0.32	0.70
1/24/07	07-019	6.20	3.78	0.12	0.25	2.05
1/24/07	07-020	11.40	6.95	0.46	2.74	1.25
9/26/08	08-104	4.20	2.90	0.05	0.75	0.99
10/17/08	08-122	7.4				
1 day O3 + V-05-015						
12/16/06	06-091	8.50	6.63	0.09	1.11	0.68
12/21/06	06-095	4.10	2.87	0.08	0.29	0.90
12/21/06	06-096	4.80	3.07	0.05	0.64	1.06
Control + V-05-013						
10/12/06	06-039	3.60	2.80	0.04	0.04	0.68
10/24/07	07-212	5.10	3.90	0.03	0.20	1.00
10/26/07	07-214	5.40	4.30	0.05	0.32	0.76
11/5/07	07-235	10.20	8.67	0.20	0.10	1.12
11/7/07	07-236	9.10	8.30	0.18	0.09	0.55
11/9/07	07-239	8.60	5.30	1.03	0.09	2.20
Control + V-05-014						
1/22/07	07-016	6.90	5.73	0.07	0.14	0.97
1/22/07	07-017	9.20	7.45	0.03	0.28	1.38
2/15/07	07-045	12.40	10.80	0.12	0.25	1.24
2/23/07	07-051	5.80	4.58	0.02	0.06	1.16
11/15/07	07-243	13.00	10.10	1.17	0.13	1.56
Control + V-05-015						
11/15/07	07-244	9.20	6.99	0.18	0.37	1.66
12/19/07	07-260	10.70	9.42	0.21	0.21	0.86
12/20/07	07-261	8.1	5.35	0.24	0.16	2.35
9/25/08	08-103	5.2	4.2	0.5	0.05	0.49

PERIPHERAL BLOOD Cell Numbers millions

Treatment Date	Code	Total	Monocytes	Lymphocytes	Neutrophils	Eosinophils
Control						
12/21/05		5.2	0.83	1.14	2.91	0.31
1/17/06		10.3	1.75	2.9	5.6	0.1
10/12/06	06-040	3.9	0.86	0.78	2.22	0.04
11/6/06	06-059	7	1.05	3.78	2.1	0.07
12/20/06	06-094	9.5	1.71	2.85	4.94	0.1
9/25/08	08-102	6.4	1.1	1.9	3.1	0.32
1 day post O3						
10/16/06	06-045 dmso	2.2	0.75	1.19	0.24	0.02
11/9/06	06-063 dmso	12.3	2.34	3.81	5.78	0.37
11/16/06	06-068 dmso	16.2	4.05	0.81	11.18	0.16
1/5/07	07-002	3.86	0.69	1.81	1.31	0.04
9/19/08	08-095	4.5	1.98	1.62	0.86	0.56
9/26/08	08-105	4.4	0.75	2	1.5	0.09
1 day O3 + V-05-013						
10/14/06	06-044	6.9	1.24	2.2	3.38	0.07
10/27/06	06-053	3.4	0.99	2	0.37	0.03
11/9/06	06-064	12.8	1.92	2.82	7.94	0.13
12/16/06	06-090	11.7	2.22	4.09	5.27	0.12
9/19/08	08-096	2.6	1.1	0.96	0.44	0.1
10/17/08	08-123	15.2	2.6	4.7	7.8	0.15
1 day O3 + V-05-014						
1/5/07	07-003	3.70	0.81	2.07	0.70	0.11
1/24/07	07-019	7.60	0.68	4.56	2.28	0.08
9/26/08	08-104	4.20	1.40	1.80	0.80	0.13
10/17/08	08-122	3.4	1.6	0.9	0.68	0.17
1 day O3 + V-05-015						
12/16/06	06-091	6.40	0.90	3.33	2.11	0.06
12/21/06	06-095	8.10	2.19	2.43	3.40	0.08
12/21/06	06-096	10.40	1.46	3.43	5.20	0.42
Control + V-05-013						
10/12/06	06-039	3.60	1.39	1.09	0.76	0.03
10/24/07	07-212	3.00	0.48	1.80	0.60	0.09
10/26/07	07-214	5.60	0.78	2.60	2.10	0.06
11/5/07	07-235	2.80	0.84	1.46	0.48	0.03
11/7/07	07-236	12.20	0.85	3.66	7.44	0.12
11/9/07	07-239	11.60	2.10	3.02	6.40	0.12
Control + V-05-014						
1/22/07	07-016	3.50	0.60	1.26	1.51	0.10
2/15/07	07-045	4.60	1.00	2.39	1.20	0.05
11/15/07	07-243	7.60	1.29	3.27	2.89	0.15
Control + V-05-015						
11/15/07	07-244	8.40	2.60	2.94	2.77	0.08
12/19/07	07-259	9.7	1.84	5.82	1.94	0.1
12/19/07	07-260	10.00	1.80	2.90	5.00	0.20
12/20/07	07-261	7.60	1.14	3.12	3.19	0.15
9/25/08	08-103	12.20	2.70	1.70	7.20	0.49

Goblet Cells/mm airway epithelium

Treatment Date	Code	GC/mm
Control		
11/6/06	06-059	22.8
12/20/06	06-094	14.8
2/15/07	07-044 dmso	25.3
2/23/07	07-050 dmso	18.9
10/26/07	07-213 dmso	31.0
11/5/07	07-234 dmso	29.4
1 day post O3		
10/16/06	06-045 dmso	4.9
11/9/06	06-063 dmso	0.9
11/16/06	06-068 dmso	17.1
8/17/06	06-011	3.7
10/14/06	06-043 dmso	7.0
1/5/07	07-002	2.3
1 day O3 + V-05-013		
10/14/06	06-044	10.5
10/27/06	06-053	5.6
11/9/06	06-064	2.2
12/16/06	06-090	1.3
9/19/08	08-096	1.7
1 day O3 + V-05-014		
1/5/07	07-003	8.5
1/24/07	07-019	2.2
1/24/07	07-020	4.8
1 day O3 + V-05-015		
12/16/06	06-091	1.7
12/21/06	06-095	1.8
12/21/06	06-096	6.0
Control + V-05-013		
10/26/07	07-214	23.19
11/9/07	07-239	16.5
11/7/07	07-236	10.6
10/12/06	06-039	18.2
Control + V-05-014		
1/22/07	07-017	16.6
2/15/07	07-045	9.1
2/23/07	07-051	14.4
11/15/07	07-243	33.4
Control + V-05-015		
11/15/07	07-244	17.3
12/19/07	07-259	15.2
12/19/07	07-260	7.3
12/20/07	07-261	9.1

C. Raw data for IL-1 receptor antagonist paper (Chapter IV)

INCREASE IN Ppi - Frequency

Treatment Date	Hz Code	2	4	6	8	10	12	15	20	25
Control										
4/13/05		2	5	5	6	10	10	25	68	105
4/20/05	C2	4	11	13	21	28	30	33	45	55
4/25/05		9	6	24	31	42	56	66	95	180
4/27/05		9	18	28	43	48	64	85	90	150
8/11/05	C3	1	2	3	6	18	38	105	155	175
11/2/05	C4	1	4	4	9	17	39	86	115	180
7/5/07		5	11	14	24	38	56	90	140	155
3 Day Post O3										
4/15/05	O1	20	60	100	135	160	240	290	360	
4/22/05		3	7	16	25	29	64	110	170	230
5/5/05		36	84	130	210	260	280	360	440	480
5/6/05		8	21	44	66	85	120	150	240	380
6/3/05 green	O4	6	16	27	46	80	125	195	280	420
3 day O3+IL-1Ra 10mg/kg										
5/20/05	OA1	3	8	15	30	52	85	140	220	260
5/27/05 (blue)	OA2	16	32	58	80	100	115	115	95	80
5/27/05 (orange)	OA3	15	68	74	130	160	220	240	300	380
6/3/05 purple		11	32	58	85	105	170	230	300	400
3 day O3+IL-1Ra 30mg/kg										
6/13/05 (blue)	OA4	9	16	23	30	42	54	66	70	120
6/13/05 (orange)	OA5	23	46	55	70	75	65	85	120	155
7/22/05 (blue)	OA6	3	8	17	22	56	60	135	170	190
7/22/05 (black)	OA7	6	20	29	54	85	95	110	140	180
Control+IL-1Ra 30mg/kg										
7/19/05 (blue)		26	60	110	150	195	200	290	390	480
7/19/05 (purple)	CA3	0	1	2	2	4	6	13	28	42
8/5/05 (pink)	CA5	0	1	3	6	4	3	10	11	10
11/9/05 (green)	CA7	3	5	12	22	26	32	48	80	135
11/9/05 (red)	CA8	9	25	42	70	100	165	210	310	380
11/13/06	06-65	4	9	14	23	38	62	100	170	220
Control+Vehicle										
8/31/05	CV1	7	19	19	39	56	65	85	145	140
9/15/05	CV2	21	39	46	80	105	160	160	170	120
7/5/07 (07-121)		5	12	18	24	38	54	85	130	105
3 day O3+Vehicle										
9/12/05 (blue)	OV	21	52	106	195	230	270	340	460	460
9/12/05 (green)	OV1	6	24	36	48	105	160	240	340	360
12/9/05	OV2	6	17	32	52	85	140	180	190	420

INCREASE IN Ppi –
Acetylcholine

Treatment Date	(ug/kg) Code	1	2	4	6	10
Control						
4/13/05	C2	2	4	10	40	250
4/20/05		13	29	60	110	310
4/25/05		11	21	30	96	250
4/27/05	C3	4	12	14	90	250
8/11/05	C4	0	2	3	20	90
3 Day Post O3						
4/15/05	O1	40	60	70	210	
4/22/05		10	14	14	90	220
5/5/05		29	130	150	310	520
5/6/05		7	13	31	120	240
6/3/05 green	O4	12	19	28	85	280
3 day O3+IL-1Ra 10mg/kg						
5/20/05	OA1	16	39	245	340	580
5/27/05 (blue)	OA2	8	12	15	48	180
5/27/05 (orange)	OA3	8	30	30	200	460
6/3/05 purple		28	92	225	380	250
3 day O3+IL-1Ra 30mg/kg						
6/13/05 (blue)	OA4	9	25	100	235	460
6/13/05 (orange)	OA5	18	54	95	250	430
7/22/05 (blue)	OA6	10	17	52	205	400
7/22/05 (black)	OA7	13	45	65	200	500
Control+IL-1Ra 30mg/kg						
7/19/05 (blue)		42	65	275	380	520
7/19/05 (purple)	CA3	2	7	50	115	155
8/5/05 (pink)	CA5	7	14	46	126	320
11/9/05 (green)	CA7	6	35	68	195	350
11/9/05 (red)	CA8	55	190	300	440	580
11/13/06	06-65	16	14	22	52	185
Control+Vehicle						
8/31/05	CV1	3	17	30	106	250
9/15/05	CV2	12	42	54	150	330
7/5/07	07-121	6	13	23	76	185
8/10/07	07-142	5	10	22	58	145
3 day O3+Vehicle						
9/12/05 (blue)	OV	18	25	110	205	340
9/12/05 (green)	OV1	7	18	32	85	160
12/9/05	OV2	7	32	65	210	380

INCREASE IN Ppi – Frequency

Treatment Date	Hz Code	2	4	6	8	10	12	15	20	25
Control										
3/20/06	C6	1	2	3	4	6	9	15	27	50
3/29/06	C7	14	23	44	60	95	125	180	200	330
8/4/06	06-006	2	5	10	20	32	40	45	70	45
8/8/06	06-007	6	12	21	28	44	70	100	155	150
8/17/06	06-010	1	3	11	14	26	40	45	80	70
1 Day Post O3										
11/23/05	1O	4	5	10	34	48	62	72	140	200
12/7/05	1O1	1	6	17	90	180	220	260	340	340
12/20/05		16	36	66	140	160	180	210	320	340
3/15/06	1O3	4	6	8	22	34	52	104	130	160
8/17/06	06-011	14	50	88	150	220	270	320	420	400
9/28/06	06-024	6	32	70	84	120	160	200	280	350
1 day O3+IL-1Ra 30mg/kg										
2/24/06	1OA1	2	4	10	12	32	80	120	180	210
3/9/06	1OA2	4	8	10	32	64	100	140	160	200
10/2/06	06-026	2	6	18	30	64	92	150	240	240
10/2/06	06-027	8	32	72	136	190	240	290	360	420

INCREASE IN Ppi - Acetylcholine

Treatment Date	(ug/kg) Code	1	2	4	6	10
Control						
3/20/06	C6	1	4	5	16	70
3/29/06	C7	7	47	110	275	360
8/4/06	06-006	0	9	30	110	180
8/8/06	06-007	0	3	8	32	110
8/17/06	06-010	3	10	22	110	240
1 Day Post O3						
11/23/05	1O	4	14	16	32	192
12/7/05	1O1	6	6	22	74	300
12/20/05		12	30	120	220	340
8/17/06	06-011	0	12	55	135	280
9/28/06	06-024	22	100	220	440	560
1 day O3+IL-1Ra 30mg/kg						
2/24/06	1OA1	4	14	52	132	260
3/9/06	1OA2	4	22	44	164	240
10/2/06	06-026	18	120	360	340	680
10/2/06	06-027	8	46	96	270	440

FALL IN HEART RATE - Frequency

Treatment Date	Hz Code	2	4	6	8	10	12	15	20	25
Control										
4/20/05	C2	5	5	10	10	11	13	15	25	125
4/25/05		7	10	12	15	23	30	38	55	205
4/27/05		0	3	5	7	10	10	140	160	175
8/11/05	C3	0	0	0	0	5	5	5	5	165
11/2/05	C4	10	25	40	95	145	175	195	215	215
3/20/06		10	35	50	60	80	100	160	165	180
8/17/06	06-010	0	5	10	15	25	30	50	95	100
3 Day Post O3										
4/15/05	O1	10	20	32	50	62	95	215	240	255
4/22/05		15	25	33	50	65	125	238	238	238
5/5/05		5	10	10	10	15	20	35	45	180
5/6/05		5	10	15	26	55	75	95	130	265
6/3/05 green	O4	15	40	55	65	80	115	115	115	115
8/18/05 black	O3	5	15	25	40	60	85	130	190	220
3 day O3+IL-1Ra 10mg/kg										
5/20/05	OA1	10	10	20	35	80	90	115	115	115
5/27/05 blue	OA2	5	15	45	70	80	125	160	190	225
5/27/05 orange	OA3	5	15	15	20	25	45	75	130	200
6/3/05 purple		20	35	60	100	130	160	185	245	245
3 day O3+IL-1Ra 30mg/kg										
6/13/05 (blue)	OA4	5	10	10	20	20	25	25		
6/13/05 (orange)	OA5	5	15	30	55	70	90	115	150	185
7/22/05 (blue)	OA6	5	7	1	10	20	15	25	135	160
7/22/05 (black)	OA7	10	20	35	55	65	85	110	145	175
Control+IL-1Ra 30mg/kg										
7/19/05 (blue)		5	20	40	60	80	60	105	135	160
8/5/05 (pink)	CA5	0	5	10	10	15	10	10	5	5
11/9/05 (green)	CA7	5	10	10	10	15	20	35	65	175
11/9/05 (red)	CA8	5	15	25	40	50	85	120	200	200
11/13/06	06-065	5	15	25	50	85	105	130	175	180
Control+Vehicle										
8/31/05	CV1	15	40	60	85	115	135	155	185	210
9/15/05	CV2	10	25	40	65	95	110	145	180	225
7/5/07	07-121	10	20	25	40	50	75	115	180	195
8/10/07	07-142	10	20	35	60	90	110	140	185	215
3 day O3+Vehicle										
9/12/05 (blue)	OV	5	10	15	20	30	30	30	35	155
9/12/05 (green)	OV1	5	10	10	20	115	125	140	160	185
12/9/05	OV2	5	20	40	65	85	120	155	230	230

FALL IN HEART RATE -
Acetylcholine

Treatment Date	(ug/kg) Code	1	2	4	6	10
Control						
4/20/05		60	70	70	70	110
4/25/05		0	0	15	20	135
4/27/05	C3	0	5	15	115	130
8/11/05	C4	10	10	40	155	165
11/2/05		15	175	195	200	235
7/5/07	07-120	0	0	10	10	140
3 Day Post O3						
4/15/05	O1	100	117	90	150	170
4/22/05		0	0	10	20	45
5/5/05		0	5	45	175	280
5/6/05		5	5	15	25	160
6/3/05 green	O4	0	20	195	195	195
3 day O3+IL-1Ra 10mg/kg						
5/20/05	OA1	25	160	220	240	240
5/27/05 (blue)	OA2	0	10	25	35	190
5/27/05 (orange)	OA3	0	5	30	140	175
6/3/05 purple		0	5	25	160	175
3 day O3+IL-1Ra 30mg/kg						
6/13/05 (blue)	OA4	0	25	115	115	115
6/13/05 (orange)	OA5	15	15	15	155	150
7/22/05 (blue)	OA6	15	10	150	170	170
7/22/05 (black)	OA7	5	10	180	195	210
Control+IL-1Ra 30mg/kg						
7/19/05 (blue)		15	25	25	145	160
8/5/05 (pink)	CA5	0	25	35	180	220
11/9/05 (red)	CA8	0	35	150	170	190
11/13/06	06-065	0	0	0	130	165
Control+Vehicle						
8/31/05	CV1	10	15	30	150	160
9/15/05	CV2	0	0	130	145	165
7/5/07	07-121	0	0	25	35	50
8/10/07	07-142	25	40	135	170	160
3 day O3+Vehicle						
9/12/05 (blue)	OV	0	10	125	130	135
9/12/05 (green)	OV1	25	70	25	135	140
12/9/05	OV2	15	35	175	235	225

FALL IN HEART RATE - Frequency

Treatment Date	Hz Code	2	4	6	8	10	12	15	20	25
Control										
3/20/06	C6	10	35	50	60	80	100	160	165	180
3/29/06	C7	20	40	75	105	130	150	175	175	175
8/8/06	06-007	5	10	15	20	45	90	110	230	250
8/17/06	06-010	0	5	10	15	25	30	50	95	100
11/6/06	06-059	10	30	35	35	35	40	55	60	165
12/20/06	06-094	5	10	15	25	30	45	75	210	245
2/15/07	07-044	7	20	35	45	65	95	125	225	225
1 Day Post O3										
11/23/05	1O	10	10	20	35	40	55	65	200	210
12/7/05	1O1	10	25	30	60	95	120	230	245	245
12/20/05		30	55	90	115	135	200	200	200	200
3/15/06	1O3	5	20	30	45	50	65	180	190	210
8/17/06	06-011	20	40	40	50	70	85	155	165	160
9/28/06	06-024	0	0	0	0	5	10	15	35	190
1 day O3+IL-1Ra 30mg/kg										
2/24/06	1OA1	5	30	50	50	95	115	130	145	160
3/9/06	1OA2	10	20	35	35	50	170	180	200	215
10/2/06	06-027	5	10	10	10	15	15	20	35	195

FALL IN HEART RATE - Acetylcholine

Treatment Date	(ug/kg) Code	1	2	4	6	10
Control						
3/20/06	C6	0	0	10	140	145
3/29/06	C7	10	10	0	125	130
8/4/06	06-006	0	0	10	15	130
8/17/06	06-010	10	15	85	100	105
7/5/07	07-120	0	0	10	10	140
1/17/06		5	5	130	165	150
10/12/06	06-040	20	25	20	130	150
1 Day Post O3						
11/23/05	1O	15	25	155	205	190
12/7/05	1O1	0	20	210	210	210
12/20/05		0	110	200	200	200
8/17/06	06-011	5	5	10	20	125
9/28/06	06-024	0	5	15	65	240
1 day O3+IL-1Ra 30mg/kg						
2/24/06	1OA1	0	5	10	135	180
3/9/06	1OA2	0	5	200	200	200
10/2/06	06-026	10	15	30	30	180
10/2/06	06-027	15	20	35	155	205

BAL Cell Numbers millions

Treatment **Date**	**macrophages**	**lymphocytes**	**eosinophils**	**neutrophils**	**total**
Control					
4/13/05	6.74	0.25	0.95	0.33	8.27
4/20/05	4.82	0.88	1.12	0.21	7.03
4/25/05	6.21	0.62	2.29	0.48	9.55
4/27/05	4.86	1.00	2.00	0.50	8.38
8/11/05	6.92	0.27	1.68	0.23	9.10
11/2/05	6.37	0.10	3.00	0.40	9.80
7/5/07	3.74	0.04	0.38	0.02	4.20
3 Day Post O3					
4/15/05	9.79	1.00	5.15	0.66	16.60
4/22/05	13.10	2.29	7.62	2.41	25.40
5/5/05	12.80	2.00	3.20	4.50	22.60
5/6/05	14.10	1.30	7.00	3.70	26.10
6/3/05	12.20	1.08	5.40	2.92	21.60
3 day O3+IL-1Ra 10mg/kg					
5/20/05	3.79	0.23	2.61	1.26	7.90
5/27/05 blue	14.10	1.34	6.59	2.32	24.40
5/27/05 orange	11.90	0.71	2.74	2.39	17.70
3 day O3+IL-1Ra 30mg/kg					
6/13/05 (blue)	22.90	2.56	5.64	3.08	34.20
6/13/05 (orange)	13.30	1.34	3.92	3.80	22.40
7/22/05 (blue)	5.62	0.64	3.82	0.53	10.60
7/22/05 (black)	10.40	1.04	8.22	1.25	20.80
Control+IL-1Ra 30mg/kg					
7/19/05 (blue)	4.25	0.30	1.24	0.90	6.70
7/19/05 (purple)	5.67	0.47	3.26	1.10	10.50
8/5/05 (pink)	7.14	1.34	2.75	0.47	11.70
11/9/05 (green)	5.70	0.56	1.10	0.14	7.50
11/9/05	4.05	0.30	3.00	0.15	7.50
06-065	2.56	0.14	0.70	0.07	3.50
Control+Vehicle					
8/31/05	10.90	0.76	1.88	0.83	13.90
9/15/05	5.70	1.01	2.02	0.46	9.20
7/5/07 (07-121)	3.90	0.05	1.35	0.02	5.40
8/10/07 (07-142)	5.35	0.49	2.03	0.16	8.10
3 day O3+Vehicle					
9/12/05 (blue)	13.20	0.93	8.00	2.70	23.20
9/12/05 (gren)	12.30	0.69	5.70	1.39	19.80
12/9/05	10.80	1.08	3.24	3.06	18.00

Blood Cell Numbers millions

Treatment **Date**	**monocytes**	**lymphocytes**	**eosinophils**	**neutrophils**	**total**
Control					
11/2/05	2.10	1.70	0.09	1.70	5.60
7/5/07 (07-120)	2.00	1.16	0.09	0.99	4.30
8/10/07 (07-141)	1.68	2.34	0.76	2.85	7.30
3 Day Post O3					
1/26/06 (blue)	0.99	2.00	0.06	2.78	5.80
4/10/06	1.19	2.38	0.04	0.50	4.10
4/14/06	1.70	3.70	0.15	1.90	7.37
Control+IL-1Ra 30mg/kg					
11/9/05 (green)	2.69	2.1	0	0.45	4.9
11/9/05 (red)	4.08	2.16	0.16	1.52	8
7/19/05 (blue)	0.5	1	0.11	0.59	2.2
7/19/05 (purple)	1.32	2.18	0.29	1.01	4.8
8/5/05 (pink)	1.19	1.36	0.41	2.78	5.8
11/13/06 (06-065)	1.58	3.67	0.2	4.36	9.9
3 day O3+IL-1Ra 30mg/kg					
6/13/05	0.31	1.43	0.31	1.1	3.1
6/13/05 (orange)	0.3	0.48	0.28	1.5	2.5
7/22/05 (blue)	1.43	0.76	0.25	1.76	4.2
Control+Vehicle					
8/25/05	0.81	0.83	0.33	1.3	3.13
9/15/05	2.1	1.5	0.1	0.84	4.4
7/5/07 (07-121)	0.83	4.57	0.17	2.74	8.3
8/10/07 (07-142)	1.34	3	0.55	3	7.9
3 day O3+Vehicle					
9/12/05 (blue)	1.4	0.64	0.54	2.6	4.9
9/12/05 (gren)	2.3	0.69	0.2	0.97	5.1
12/19/05	2.4	2.2	1.2	1.6	7.4

BAL Cell Numbers millions

Treatment **Date**	**Total**	**eosinophils**	**macrophages**	**neutrophils**	**lymphocytes**
Control	10.3	0.94	8.86	0.04	0.41
3/20/06	17.5	2.1	8.2	1.2	5.8
3/29/06	5.2	0.68	4	0.36	0.16
8/4/06	7.5	2.25	4.7	0.15	0.3
8/8/06	8.7	0.5	7.4	0.5	0.3
8/17/06					
1 Day Post O3					
11/23/05	4	0.8	1.52	1.48	0.2
12/7/05	8.5	1.45	3.91	2.89	0.34
12/20/05	17.2	2.24	8.08	6.02	0.86
3/15/06	8.1	1.62	3.97	2.43	0.08
8/17/06	12	2.76	7.44	1.44	0.36
9/28/06	4.8	1.25	2.4	1.06	0.14
1 day O3+IL-1Ra 30mg/kg					
2/24/06	14.2	2.27	9.66	1.85	0.43
3/9/06	9.6	2.4	4.9	1.92	0.38
10/2/06	3.9	0.78	1.79	1.21	0.08
10/2/06	6.1	0.79	4.15	1.1	0.04

Blood Cell Numbers millions

Treatment **Date**	**Total**	**eosinophils**	**monocytes**	**neutrophils**	**lymphocytes**
Control					
3/20/06	3.4	0.1	0.54	1.77	0.95
3/29/06	4.5	0.09	2	0.86	1.5
8/4/06	4.2	0.08	0.76	1.43	1.93
8/17/06	5	0.02	2.1	1.5	1.4
1 Day Post O3					
11/23/05	5.68	0.28	2.31	1.42	1.7
12/7/05	4.4	0.04	0.7	2.6	1
12/20/05	4.6	0.05	0.55	3.36	0.64
3/15/06	9.1	0.09	2.46	4	2.55
8/17/06	2.2	0.02	0.5	0.2	1.5
9/28/06	5	0.05	1.75	0.85	2.4
1 day O3+IL-1Ra 30mg/kg					
2/24/06	3.9	0.16	2.22	0.51	0.98
3/9/06	3.1	0.03	1.05	1.02	0.99
10/2/06	4	0.12	1.28	1.52	1.08
10/2/06	7.4	0.07	1.7	3.55	1.99