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MODULATION

OF

OZONE-INDUCED AIRWAY HYPERRESPONSIVENESS

BY

CYCLOOXYGENASE AND NITRIC OXIDE SYNTHASE

Written By

EDWARD GEORGE (TED) WATSON

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctorate of Philosophy

McMaster University

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CYCLOOXYGENASE AND NITRIC OXIDE SYNTHASE IN THE AIRWAY

Descriptive Note

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Hamilton, Ontario

TITLE: Modulation of Ozone-Induced Airway Hyperresponsiveness by Cyclooxygenase and Nitric Oxide Synthase.

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Abstract

A large number of studies have identified that bronchoconstricting agents have increased potency and effect following inhaled ozone, but the contribution of bronchodilatory mediators to attenuation of the airway hyperresponsiveness observed following inhaled ozone remains unclear. The purpose of this investigation was to identify the contribution of nitric oxide synthase (NOS) and cyclooxygenase (COX) to the regulation of ozone-induced airway hyperresponsiveness. These mediators are derived largely from the airway epithelium and ozone exposure is likely, through epithelial damage, to alter the effect of NOS and COX in the airway. Using an ozone exposed guinea pig model of airway hyperresponsiveness, the *in vivo* responses to inhaled histamine were compared to responses in sham treated animals. Administration of the NOS inhibitor L-NAME (5120 μg/mL) increased the histamine sensitivity after ozone treatment. The non-selective COX inhibitor indomethacin (10 mg/kg) also increased the histamine sensitivity after ozone treatment. The COX-2 selective inhibitor DFU (1 or 10 mg/kg) caused a 2-fold leftward shift after ozone exposure, similar to that observed with indomethacin. The thromboxane antagonist, SQ 29,548 (1 mg/kg i.p.) attenuated the histamine responsiveness in a time dependent manner. The combination of COX and NOS inhibition produced a histamine-induced airway hyperresponsiveness greater than that observed with either NOS or COX inhibition alone. Pre-administration of dexamethasone inhibited the ozone-induced histamine hyperresponsiveness at all time points. Biochemical measurements of NOS activity identified an increase in NOS

enzyme activity following ozone. Bronchoalveolar lavage revealed an inflammatory cell profile that did not correlate to the *in vivo* airway hyperresponsiveness. *In vitro* tissue bath experiments identified the presence and biological activity of NOS in the guinea pig trachea. Liquid chromatographic detection of nitric oxide metabolites and Western blot detection of COX-2 and NOS isoforms did not provide reliable data. This thesis demonstrates that histamine airway responsiveness following ozone changes rapidly and that both NOS and COX are upregulated a few hours after ozone exposure and, through functional antagonism, modulate ozone-induced histamine airway hyperresponsiveness.

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List of Abbreviations

5-HPETE – 5-L-hydroperoxy-5,8,10,14-eicosatetraenoic acid
5-LO – 5-lipoxygenase
AHR – airway hyperresponsiveness
ANOVA – analysis of variance
ASM – airway smooth muscle
β-NADPH - β-nicotinamide adenine dinucleotide phosphate (reduced form)
BAL – bronchoalveolar lavage
BH_4 - 6R-5,6,7,8-tetrahydrobiopterin
Ca^{2+} - calcium ion
CaM - calmodulin
CD – cluster of differentiation
cGMP - cyclic-3,5-guanine monophosphate
COX - cyclooxygenase
DFU - 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furan one
DMSO – dimethyl sulfoxide
DPM – disintegrations per minute
EAR – early asthmatic response
eNOS - endothelial (calcium-dependent) nitric oxide synthase
ECP – eosinophil cationic protein
EIA – exercise-induced asthma
EPO – eosinophil peroxidase
FEV – forced expiratory volume
FLAP – five lipoxygenase activating protein
GM-CSF – granulocyte-macrophage colony stimulating factor
HIA – hyperventilation-induced asthma
HPLC – high performance liquid chromatography
ICAM – intracellular adhesion molecule
IFN-γ - interferon-γ
IgG - immunoglobulin G
IL - interleukin
iNOS - inducible (calcium-independent) nitric oxide synthase
IP_3 - inositol triphosphate
LAR – late asthmatic response
L-NAME - L-nitroarginine methyl ester
L-NMMA - L-Nw-monomethylarginine
L-NNA - L-N^{ω}-nitroarginine
L-NOARG – see L-NNA
LPS - bacterial lipopolysaccharide
LT - leukotriene
MBP - major basic protein
MHC – major histocompatibility complex
NADH - nicotinamide adenine dinucleotide (reduced form)
NANC - non-adrenergic, non-cholinergic
NaNP – sodium nitroprusside
NBT - nitroblue tetrazolium
NKA - neurokinin A
NK1 - neurokinin 1
NK2 - neurokinin 2
nNOS - neural (calcium-dependent) nitric oxide synthase
NO - nitric oxide
NOS - nitric oxide synthase
O_3 - ozone
OA - ovalbumin
P_{enh} - enhanced Pause
PAF – platelet activating factor
PAGE - polyacrilamide gel electrophoresis
PBS – phosphate-buffered saline
PC_5 – provocation concentration elevating P_{enh} to a value of 5
PDE - phosphodiesterase
PEP – peak expiratory pressure
PG - prostaglandin

PIP – peak inspiratory pressure
PKA – protein kinase A
PKC – protein kinase C
PLC – phospholipase C
PMN - polymorphonuclear
PMSF - phenylmethylsulphonyl fluoride
PPD - para-phenylenediamine
ROS – reactive oxygen species
SDS - sodium dodecyl sulphate
SMB - sucrose MOPS buffer
SNP - sodium nitroprusside
SP – substance P
TBS - tris buffered saline
TDI – toluene diisocyanate
Te – time for expiration
TNF-α - tumor necrosis factor-α
Tr – time for relaxation
TTBS - Tween-20 containing tris buffered saline
TX - thromboxane
UV - ultraviolet
VCAM – vascular adhesion molecule
VIP – vasoactive intestinal polypeptide
VLA – very late antigen
WBP – whole body plethysmography

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1. INTRODUCTION

Asthma

Asthma is a chronic disease that is characterized by reversible narrowing of the airways and periodic, acute exacerbations (McFadden, Jr. and Gilbert, 1992). Although extensively studied, asthma remains to be accurately defined. The definition of asthma must take into consideration the symptoms of the disease (including wheezing, dypsnea and cough (McFadden, Jr. and Gilbert, 1992)), physiological abnormalities, histological changes and etiology. An International Panel was assembled in an attempt to define human asthma (NHLBI/WHO Workshop Report, 1993) and concluded that asthma is a lung disease with the following characteristics: i) widespread but variable airflow obstruction that is at least partly reversible either spontaneously or with treatment, ii) chronic airway inflammation, iii) an increase in airway responsiveness to a variety of stimuli.

Early and Late Asthmatic Responses

Upon exposure of the asthmatic lungs to inhaled inflammatory stimuli, such as allergens, there are two distinct types of bronchoconstriction that occur sequentially in many patients, the early asthmatic response (EAR) and the late asthmatic response (LAR) (Figure 1). The identification of the LAR was first documented by Herxheimer

(1952) and has since created a great deal of attention on asthma, its mechanisms and its pathophysiology.

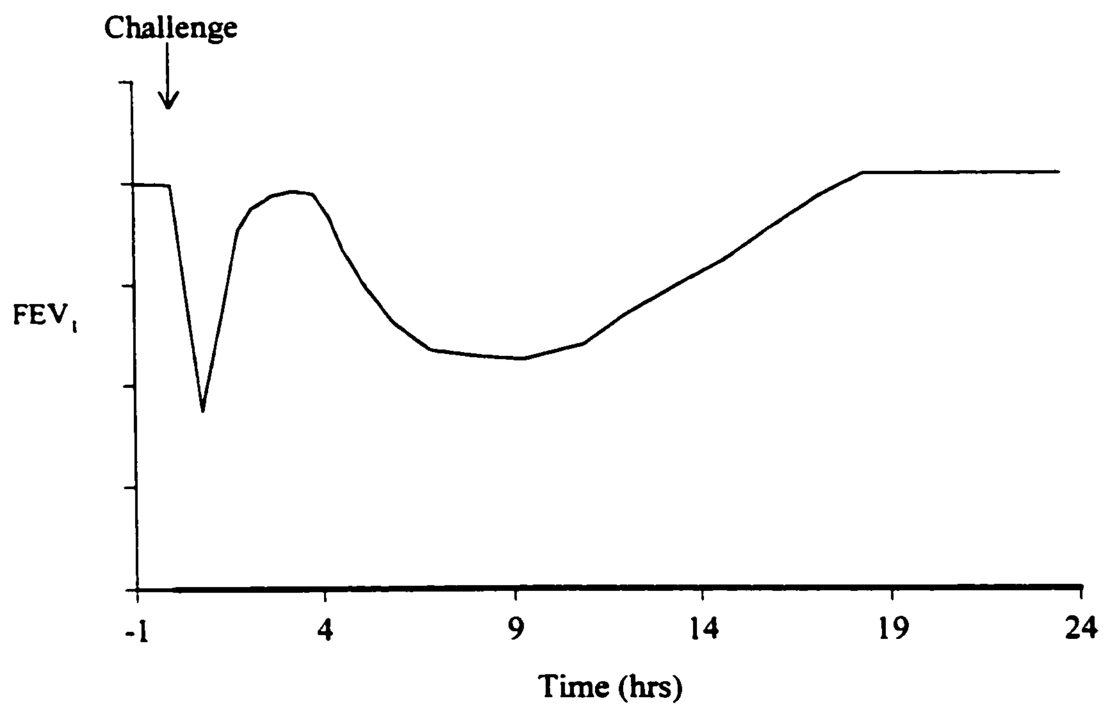


Figure 1: Representative graph showing the changes in FEV_1 for the EAR and LAR.

Early Asthmatic Response

The EAR occurs within 5 to 10 minutes following allergen challenge with a gradual recovery over the following 2 hours post-challenge. This bronchoconstriction is predominately mediated by mast cells via release of inflammatory mediators. The activation of mast cells is dependent upon the cross-linking of the cell surface receptor IgE, found on mast cells of the epithelial surface and lung connective tissue. Allergen challenge or anti-IgE produces a cross-linking of the IgE receptors (Kimura et al., 1974), stimulating the release of histamine (Lowman et al., 1988a; Siraganian and Hazard, 1979), leukotrienes (MacDonald et al., 1989), prostaglandins (Pele et al., 1989) and several chemotactic factors (Plaut et al., 1989; Wodnar-Filipowicz et al., 1989), which contribute to the development of the late asthmatic response. The EAR is reduced by histamine (H_1) antagonists (Roquet et al., 1997a) and by leukotriene antagonists (Diamant et al., 1999; Jonsson and Dahlen, 1994).

Late Asthmatic Response

The late asthmatic response (LAR) is identified by bronchoconstriction occurring approximately 2 to 4 hours following the initial challenge which may resolve within 24 hours but has taken several days in some instances (Pepys and Hutchcroft, 1975). Although mast cell mediators do not play a direct role in generation of the LAR, it is clear that the inflammatory mediators IL-1, IL-3, IL-4, IL-5, IL-6, IL-13, TNF-α, IFN-γ and granulocyte-macrophage colony stimulating factor (GM-CSF) released during

the EAR initiate an infiltration of immune cells to the lung tissues (Kaltreider, 1991; Terr, 1997). Once released, the chemotactic cytokines produce an influx of eosinophils (Gleich and Adolphson, 1991), neutrophils (Abramson et al., 1991), basophils (Pearce, 1988), macrophages (Hogg and Doerschuk, 1995; Poulter et al., 1994) and T-lymphocytes (Kaltreider, 1991) into the lung tissue. It is this immune cell infiltrate that is now characteristically described as a hallmark of asthma.

Airway Hyperresponsiveness

Definition

Airway responsiveness refers to the capacity of the airways to constrict to chemical or physical stimuli (Cockcroft and Hargreave, 1991). Airway hyperresponsiveness (AHR) refers to the exaggerated bronchoconstrictor response of the airways to those same stimuli (Cockcroft and Hargreave, 1991). Airways hyperresponsiveness is characterized both clinically and in animals by a leftward shift in the concentration-response curves as well as an increased maximum response to a particular bronchoconstrictor, when compared to baseline (Sterk et al., 1985; Woolcock et al., 1984). The degree of shift that defines airway hyperresponsiveness is not clear as airway responsiveness has a unimodal distribution in the general population, and encompasses asthmatics after challenge (Cockcroft et al., 1983), but empirical values for the degree of shift classifying an individual as having airway hyperresponsiveness have been established (Cockcroft et al., 1983; Dolovich et al., 1992).

Clinical Assessment of Airway Hyperresponsiveness

Clinical assessment of asthma and its associated airway hyperresponsiveness is accomplished with standardized methods of provocation (Cockcroft and Hargreave, 1991). Pulmonary function assessment is typically measured using the volume of air expired in one second during forced expiration (FEV_1). Airway hyperresponsiveness is determined by the concentration of provocation mediator required to reduce the FEV_1 value by 20% from its baseline value (PC_{20}). Histamine and methacholine are currently used clinically as tools for direct assessment of airway hyperresponsiveness. The characteristics of each are discussed below.

Histamine

Since it was first identified as having the capability to mimic anaphylactic bronchoconstriction (Dale and Laidlaw, 1910) there has been a great deal of interest in histamine and its control of the airways. Histamine has been a leading candidate as a major mediator in the pathogenesis of airway disease. Elevated levels of histamine have been found in both bronchoalveolar lavage fluid (Agius et al., 1985; Casale et al., 1987; Cooper, Jr. et al., 1989; Jarjour et al., 1991) and plasma (Howarth et al., 1987; Phillips et al., 1990) of asthmatic patients. It has been shown to contract bronchial smooth muscle (Armour et al., 1984; Hulbert et al., 1985), induce airway mucus secretion (Shelhamer et al., 1980), mediate inflammatory mediator release (Takizawa et al., 1995) and induce vascular leakage (Owen et al., 1984). *In vivo*, the role of histamine in the asthmatic airways has been identified by the use of histamine antagonists. Selective antagonism of

the histamine H_1 receptor subtype attenuates the acute bronchoconstrictor response (EAR) in asthmatics (Crimi et al., 1993) suggesting that histamine is released in acute exacerbations of asthma and partially mediates bronchoconstriction. Its usefulness as a tool for assessment of airway hyperresponsiveness has been established in clinical studies (Cockcroft et al., 1977; Hargreave et al., 1981).

Methacholine

Methacholine is an analogue of acetylcholine that lacks nicotinic activity (muscarinic receptor selective) and is poorly metabolized by acetylcholinesterases (Day, 1979). The primary mechanism by which methacholine stimulates bronchoconstriction is the activation of muscarinic M_3 receptors found on smooth muscle cells (Mak and Barnes, 1990). The M_2 receptor subtype is localized to postganglionic, prejunctional neurons in several species including humans (Patel et al., 1995a) and airway smooth muscle (Sankary et al., 1988). This M_2 receptor is an inhibitory autoreceptor that regulates the release of acetylcholine (Menzel, 1994) and attenuates responses to muscarinic stimulation. The M_2 receptor appears to be dysfunctional in asthmatics (Minette et al., 1989) and is believed to contribute to the phenomenon of exaggerated cholinergic bronchoconstriction observed with non-selective anticholinergics (Barnes, 1992). Like histamine, methacholine has been used extensively in the clinical assessment of airway hyperresponsiveness (Adelroth et al., 1986; Hargreave et al., 1981; Ramsdale et al., 1984; Sterk et al., 1985) and is a valuable tool in evaluating pulmonary function.

Cellular Physiology of the Lung

Inflammation

One of the characteristics of human asthma is the underlying presence of inflammation within the airways (NAEP, 1991). Inflammation of the airways in asthma has been demonstrated histochemically, pathologically, biochemically and clinically many times (Busse, 1993; Ferguson and Wong, 1989; Fick et al., 1987; Foresi et al., 1990; Poston et al., 1992). This close relationship between airways inflammation and asthma has led to a large body of research examining the contribution of specific inflammatory cell types and inflammatory cell mediators to the pathogenesis of asthma. Of the inflammatory cell types produced in the body, eosinophils, macrophages, T-lymphocytes and neutrophils have received most of the attention, most probably due to the correlation of their presence in the airway and asthma itself (Pauwels et al., 1990; Snapper and Brigham, 1984).

The association of eosinophils in the lung with asthma is certain (Bentley et al., 1992; Bradley et al., 1991; Laitinen et al., 1993; Poston et al., 1992; Rossi et al., 1991; Tanizaki et al., 1993). Eosinophils are circulating granulocytes that participate in allergic reactions and helminthic parasite infections (Gleich and Adolphson, 1991; Holgate et al., 1991). They produce reactive oxygen metabolites through the activity of eosinophil peroxidase (EPO) and also several basic proteins, including eosinophil cationic protein (ECP) and major basic protein (MBP) (Frigas et al., 1980; Frigas et al., 1981; Gleich et

al., 1979; Holgate et al., 1991). While both ECP and MBP are released by activated eosinophils the action of MBP has received the greatest attention and has been implicated as a potential mediator of cytotoxic damage in asthma (Holgate et al., 1991; Kay and Corrigan, 1992). Major basic protein is released by eosinophilic granules upon chemical stimulation. Toxic levels of MBP, as seen in eosinophilic diseases (Gleich et al., 1979), may contribute to host tissue damage (Frigas et al., 1980; Frigas et al., 1981). Major basic protein also initiates histamine release from mast cells (O'Donnell et al., 1983), which may lead to localized elevations of histamine within the airways. The finding that MBP is present in the sputum of asthmatics (Frigas et al., 1981) lends support to the idea that the production of MBP by eosinophils in the lung is a contributing factor in the pathogenesis of asthma.

Alveolar macrophages are the predominant non-parenchymal cell in the lung (Crystal, 1991) and contribute to airway function by phagocytizing foreign bodies (Bailie et al., 1996), actively participating in immune responses (Blyth et al., 1996) and through release of chemical mediators, recruit and activate other immune cells (Kambayashi et al., 1995; Ohara et al., 1998; Zhou et al., 1995). Alveolar macrophages are present in increased numbers in asthmatics and, when activated, contribute to airway dysfunction (Adelroth et al., 1990; Boulet et al., 1990; Ferguson and Wong, 1989).

Neutrophils, or polymorphonuclear (PMN) cells, are also associated with asthma. Neutrophils are phagocytic cells circulating in the bloodstream and are capable of

translocating to sites of tissue injury (Hogg and Doerschuk, 1995). These cells contain storage granules filled with bactericidal and lysosomal enzymes used to digest foreign microorganisms (Abramson et al., 1991). Neutrophils also produce highly reactive oxidative species that react with elements of the infecting microorganism's structure and contribute to its destruction. NADPH-dependent oxidases produce hydrogen peroxide, which is converted to hypochlorous acid by myeloperoxidase (Abramson et al., 1991). The highly reactive hypochlorous acid reacts with amines, thiols, proteins and nucleic acids to eliminate the microorganism (Abramson et al., 1991). Another oxidative product from neutrophils is nitric oxide (Carreras et al., 1994). This molecule is reactive toward many biomolecules and also combines with reactive oxygen species to form peroxynitrite, a compound highly toxic to many pathogens including bacteria and viruses (Sadeghi-Hashjin et al., 1998; Saleh et al., 1998; Szabó, 1996).

In situations of prolonged or intense inflammatory reactions, the enzymes and reactive oxygen species may accumulate in the surrounding tissue and result in host cell damage (Abramson et al., 1991). Elevated levels of neutrophils have been confirmed in the bronchoalveolar lavage fluid (Balmes et al., 1997; Laitinen et al., 1993; Smith et al., 1992) and bronchial biopsies (Ohashi et al., 1992a) of asthmatics. When activated, neutrophils also produce several extracellular matrix enzymes and membrane derived lipid mediators (Abramson et al., 1991). The release of the extracellular matrix enzymes elastase and collagenase within the lung tissues would degrade the connective tissue structure and contribute to the epithelial shedding found in asthmatic airways (Arm and

Lee, 1992; Foresi et al., 1990). This epithelial shedding has been implicated as the mechanism by which sub-epithelial neurons and smooth muscle are exposed to the airways lumen (Arm and Lee, 1992). The production and release of membrane derived lipid mediators by neutrophils (Lewis and Austen, 1988), the ability of these lipid mediators to modulate bronchoconstriction (Abraham et al., 1992; Bjorck and Dahlen, 1993; Nowak et al., 1993) and the production of reactive oxidative molecules suggests that neutrophil-mediated tissue damage and release of pro-inflammatory mediators may contribute to the pathophysiology of asthma.

Lymphocytes are a group of cells comprised of T-cells, B-cells and Natural Killer (NK) cells and are the central component of the adaptive immune response which confers pathogen-specificity yet does not recognize or damage self-antigens. B-cells are identified by the expression of antigen-specific immunoglobulin on their cell surface and secrete this antigen-specific immunoglobulin upon stimulation (Pearlman, 1999). Natural Killer cells differ from both T-cells and B-cells due to the lack of a specific antigen recognition receptor on its cell surface. This cell type is highly granular as it contains several lytic enzymes.

All T-cells express the cell surface marker CD3, a component of the T-cell receptor complex. T-cells can be further sub-classified according to the expression of cell surface markers into three groups; CD4+, CD8+ and CD4-/CD8- (Miceli and Parnes, 1991). The association of the cell surface markers confers the specificity of antigen

recognition upon each T-cell subpopulation for specific antigens and major histocompatibility complexes (MHCs) as presented by antigen-presenting cells (APCs) (Kaltreider, 1991). As a general rule, CD8+ cells recognize antigens presented by MHC I molecules on APCs. The most common MHC I associated antigens are viruses, intracellular parasites and mycobacteria which elicit an immune response from CD3+/CD8+ lymphocytes. CD4+ cells recognize antigens presented by MHC II molecules on APCs and regulate immune responses through the secretion of regulatory cytokines (Kaltreider, 1991). The role of the CD4+ (T-helper, Th) cell in asthma garnered additional attention when it was found that there existed two distinct Th cell subtypes with different profiles of secreted cytokines (Mossmann et al., 1986). Both Th cell subtypes secrete IL-3 and GM-CSF but otherwise differ in the cytokine profile. The Th1 phenotype mediates the delayed-type hypersensitivity reactions and secretes (in addition to those already mentioned) IL-2 and IFN-γ. The Th2 phenotype secretes IL-4, IL-5 and IL-13 and plays a key role in IgE production (Mossmann et al., 1986). It is the presence of the Th2 phenotype in the lung tissue of asthmatics that is believed to contribute significantly to the chronic inflammation seen in asthmatics and the associated late asthmatic response observed following antigen challenge. Both IL-4 and, to a lesser extent, IL-13 stimulate IgE production by B-cells. IL-5 plays a critical role in the recruitment and activation of eosinophils following antigen challenge (Nakajima et al., 1992). The preponderance of the Th2 phenotype of T-cells in the asthmatic lung (Bentley et al., 1992; Robinson et al., 1993) indicates that this cell type may play a central role in the pathogenesis of the disease.

Inflammatory Cell Localization to Lung Tissue

In the normal state, leukocytes (lymphocytes, monocytes and granulocytes) circulate within the bloodstream and occasionally exit the plasma to reside temporarily in surrounding tissue. In the presence of a foreign antigen the small number of residing leukocytes begin the inflammatory process with the release of cytokines, including interleukin (IL)-1, interleukin (IL)-8 (Devlin et al., 1994), interferon-γ (IFN-γ) and tumour necrosis factor (TNF)-α and chemokines, including eotaxin (Jose et al., 1994; Rothenberg et al., 1995), and RANTES (Ying et al., 1996). This signal of cytokine release acts, in part, to recruit additional leukocytes to the site of the antigen. The recruitment of these leukocytes from the bloodstream is site-specific; the accumulation of inflammatory cells occurs only at the site of cytokine release. To accomplish this site-specificity a collection of proteins referred to as adhesion molecules is necessary.

Investigations in the area of cell-cell interaction have identified that epithelial cells (Patel et al., 1995b) and endothelial cells (Khan et al., 1996) express proteins that mediate intercellular adhesion (Bennett et al., 1996; Khew-Goodall et al., 1996; Sriramarao et al., 1994). The adhesion molecules identified to date can be divided into three distinct structural groups: the immunoglobulin (Ig) superfamily; the integrins and the selectins (reviewed in (Hogg and Doerschuk, 1995; Letts et al., 1994; Schleimer and Bochner, 1998)). The immunoglobulin superfamily is characterized by repetitive extracellular Ig-like domains followed by a transmembrane domain and a short

cytoplasmic sequence. The members of the Ig-superfamily that are involved in intercellular adhesion are: intercellular adhesion molecule-1 (ICAM-1, CD54), ICAM-2 and vascular cell adhesion molecule-1 (VCAM-1). ICAM-1 has five extracellular domains (Staunton et al., 1988) and is constitutively expressed on lymphocytes and monocytes and is expressed at low levels on endothelial cells, epithelial cells and fibroblasts. ICAM-1 is also inducible in several non-hematopoietic cells including endothelial cells, epithelial cells and fibroblasts when stimulated by cytokines (Lee et al., 1994; Leung et al., 1991). ICAM-2 has two extracellular repeat sequences, in contrast to the five of ICAM-1, and is expressed on endothelial cells, lymphocytes and monocytes (de Fougerolles et al., 1991). ICAM-2 is not found on neutrophils. It is constitutively expressed at levels ten to fifteen times higher than ICAM-1 but its expression is not upregulated upon exposure to cytokines. VCAM-1 has six extracellular domains and is expressed on endothelial cells. VCAM-1 is not constitutively expressed on endothelial cells, but is induced by IL-1 and TNF-α (Lee et al., 1994; Masinovsky et al., 1990).

The second group of molecules involved in intercellular adhesion is referred to as the integrins. Composed of both a α- and a β-subunit, the integrin family is composed of 16 different α subunits and 8 different β subunits (Schleimer and Bochner, 1998). There have been twenty-three different combinations of α/β subunits identified to this point and the most comprehensively defined groups are the β_1, β_2 and β_3 subfamilies. The β_1 and β_2 subfamilies play a well-defined role in cellular adhesion and will be discussed here. The β_1 subfamily shares identical β subunits and consists of six different α subunits. This

subfamily is known as the VLA (very late antigen) proteins and they are named VLA-1 to VLA-6. The VLA proteins provide receptors for the extracellular matrix (ECM) proteins laminin, fibronectin and collagen (Hemler, 1990) and play an important role in tissue organization and the movement of inflammatory cells across the interstitial spaces between the blood vessels and sites of foreign antigen (Bednarczyk and McIntyre, 1990). One member of this subfamily (VLA-4) is expressed on most leukocytes and binds to VCAM-1, the endothelial ligand mentioned above (Elices et al., 1990). The β_2-integrins (also referred to as CD18 molecules) have three known α subunits (CD11a, CD11b, and CD11c) combining to form lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18), macrophage antigen-1 (mac-1, CD11b/CD18) and p150/95 (CD11c/CD18) (Schleimer and Bochner, 1998). The CD18 molecules are expressed on leukocytes and vascular endothelium and play an important role in site-specific immunity (Rosen and Gordon, 1989).

The third group of molecules involved in intercellular adhesion is the selectins (Tedder et al., 1995). This family differs from the other two in that the determinant for recognition is a carbohydrate, in contrast to the Ig-superfamily and integrins, which recognize proteins. The extracellular terminal end is comprised of a C-type lectin (Drickamer, 1989). Adjacent to this is a sequence homologous to epidermal growth factor and next to this are a number of domains (from 2 to 9) of a homologous complement regulatory protein. There is a transmembrane sequence following this and finally a short cytoplasmic tail. The only three known members of this family are E-

selectin (ELAM-1), L-selectin (Mel-14) and P-selectin (CD62, GMP 140). E-selectin is restricted to endothelial cells and is expressed only after stimulation by IL-1 or TNF-α (Bevilacqua et al., 1987). This expression is maximal at approximately four hours and returns to baseline after eight hours. This time course coincides with neutrophilic adhesion, in the absence of CD18 integrins, strongly suggesting that E-selectins play an important role in granulocyte/endothelial adhesion (Carlos et al., 1991; Leeuwenberg et al., 1992). E-selectin will bind the specific carbohydrate moieties sialyl-Lewis X (SLe^x) and sialyl-Lewis A (SLe^a) but it will also bind L-selectin, another member of the selectin family, found on all types of blood leukocytes. L-selectin, like E-selectin, interacts with SLe^x and SLe^a found on endothelial cells. L-selectin is found on unstimulated leukocytes but is rapidly shed following stimulation of the leukocyte by IL-1 or TNF-α (Griffin et al., 1990; Tedder et al., 1990). L-selectin facilitates the rolling or margination of monocytes and granulocytes along endothelial cells during the inflammatory response. P-selectin is found on blood platelets and Weible-Palade bodies. It is not constitutively expressed but is stored in granules within leukocytes (Lorant et al., 1991). P-selectins are rapidly (5-30 minutes) distributed on the cell surface when stimulated by the leukotrienes B_4 and C_4, histamine, TNF-α (Bevilacqua et al., 1987) or thrombin.

The diversity of adhesion molecules produces a complex, coordinated series of interactions necessary to translocate a leukocyte from the bloodstream to the site of the foreign antigen. The release of cytokines by resident tissue macrophages initiates the increased expression of ICAM-1, L-selectin, P-selectin and E-selectin on the surface of

endothelial cells, as discussed above. This, in addition to the presence of VCAM-1 on the endothelial surface, increases the number of leukocytes, particularly neutrophils, which begin the process of margination. By increasing the number of cells marginating, the probability that leukocytes will bind to the endothelium is significantly increased. Once bound to the endothelium the leukocytes undergo activation and as a result, intracellular calcium concentrations increase activating protein kinases, which convert inactive β_2 integrin molecules to their active form. The active CD11a/CD18 molecule binds to ICAM-1 as the L-selectin molecule is shed by the leukocyte. It is thought that the release of the L-selectin at this time facilitates a more intimate association between the CD11a/CD18 molecule and ICAM-1. The binding of CD11a/CD18 and CD11b/CD18 to ICAM-1 produces a stretching or flattening of the leukocyte along the endothelial cell due to a large number of receptor/ligand interactions (Smith et al., 1988). The leukocyte also migrates laterally along the endothelium to the edge of the cell. The mechanism by which the leukocyte stops at the edge of the endothelial cell is as yet unclear but its presence at this point facilitates transendothelial migration as the leukocyte can pass with little resistance between endothelial cells (Furie et al., 1987).

The sequence of events described above occurs in all instances of inflammation in the body including responses to self-antigens. Asthma, in all instances, is characterized by airway hyperresponsiveness and inflammation (Barnes, 1998). The hyperresponsiveness, once attributed to an increased sensitivity of airway smooth muscle

to contractile agonists, is now understood to encompass the increased airway resistance produced by the luminal narrowing seen in inflammation (Liu et al., 1998).

Allergen-induced asthma is a chronic condition due to the ubiquity of one causative antigen (e.g. the dust mite) so that the lungs are continuously in a state of inflammation. This poses a particular problem in asthma, in contrast to other types of inflammation, because the airway epithelium, a cytoprotective barrier in the lungs, is easily damaged by cells involved in the immune response. The two major contributors to the late asthmatic response are neutrophils and eosinophils (Blyth et al., 1996; Dunn et al., 1988; Santing et al., 1994; Wood et al., 1998). The strongest evidence points to the eosinophil as the cell responsible for allergen-induced hyperresponsiveness. The presence of the eosinophil is temporally associated with the presence of airway hyperresponsiveness. Inhibition of airway eosinophilia, using gene knockout mice, eliminates airway hyperresponsiveness (Danzig and Cuss, 1997; Hamelmann et al., 2000; Mould et al., 2000). Eosinophils also release major basic protein and eosinophilic cationic protein upon the binding of ICAM-1 to CD11a/CD18 and CD11b/CD18. The release of MBP and ECP results in local cytotoxic levels of these compounds, which disrupt epithelial cells and leads to cellular necrosis. The resulting epithelial desquamation is believed to augment airway hyperresponsiveness (Hargreave et al., 1986; Rennard et al., 1991). The epithelium provides a barrier and also produces airway smooth muscle inhibitory mediators (PGE_2, PGI_2) both of which are lost with the destruction of the epithelial cells.

Smooth Muscle Reactivity

The submucosal layer of the airways consists primarily of smooth muscle cells at most levels of the respiratory tree (Weibel and Taylor, 1988). The airway smooth muscle (ASM) is regulated by myogenic and neural pathways and the smooth muscle tone is determined by the sum total of bronchodilatory and bronchoconstrictive signals (Janssen et al., 1998).

A unique characteristic of the airway smooth muscle of both the human and guinea pig airways is the spontaneous activity of the smooth muscle, referred to as slow waves. The myogenic properties of smooth muscle are affected by the numerous gap junctions found between individual smooth muscle cells (Daniel et al., 1986; Janssen et al., 1998). Action potentials radiate to neighbouring cells via gap junctions (Janssen et al., 1998), creating a system of rapid signal transmission in the absence of dense innervation (Kannan and Daniel, 1980). There is some evidence that prostaglandin E_2 can augment the gap junction density (Li et al., 1992) and perhaps the prostanoids contribute to airway hyperresponsiveness through regulation of gap junction formation in the smooth muscle. Additional investigations into this mechanism of smooth muscle hyperresponsiveness are needed to confirm this hypothesis.

The neural regulation of the ASM consists of an adrenergic, a cholinergic, and a non-adrenergic non-cholinergic (NANC) system (Barnes, 1991). Muscarinic agonists act

via the M_3 receptors on smooth muscle cells (Mak and Barnes, 1990) to contract ASM. Activation of α-adrenoceptors, in the presence of β-adrenoceptor blockade, has a limited capacity to induce bronchoconstriction and is not likely to contribute in any substantial manner to bronchoconstriction in normal or asthmatic airways (Leff et al., 1986). The most potent group of NANC neuromodulators is the tachykinins. Originating as post-translational modifications of the same precursor, substance P (SP) and neurokinin A (NKA) have both been localized to nerves within the human airways (Maggi et al., 1995; Piedimonte, 1995). While both are released from sensory nerves within the airways, neurokinin A (NKA), acting via NK_2 receptors, initiates bronchoconstriction after either aerosol inhalation (Joos et al., 1987) or intravenous infusion (Evans et al., 1988) whereas substance P has the greatest potency at NK_1 receptors where it stimulates mucus secretion (Kuo et al., 1990; Rogers et al., 1989), vascular leakage (Rogers et al., 1988) and immune cell degranulation (Lowman et al., 1988b).

Mediators from both the adrenergic and iNANC nervous systems regulate bronchodilation. The stimulation of ASM β-adrenoceptors by aerosol agonists produces significant bronchodilation and is the basis for several anti-asthma drugs currently marketed. Receptor localization studies have identified the receptor stratification of the β_2 receptors along the respiratory tree with the greatest density of these receptors in the central airways and decreasing to low levels in the peripheral airways (Ind and Barnes, 1988). This stratified distribution of nerves suggests that the adrenergic regulation of the

airways acts predominately on the central airways, thereby regulating the greatest percentage of airway resistance in the lung.

Two different iNANC neurotransmitters have been identified within the last few decades that have provided additional insight into ASM regulation. The first, vasoactive intestinal polypeptide (VIP), has been identified as a potent bronchodilator in several animal species (Diamond and O'Donnell, 1980; Goyal and Rattan, 1980; Li and Rand, 1991; Matsuzaki et al., 1980) and has been localized to nerves throughout the tracheobronchial tree (Uddman et al., 1997). Its effectiveness as a bronchodilator in humans is limited. Administration of VIP to either normal or asthmatics subjects produces no change in airway function (Barnes and Brown, 1981; Palmer et al., 1986). The inhibition of VIP activity by enzymatic degradation of VIP does not alter the iNANC inhibitory responses of human trachea *in vitro* (Belvisi et al., 1992). This is in contrast to the attenuation of the iNANC response by approximately 50% in guinea pig trachea (Ellis and Framer, 1989).

The second, nitric oxide (NO), has been the subject of intense research since its initial identification in pulmonary vasculature (Ignarro et al., 1987b; Ignarro et al., 1987a). The neuronal isoform of nitric oxide synthase (NOS) is localized to the peripheral nerves in human airways (Ward et al., 1995) and *in vivo* evidence suggests that NO may be the neurotransmitter of inhibitory NANC transmission (Belvisi et al., 1995). The relative contribution of neuronal NO to asthma is difficult to define as it is a

ubiquitous mediator found in almost all tissue types in the asthmatic airways, as described below.

There are several humoral mediators that contribute to the regulation of airway function including catecholamines, angiotensin II, endothelins, cortisol, and inflammatory mediators such as histamine, thromboxane and leukotrienes (Thomson, 1998).

The circulating catecholamines adrenaline, noradrenaline and dopamine do not contribute significantly to regulation of the airways in normal patients (Maggi et al., 1988). The asthmatic airways, unlike normal patients, are sensitive to β-adrenoceptor blockade which can induce severe bronchoconstriction (Gayrard et al., 1975). Adrenaline, when released from the adrenal medulla, activates both α- and β-adrenoceptors. β-Adrenoceptor activation by adrenaline during exercise equally bronchodilates the airways in both normal and asthmatic patients (Berkin et al., 1988) but circulating levels of adrenaline do not correlate with the severity of asthma (Ind et al., 1985). Neither noradrenaline, circulating in the bloodstream, (Berkin et al., 1985) nor dopamine (Thomson and Patel, 1978) have any *in vivo* effect on airway regulation.

Angiotensin II is a component of the renin-angiotensin system that regulates fluid and electrolyte balance within the body. The intravenous administration of angiotensin II does elicit bronchoconstriction in mild asthmatics (Millar et al., 1994). Angiotensin II

also potentiates methacholine-induced bronchoconstriction (Millar et al., 1995) but is without effect on histamine-induced bronchoconstriction (Ramsey et al., 1997) suggesting a mediator-dependent effect on airway regulation.

Endothelin-1 is a potent bronchoconstrictor in asthmatics with an increased sensitivity compared to normal patients (Chalmers et al., 1997). In addition to the humoral circulation, several cell types produce endothelin-1 within the airways of asthmatics (Dinh-Xuan et al., 1994; Howarth et al., 1995; Lamas et al., 1992a). The overall contribution of the humoral supply of endothelin-1 to regulation of the asthmatic airways remains unknown.

Physiological concentrations of cortisol do not have any demonstrated direct contribution to the pathogenesis of airway dysfunction in asthmatic patients (Barnes et al., 1980; Ramsdell et al., 1983). Histamine, thromboxane and leukotrienes induce bronchoconstriction in normal and asthmatic patients (Howarth et al., 1987; Jones et al., 1987; Robinson and Holgate, 1986; Woolcock et al., 1984). The contribution of circulating levels of these mediators to the asthmatic airway is difficult to determine, as these mediators are significantly elevated within the lung tissue itself (Agius et al., 1985; Bakhle, 1981; Bjorck and Dahlen, 1993; Casale et al., 1987; de Monchy et al., 1985; Seltzer et al., 1986). A detailed discussion of these mediators is provided below.

Key Cellular Components of the Airways

Epithelium

As the first line of defence for protecting the body from xenobiotic substances and inhaled toxins, like ozone, the epithelium plays a prominent and essential role in preserving homeostasis within the respiratory tract. The epithelium provides both a physical barrier to all inhaled substances and a primary signalling pathway capable of initiating cell and tissue defence mechanisms when necessary. The epithelial layer is composed of several different cell types that act in concert to regulate the airways. The epithelial surface of the trachea and large bronchi is defined as a pseudostratified structure in which all cells are attached to the basement membrane, or basal lamina, but not all reach the lumen of the airway. The cellular composition of the tracheal epithelium consists predominately of ciliated cells and goblet cells with serous cells, basal cells, Clara cells, and neuroendocrine cells present in comparatively smaller numbers (Forrest and Lee, 1991; Plopper et al., 1991; Weibel and Taylor, 1988). In the lower order airways the epithelium changes to a simple cuboidal and changes again in the alveoli to a simple squamous structure. The alveoli are lined with one or both of only two different cell types, referred to as alveolar Type I and Type II cells (Rennard et al., 1991). The type I cell is flat and provides the greatest surface area coverage for the alveolar sacs. The type I cells are approximately 25% of the total cell number in the alveoli but cover over 95% of the surface area. The alveolar type II cells are considerably thicker than the type I cell and produce surfactant, which covers the surface of the alveoli.

In the alveoli, the diffusion distance that the exchange gases must travel is only two cells thick. Oxygen in the lumen of the alveoli must cross the thin alveolar type I cell and then the single cell wall of the alveolar capillary. This diffusion distance is approximately 2 μm in thickness and provides little resistance to gas exchange. The varying composition of cells within the epithelial layer suggests that the airways from different areas of the respiratory tree will, in fact, respond differently to the same stimulus. The distribution of mast cells shows a similar regional variation in distribution with the percentage of mast cells increasing toward the alveoli with approximately 100 times more mast cells per unit area in the bronchioles than the trachea (Pearce, 1988).

The epithelium plays an important role as a barrier between the external environment and the sub-epithelial tissues. Ozone exposure causes epithelial cell damage, leading to a general loss of surface epithelial cells (Boatman et al., 1974; Easton and Murphy, 1967; Fedan et al., 2000; Nikula and Wilson, 1990; Pinkerton et al., 1993). Loss of epithelial function has several consequences: increased epithelial permeability (Bromberg et al., 1991; Miller et al., 1986; Stutts and Bromberg, 1987), decreased mucus production (Fedan et al., 2000), inflammation (Bhalla, 1999; Fabbri et al., 1984) and a decreased capacity for the production of bronchoactive mediators (Jones et al., 1988a; Leikauf et al., 1995). The epithelium produces both prostaglandins and nitric oxide, the loss of which can have a significant impact on airway function.

Airway Smooth Muscle

Airway hyperresponsiveness is the major pathophysiological finding in asthmatics and abnormalities in the constriction of airway smooth muscle contribute to generation of the hyperresponsiveness. It is therefore relevant that a discussion on the changes in airway smooth muscle structure and function in asthmatics be included here. Huber and Koessler (1922) initially made the finding of increased muscle mass in asthmatics. Since then, several other investigators have confirmed an increase in muscle mass in asthmatic lungs when compared to non-asthmatic lungs (Dunnill et al., 1969; Heard and Hossain, 1973). The increase in muscle mass may be a result of smooth muscle cell hyperplasia, smooth muscle hypertrophy or both (Ebina et al., 1993) and contributes to the narrowing of the airways in two ways. The first is the increased muscle mass may have an increased capacity for shortening (increased force generation) producing a greater constriction of the airways to any given bronchoconstrictor. The evidence for increased smooth muscle shortening is conflicting. Evidence from asthmatic tissues has demonstrated increased force generation by some laboratories (Bramley et al., 1994) and no change in force generation by others (Roberts et al., 1985). One possible explanation has been put forth (Seow et al., 1998) suggesting that the individual laboratories utilized different methods of evaluating force generation while not taking into account the tissue cross-sectional area or the length-tension relationship of smooth muscle from asthmatic airways.

Airway Edema and Mucus Hypersecretion

A contributing factor to the narrowing of the airways observed in asthma is the accumulation of excess fluid within the airway lumen (Jeffery, 1992). This excess fluid can be derived from vascular leakage, producing airway tissue edema, or from mucus hypersecretion.

Vascular permeability is regulated by several different mediators including bradykinin (Hulstrom and Svensjo, 1979), histamine (Owen et al., 1984), PAF (Evans et al., 1989; Lotvall et al., 1991), substance P (Lundberg et al., 1983), leukotrienes (Arakawa et al., 1993; Henderson, Jr., 1994) and thromboxane (Lotvall et al., 1992). Upon stimulation, plasma extravasation occurs quickly with extravasated fluid reaching the epithelial surface in less than 1 minute (Persson, 1998). The release of many, if not all, of these mediators within the lung of asthmatics leads to an increase in vascular permeability and tissue edema. Support for this observation comes from several studies demonstrating edema formation in asthmatics (Djukanovic et al., 1990). The significance of this edema and its relation to airway narrowing is explained below.

Mucus hypersecretion contributes to both an increase in the viscosity and volume of the periciliary fluid (Liu et al., 1998). Produced by both goblet cells and submucosal glands, mucus secretion is upregulated by many of the same mediators contributing to plasma exudation. The idea that mucus hypersecretion contributes to the

pathophysiology of asthma is supported by histological finding of mucus plug formation and goblet cell hyperplasia in patients who died of asthma (Liu et al., 1998).

The mechanism by which the increase in airway luminal fluid contributes to asthma is based upon Poiseuille's law regarding resistance to flow in tubes. For a tube (trachea or bronchi) of a given radius, bronchoconstriction increases the resistance to airflow by a factor equivalent to the inverse of the new radius to the fourth power. A theoretical explanation is provided by Liu et al (1998) and is shown in figure 2. This dramatic effect on airway diameter indicates that the elevated level of fluid in the airways is a contributing factor to the pathophysiology of asthma.

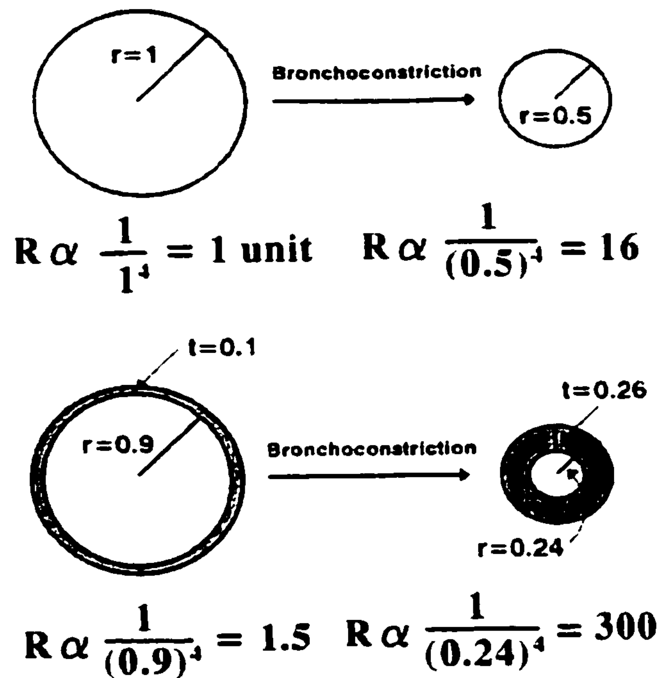


Figure 2: Effect of increased airway thickness on airway resistance.

The top portion of the figure illustrates the change in resistance (R) for a given cylinder with a radius of r. The lower portion illustrates that the same cylinder, with the additional parameter of wall thickness (t), and the significant increase in effect that airway constriction may have on airflow following a reduction in radius (bronchoconstriction) combined with an increase in airway wall thickness (edema). From the book entitled "Asthma: Basic Mechanisms and Clinical Management", 3rd edition, edited by PJ Barnes, NC Thomson and IW Rodger (Copyright © 1998 by Academic Press; reprinted with permission).

Mediators of Airway Function

Leukotrienes

Leukotrienes are members of the arachidonic acid cascade of mediators, which includes the prostaglandins and thromboxanes (Voelkel et al., 1989). Arachidonic acid is a 20 carbon polyunsaturated fatty acid with four double bonds found as a component of membrane phospholipids. Free arachidonic acid, commonly released from cellular membranes by phospholipase A_2 (Henderson, Jr., 1994), enters the lipoxygenase branch of the arachidonic acid cascade by its peroxidation to 5-L-hydroperoxy-5,8,10,14-eicosatetraenoic acid (5-HPETE) catalyzed by 5-lipoxygenase (5-LO) (Yamamoto, 1983). 5-LO is found in the cytosolic portion of most cells and does not produce leukotrienes until activated by 5-LO activating protein (FLAP), located on the nuclear membrane. The next step in this process is catalyzed by LTA_4 synthetase which converts 5-HPETE to the epoxide LTA_4, an unstable intermediate, which can be enzymatically converted by either LTB_4 synthase to produce LTB_4 or by glutathione-S-transferase to produce LTC_4; the first of three sulphur containing leukotrienes referred to as cysteinyl or sulfidopeptide leukotrienes (Samuelsson et al., 1987). Successive shortening of the LTC_4 side chain by gamma-glutamyl-transpeptidase produces LTD_4 with further cleavage of the side chain resulting in LTE_4 formation (Peskar, 1989).

Leukotriene biosynthesis occurs in a number of myeloid cells including: neutrophils, eosinophils, monocytes, macrophages, mast cells and B lymphocytes where the enzyme 5-LO is localized (Henderson, Jr., 1994; Yamamoto, 1983). With the

exception of macrophages, these cells produce measurable quantities of either LTB_4 or LTC_4 but not both (Henderson, Jr., 1994). Eosinophils and mast cells have been shown to release LTC_4 while neutrophils produce LTB_4 (Henderson, Jr., 1994; Yamamoto, 1983). Strong evidence of transcellular biosynthesis of eicosanoids has been presented by several groups (Feinmark, 1992; Maclouf, 1993). Endothelial cells, mast cells, red blood cells, smooth muscle cells and platelets lack 5-LO but *in vitro* experiments have demonstrated that those cells can convert LTA_4, released from neutrophils and eosinophils, to LTC_4 (in endothelial cells, smooth muscle cells, platelets) or LTB_4 (in red blood cells) (Feinmark, 1992; Maclouf, 1993). Distinct receptors have been identified for LTB_4 and the cysteinyl leukotrienes C_4, D_4, and E_4. The cysteinyl leukotrienes act on the same receptor, referred to as the $CysLT_1$ receptor. This receptor is found in several tissues including airway smooth muscle, vascular smooth muscle, eosinophils and vascular endothelium (Henderson, Jr., 1994; Samuelsson et al., 1987; Shaw and Krell, 1991). Leukotriene receptor signal transduction is believed to consist of a G-protein coupled receptor-operated channel (Crooke et al., 1991; Henderson, Jr., 1994). Activation of the leukotriene receptor produces an intracellular calcium influx (Chan et al., 1994) resulting in contraction of the arterial smooth muscle and increases in post-capillary permeability (Joris et al., 1987).

Nitric Oxide and Nitric Oxide Synthase

Endothelium-derived relaxing factor was first identified as nitric oxide in 1987 by three different laboratories, lead by Ignarro, Murad, and Furchgott, for which they were

awarded the Nobel prize in physiology and medicine in 1998. This diatomic molecule has a half-life of approximately ten seconds in biological environments and is quickly converted by oxygen, oxygen radicals and/or water into nitrite (NO_2^-) and nitrate (NO_3^-) (Marletta et al., 1988). This transient property makes nitric oxide a unique candidate as a neurotransmitter. Classically, a neurotransmitter is defined as a compound that is stored in synaptic vesicles and released by the appropriate nerves, its actions can be mimicked by exogenous application of the potential transmitter and its physiological effects can be competitively antagonized by agents acting at the same receptor. Unlike classically defined neurotransmitters, no storage sites have been identified for NO. One possible source of stored nitric oxide comes from recent evidence suggesting that vascular NO persists in the circulation as an S-nitroso adduct of serum albumin, increasing the half-life of NO to several hours and providing a form of storage and transport for this short-lived gas (Jia et al., 1996; Stamler et al., 1992; Wolzt et al., 1999).

Nitric oxide also differs from classical transmitters with respect to its receptor which is not a cell membrane protein coupled to a signal transduction mechanism but is, at least in part, the cytosolic enzyme guanylyl cyclase (Bredt and Snyder, 1989; Fleming et al., 1991a; Gold et al., 1990; Schini and Vanhoutte, 1989; Waldman and Murad, 1988). NO is both lipid and water soluble and is able to pass through cell membranes unimpeded; it binds to the central iron atom of the heme group in guanylyl cyclase and causes a conformational change in the enzyme which increases its activity and leads to an increase in cGMP (Snyder and Bredt, 1992).

Nitric oxide can also interact with reactive oxygen species (ROS), forming more reactive species such as peroxynitrite (Beckman, 1994; Lipton et al., 1993; Miller et al., 1995), and promoting widespread tissue damage (Morley et al., 1991). This damage frequently results from lipid peroxidation and is implicated in the disease processes of ischemia/reperfusion injury (Szabó, 1996), gastric mucosal necrosis (Lamarque and Whittle, 1996) and pulmonary dysfunction (Haddad et al., 1993).

NO has been demonstrated by several groups to act in an autocrine or paracrine fashion to activate guanylyl cyclase (Mayer, 1994; Murad, 1994; Rengasamy et al., 1994; Schmidt et al., 1992). The resultant elevation in cellular cGMP has been shown to stimulate secretion and particle internalization in alveolar macrophages (Foster, 1980; Wirth and Kierszenbaum, 1983); regulate fluid balance (Geary et al., 1993), transepithelial ion transport (Jain et al., 1998; Schwiebert et al., 1997) and ciliary beat frequency (Wyatt et al., 1998) in lung epithelial cells; attenuate cardiac muscle contractility (Flesch et al., 1997; Kojda et al., 1995); induce relaxation of vascular smooth muscle (Mollace et al., 1991), bronchial smooth muscle, and tracheal smooth muscle (Duncan et al., 1980; Kannan and Johnson, 1995; Pyne and Pyne, 1993); and has multiple effects in neurons (Knowles et al., 1989; Reiser, 1990), and vascular endothelial cells (Sertl et al., 1987), although the level of cGMP increase in bronchial and vascular smooth muscle may not be physiologically relevant in response to inhaled spasmogens (Sertl et al., 1987).

Two classes of the enzyme producing nitric oxide, referred to as nitric oxide synthase (NOS), have been identified (Forstermann and Kleinert, 1995). The first is a calcium-dependent group in which two distinct isozymes have been delineated and are referred to as the endothelial (type III) NOS (eNOS) and the neural (type I, brain) NOS (nNOS) (Forstermann and Kleinert, 1995). The second is an inducible, calcium-independent isoform referred to as inducible (type II) nitric oxide synthase (iNOS) (Forstermann and Kleinert, 1995). Both calcium-dependent enzymes have been sequenced in mouse (Lyons et al., 1992), rat (Bredt et al., 1991; Lyons et al., 1992), cow (Lamas et al., 1992b), rabbit (Teng et al., 1998), and human (Hall et al., 1994). There is only one member of the second class of NOS enzymes and that is iNOS. This isoform has been sequenced in mouse (Xie et al., 1992), and guinea pig (Shirato et al., 1998). The NOS enzyme is a dimer with a subunit mass of approximately 150 kDa (Evans et al., 1992; Forstermann et al., 1991a; Iida et al., 1992; Klatt et al., 1992a; Lamas et al., 1992b; Schmidt et al., 1991) and contains a heme moiety (Klatt et al., 1992a). It has sequence similarities to the enzyme cytochrome P450 reductase (Bredt et al., 1991) and has been described as the first example of a soluble cytochrome P450 in eukaryotes (White and Marletta, 1992). The bovine endothelial NOS consists of 1205 amino acids and possesses a molecular mass of 133 kDa (Lamas et al., 1992b; Sessa et al., 1992). The cerebellar NOS has an amino acid sequence of 1429 and a mass of 160 kDa, the macrophage NOS has an amino acid sequence of 1144 (Lowenstein et al., 1992) and the human endothelial NOS is 1294 amino acids in length (Janssens et al., 1992). All sequences have a cofactor binding region for FAD, FMN

and NADPH (Bredt et al., 1991; Janssens et al., 1992; Lamas et al., 1992b; Lowenstein et al., 1992; Sessa et al., 1992) homologous to that of cytochrome P450 reductase (Bredt et al., 1991). Calmodulin binding sites exist in both the brain (Bredt et al., 1991; Vorherr et al., 1993) and endothelial (Lamas et al., 1992b; Sessa et al., 1992) nitric oxide synthase sequence, supporting the idea that these isoforms are regulated by cellular calcium levels. It has also been determined that both cNOS and iNOS require BH_4 for optimal activity but the role of this cofactor was difficult to identify (Gross and Levi, 1992; Klatt et al., 1992b; Mayer et al., 1990; Radomski et al., 1990; Tayeh and Marletta, 1989; Werner-Felmayer et al., 1993). Experiments measuring BH_4 levels during NOS activity and inhibiting BH_4 synthesis, using the dihydrofolate reductase inhibitor methotrexate, demonstrated that BH_4 is not recycled in the cytosol of the rat cerebellum (Giovanelli et al., 1991). It was theorized that BH_4 had an allosteric effect or perhaps maintained specific groups in a reduced state necessary for NO synthesis (Giovanelli et al., 1991). The function of BH_4 may have been explained by Hevel and Marletta (1992) who found that it is tightly bound to NOS in a 1:1 ratio and may function as a redox element that is recycled while bound to NOS and not in solution.

The cNOS isoforms are activated via increases in intracellular calcium levels (Busse and Mulsch, 1990; Mulsch et al., 1989; Mulsch and Busse, 1991). The elevated calcium binds calmodulin and the calcium/calmodulin complex binds to the NOS enzyme resulting in a conformational change and enzyme activation. The two calcium-dependent isoforms of NOS were originally differentiated by their cellular location. The neural

isoform was found in the cytosolic portion of the cell and the endothelial isoform, due to a myristylation at its N-terminus (Pollock et al., 1992), is membrane bound (Boje and Fung, 1990; Forstermann et al., 1991b; Sessa et al., 1993). Additional research has shown that the neuronal isoform is present in both cardiac and skeletal muscle and is localized to cellular membranes (Kobzik et al., 1994; Xu et al., 1999). As additional information regarding the tissue distribution and functional role of nitric oxide synthase becomes available it is likely that the original designation of nNOS, eNOS and iNOS may require re-classification but in what manner remains to be determined.

Nitric oxide synthase converts the free amino acid L-arginine to L-citrulline and NO (Bush et al., 1992a; Bush et al., 1992b; Iyengar et al., 1987; Marletta et al., 1988; Palmer et al., 1988). L-Arginine is first converted to N^G-OH-L-arginine in the presence of NADPH and molecular oxygen (Leone et al., 1991; Marletta et al., 1988; Marletta, 1993; Stuehr et al., 1991; Zembowicz et al., 1992). The intermediate N^G-hydroxy-L-arginine is formed by NOS (Leone et al., 1991; Pufahl et al., 1992; Stuehr et al., 1991) and this hydroxylation mimics the reaction catalyzed by cytochrome P450 reductase (White and Marletta, 1992). The conversion of N^G-OH-L-arginine to L-citrulline and NO by cytochrome P450 reductase provides convincing evidence of the enzymatic similarity (Renaud et al., 1993). The hydroxylation occurs at the guanidino nitrogen of arginine (Marletta et al., 1988; Marletta, 1993; Nathan, 1992; Palmer et al., 1988) and this intermediate may then interact with the iron-protoporphyrin IX moiety of NOS using an additional molecule of oxygen and ½NADPH (Stuehr et al., 1991) to release NO from N^G-OH-L-arginine and form L-citrulline

(Marletta, 1993). This formation of nitric oxide and citrulline occurs in a 1:1 ratio (Bush et al., 1992a). This enzymatic conversion is stereospecific, and only the L-, and not D-, isomer of arginine can act as the precursor. L-arginine is the only amino acid that can serve as a substrate for NOS activity (Iyengar et al., 1987; Palmer et al., 1988).

Mammalian cells are capable of transporting L-arginine from the extracellular space to the cytoplasm to compensate for the increased demand for NOS substrate (White, 1985). Studies investigating the kinetics of this transport system have revealed that some arginine analogues used to inhibit NOS in intact cell preparations are also capable of inhibiting arginine transport by those particular cells (Baydoun et al., 1993; Schmidt et al., 1990; Westergaard et al., 1993) although both L-NAME and L-NOARG have been shown to inhibit only NOS and not the arginine transport system (Bogle et al., 1996; Closs et al., 1997). Arginine transport is critical for nitric oxide synthase activity as the inhibition of arginine transport, specifically the y+ carrier CAT2B, can attenuate nitric oxide production without direct inhibition of nitric oxide synthase (Hammermann et al., 1999; Ookawauchi et al., 1998; Wileman et al., 1995) This suggests that the inhibition observed with some arginine analogue inhibitors may be a result of arginine transport inhibition and not direct inhibition of the enzyme itself. Both L-NAME and L-NOARG are useful tools for examining the direct effect of NO inhibition as they do not affect arginine transport. A second source for arginine involves the recycling of citrulline to arginine via the argininosuccinate synthase/argininosuccinate lyase pathway. Both enzymes are co-induced with nitric oxide synthase following LPS exposure *in vivo* in the lungs, heart and liver of

mice (Nagasaki et al., 1996), the enteric neurons of the dog (Shuttleworth et al., 1995), the vasculature of several species (Xie et al., 2000) and are co-expressed with nitric oxide synthase *in vitro* in murine macrophages (Nussler et al., 1994). This co-regulation is supported by the finding that glucocorticoids inhibit the expression of both NOS and argininosuccinate synthase (Simmons et al., 1996) suggesting that NOS activity is regulated by both enzyme production and substrate availability.

One of the first inhibitors identified was the competitive antagonist, N-omega-arginine methyl ester (L-NAME). Several new antagonists have been identified since L-NAME but all of them are competitive antagonists. Almost all of these inhibitors are modified arginine structures, which mimic the substrate binding to the enzyme, but are inert within the active site and inhibit access of arginine to the active site. There have been attempts to produce a selective inhibitor for either the calcium-dependent or calcium-independent isozymes and there has been some success with aminoguanidine, guanidinoethyldisulfide (GED), S-methylisothiourea (SMT), S,S'-1,3-Phenylene-bis(1,2-ethanediyl)-bis-isothiourea (PBITU), L-N6-(1-Iminoethyl)lysine (L-NIL) as selective inhibitors of iNOS *in vitro* but all inhibitors inhibit to some degree both calcium-dependent and calcium-independent NOS.

The gene for inducible nitric oxide synthase was cloned in 1992 and found to contain calmodulin as a subunit of iNOS with the capability of binding Ca^{2+} at basal cytosolic Ca^{2+} concentrations (Lowenstein et al., 1992). The upregulation of iNOS is

capable of producing significantly greater amounts of NO than the calcium-dependent isoforms and it is this large NO production, characteristically seen in inflammatory reactions, that is believed to result in the formation of highly reactive molecules such as peroxynitrite and generalized tissue destruction. While examining the excretion of nitrate levels greater than dietary intake levels, Tannenbaum et al. (1983) found that treatment of rats with *Escherichia coli* lipopolysaccharide raised urinary NO_3^- output by approximately 10-fold and the greatest source of this nitrate production was found to be macrophages (Stuehr and Marletta, 1985). Macrophages have also been found to increase nitrite and nitrate production after exposure to INF-γ, IL-1 and TNF-α (Eizirik et al., 1992; Kilbourn et al., 1992; Kosaka et al., 1992). The increase in nitrite and nitrate levels observed after stimulation of macrophages by the aforementioned activators results from increased levels of NO by iNOS, due to upregulation of the DNA transcription. The induction of NOS can be inhibited by glucocorticoids, actinomycin D (a mRNA synthesis inhibitor) and cycloheximide (a protein synthesis inhibitor) (Galea et al., 1992; Moritoki et al., 1992; Radomski et al., 1990; Rees et al., 1990). Thus, synthesis of both mRNA and protein is necessary for iNOS production (Moritoki et al., 1992).

Nitric oxide has been demonstrated to act directly on NOS in a reversible, auto-inhibitory manner. Because NOS is a heme-containing enzyme (Klatt et al., 1992a) it is possible that NO binds to the heme prosthetic group, inhibiting the transfer of electrons to the heme and halting the chain of reactions responsible for NO synthesis (Rogers and Ignarro, 1992). In addition to this negative feedback inhibition, NOS activity is also

inhibited by phosphorylation. Both protein kinase A (PKA) and protein kinase C (PKC) phosphorylate NOS at serine residues (Bredt et al., 1992). Phosphorylation by PKA produces no functional alteration of catalytic activity but PKC phosphorylation inhibits NO production by 50-70% (Bredt et al., 1992). The activation of the PIP_2 pathway produces both diacylglycerol and inositol trisphosphate providing antagonistic regulation of cNOS. Diacylglycerol enhances PKC activity and inhibits NO production by cNOS; IP_3 stimulates the release of Ca^{2+} from internal stores augmenting cNOS activity (Bredt et al., 1992). This antagonistic regulation via the PIP_2 pathway is not likely with iNOS as increased intracellular Ca^{2+} levels do not contribute to the rate of NO production.

Pathophysiological Role of iNOS

Inducible NOS has been localized to alveolar macrophages (Blackford, Jr. et al., 1994), neutrophils (Blackford, Jr. et al., 1994), airway epithelial cells (Punjabi et al., 1994; Robbins et al., 1994), vascular endothelial cells (Radomski et al., 1990) and plays an essential role in eradication of xenobiotic organisms and tumours (Condino-Neto et al., 1993; Cunha et al., 1993; Palacios et al., 1992). Exposure of these cells to foreign substances, including endotoxin, elicits induction of iNOS with a concomitant increase in NO production. The NO acts in a dexamethasone- and NOS inhibition-sensitive manner (Cunha et al., 1993) to kill invading organisms suggesting that NO is responsible for a portion of non-specific immunity.

Excess production of NO by immune cells may be the cause of hypotension and vascular collapse that is characteristic of endotoxic shock (Editorial, 1991; Rees et al., 1990). Bacterial infections can proliferate after abdominal surgery or physical injury, which expose the natural flora of the intestine to structures inside the body. The bacteria will eventually migrate to the bloodstream and proliferate. The exposure of macrophages to this endotoxin results in upregulated NO production to destroy the foreign body. The high levels of NO produced also act non-specifically to elicit vasodilation.

Prostaglandins, Thromboxane and Cyclooxygenase

Cyclooxygenase

The cyclooxygenase enzyme, also known as prostaglandin G/H synthase or PGHS, converts free arachidonic acid into several different products including prostaglandins (PGs) D_2, E_2, $F_{2\alpha}$, prostacyclin (PGI_2) and thromboxane (Tx) A_2 and B_2 (Ohashi et al., 1992b). This two-step enzymatic process forms the endoperoxide PGG_2, followed by a reduction to PGH_2. It is this PGH_2 that is the starting compound for formation of the prostaglandins, prostacyclin and thromboxanes. Two different isoforms of cyclooxygenase have been identified. The first is known as COX-1 and is a constitutive enzyme expressed in almost all tissues examined. The other known isoform of cyclooxygenase is COX-2, an inducible form whose expression is regulated at the gene transcription level and is expressed in several cell types including airway smooth muscle cells (Belvisi et al., 1997), vascular smooth muscle cells (Ermert et al., 1998), alveolar macrophages (Ermert et al., 1998; Ohara et al., 1998), mast cells (Ermert et al., 1998;

Moon et al., 1998), and airway epithelial cells (Alpert et al., 1997; Newton et al., 1998). The cyclooxygenase enzyme is membrane bound and in many cases localized to the endoplasmic reticulum and nuclear membrane (Ermert et al., 1998). The apparently clear distinction between the two cyclooxygenase isoforms based upon the idea that the constitutively expressed isoform performs modulatory "housekeeping" functions and the inducible isoform participates in pro-inflammatory reactions has come under scrutiny as several recent findings have suggested that this delineation in function is not accurate. The COX-1 enzyme can be induced under certain experimental conditions (Cohn et al., 1997) and can contribute, in the absence of a functioning COX-2 enzyme, to pro-inflammatory reactions (Wallace et al., 1998). Evidence that COX-2 contributes to physiological homeostasis comes from findings that COX-2 knockout mice demonstrate deficiencies in bone homeostasis (Wolfe et al., 1999), kidney function (Morham et al., 1995) and reproduction (Lim et al., 1997). In light of this evidence it is clear that a better understanding of the tissue distribution, regulatory factors and physiological actions of both COX isozymes is needed before a re-classification of the COX enzymes can be undertaken.

Prostaglandins

These are a family of lipid mediators derived from arachidonic acid and possessing multiple and in some cases opposite biological activities (Henderson, Jr., 1987). Upon formation of PGH_2 there are several enzymatic and non-enzymatic pathways that lead to formation of the prostaglandins. PGH_2 can be degraded to PGD_2

and to PGE_2 in a non-enzymatic fashion. Alternatively, there are several enzymes that are tissue specifically expressed and can convert PGH_2 into various mediators. PGH-PGD isomerase will form PGD_2 enzymatically, PGH-PGE isomerase will form PGE_2, PGFα reductase converts PGH_2 into $PGF_{2\alpha}$, and prostacyclin synthase activity results in the formation of PGI_2. It is the distribution of these enzymes that dictates the cell specific formation of the various products of cyclooxygenase.

Prostaglandin Receptors and their Distribution within the Airway

Considerable research has been conducted to identify the individual receptors involved in the signal transduction pathway initiated by the various prostaglandins (See reviews by (Coleman et al., 1994; Kennedy et al., 1982). Advances in molecular biology have permitted the identification and sequencing of five different classes of prostaglandin receptors as follows: the PGD_2 selective DP receptor, the thromboxane selective TP receptor, the prostacyclin selective IP receptor, the $PGF_{2\alpha}$ selective FP receptor and the PGE_2 selective EP receptor, of which there are four known subtypes (Coleman et al., 1994).

Mechanism(s) of Action

The mechanism of action of the prostanoids has been reviewed by Robinson and Holgate (1991) and is summarized here. Prostacyclin is mainly produced by endothelial cells of the vascular system to inhibit platelet aggregation and stimulate vasodilation in

the pulmonary vascular bed (Coleman et al., 1994). Its membrane receptor is designated IP and its signal transduction system involves multiple G-protein coupling. Prostacyclin activates adenylyl cyclase (AC) via a G_s-mediated mechanism (Hashimoto et al., 1990) and elevates cytosolic calcium (Vassaux et al., 1992; Whittle et al., 1979) through activation of a G_q protein with subsequent activation of phospholipase C (PLC).

PGD_2 is produced from numerous cell types and acts via the DP receptor (Giles and Leff, 1988) to induce both vascular and non-vascular smooth muscle relaxation via activation of a G_s protein and intracellular activation of adenylyl cyclase. The DP receptors are found in ileal, lung, liver, stomach and human uterine tissue (Sanger et al., 1982) and in all cases elevate intracellular cAMP levels (Simon et al., 1980).

The $PGF_{2\alpha}$ receptor is referred to as the FP receptor (Coleman et al., 1994) and its activation produces smooth muscle contraction including bronchoconstriction and vasoconstriction. This receptor is coupled to a G_q protein and elevates intracellular calcium in a PLC-dependent manner. The FP receptor is found in ovarian tissue (Dukes et al., 1974), kidneys (Mene et al., 1987), stomach, heart and lungs (Wasserman, 1975).

The group of EP receptors, all of which share similar affinities for PGE_2 are different in both their tissue distribution and second messenger coupling (Coleman et al., 1994). The EP_1 receptor is believed to be coupled to a calcium ion channel, as the action of PGE_2 on this particular receptor is absolutely dependent on extracellular calcium. The

activation of the EP_1 receptor does involve increased PLC activity but whether this is prior to or a consequence of calcium influx is unclear. EP_1 receptors are found in tissues of the lung (Watabe et al., 1993), gastrointestinal tract, uterus (Senior et al., 1991) and bladder. The EP_3 receptor is coupled to a G_i protein and inhibits adenylyl cyclase and activates PLC and mediates excitatory effects on several tissues. This particular subtype of EP receptor mediates gastric acid secretion (Reeves et al., 1988), lipolysis in adipose tissue, catecholamine release from the adrenal gland, and modulation of neurotransmitter release (Coleman et al., 1987). No EP_3 receptor subtypes have been found in any lung tissues. In contrast to the EP_1 and EP_3 receptors, the EP_2 and EP_4 receptors predominately mediate inhibitory activities and accomplish this by stimulating adenylyl cyclase activity via a G_s coupled mechanism (Hardcastle et al., 1982). The EP_2 receptors are known to mediate relaxation of tracheal, ileal and vascular tissue. Activation of the EP_4 receptor on mast cells produces a PGE_2 mediated inhibition of histamine release via cAMP elevation. The EP_4 receptor is also found in the vasculature (Lawrence and Jones, 1992), airway (Lydford and McKechnie, 1994) and uterus (Yeardley et al., 1992).

Thromboxanes

In addition to the production of prostaglandins, cyclooxygenase can also lead to the production of thromboxane through the action of thromboxane synthase on the common precursor PGH_2. As a potent pulmonary vasoconstrictor (Dorn et al., 1992) and bronchoconstrictor the release of thromboxane following an inflammatory response may play an important role in modulating airway function. Indirect measurements of

thromboxane have shown an elevation of its release in animal models of airway hyperresponsiveness (Aizawa et al., 1985; O'Byrne and Fuller, 1989), ozone-exposed human subjects (Seltzer et al., 1986) and asthmatic humans (Fujimura et al., 1986). Studies directed at localizing the production of thromboxane within the lung have identified alveolar macrophages, monocytes, epithelial cells, neutrophils and platelets as the predominant sources (Devillier and Bessard, 1997; Henderson, Jr., 1991; Higgs et al., 1983).

Histamine

The actions and contribution of histamine to regulation of airway tone, inflammation and airway disease have been reviewed extensively (Hill et al., 1997). Histamine is produced by decarboxylation of histidine by the enzyme L-histidine decarboxylase found in mast cells and basophils (O'Donnell et al., 1983). Histamine is stored in its preformed state and released from mast cells (Dvorak, 1997) in response to several pro-inflammatory stimuli including aerosolized antigen and IgE.

Histamine has been a leading candidate as a major mediator in the pathogenesis of airway disease (Holgate, 1999). Support for this role comes from considerable evidence demonstrating that histamine is produced rapidly following epithelial insult by several different groups of stimuli including toxic gases, exercise, aspirin, alcohol, viral infection and various organic antigens such as animal dander and plant pollen. This histamine release occurs locally with significant increases found in bronchoalveolar lavage in

asthmatics (Agius et al., 1985; Casale et al., 1987; Cooper, Jr. et al., 1989; Jarjour et al., 1991) and plasma levels (Howarth et al., 1987; Phillips et al., 1990).

Three distinct histamine receptors have been identified pharmacologically and are identified as H_1, H_2 and H_3 (Hill et al., 1997). Development of the first histamine antagonists, later identified as H_1 selective antagonists, demonstrated inhibition of histamine-stimulated contractions of the guinea pig ileum (Ash and Schild, 1966; Leurs et al., 1991a), vascular smooth muscle (Matsumoto et al., 1986), and airway smooth muscle (Bjorck and Dahlen, 1993; Crimi et al., 1993; Krell, 1979; Roquet et al., 1997b). The H_1 receptor distribution has been determined by radioligand binding and H_1-receptors have been found on macrophages, endothelial cells, smooth muscle cells, brain, and the adrenal medulla (Hill et al., 1997). H_1-receptor activation stimulates intracellular phospholipase C activity leading to increased inositol phosphate production and elevated intracellular calcium levels. Activation of the H_1 receptor stimulates smooth muscle contraction (Bjorck and Dahlen, 1993; Crimi et al., 1993; Krell, 1979; Roquet et al., 1997b), production of cyclooxygenase products (Schuligoi et al., 1998), stimulation of nitric oxide formation (Leurs et al., 1991b), and increased vascular permeability (White et al., 1987). The distribution of the H_2 receptor is coincident with the H_1 receptor in both vascular and bronchial smooth muscle (Bjorck and Dahlen, 1993), brain, and heart. The H_2 receptors are also found on gastric parietal cells, neutrophils and the rat uterus (Hill et al., 1997). The H_2 receptor is coupled to adenylate cyclase via a Gs subunit whose activation results in increased cAMP levels within the cell. Acting through H_2

receptors histamine increases gastric acid secretion, airway mucus secretion (White et al., 1987) and smooth muscle relaxation (Drazen et al., 1979; Okpako et al., 1978).

Both activation of histamine release by any one of these stimuli or administration of exogenous histamine produces, in both humans and animals, an airway bronchoconstriction and a state comparable to that observed in the human condition referred to as asthma. In addition to the direct effect of histamine on airway function, histamine is also implicated in the stimulation of additional mediator release within the airway tissue and activation of vagal cholinergic bronchoconstriction (Hulbert et al., 1985). Studies in both humans and guinea pigs have revealed a histamine-stimulated release of both prostaglandin E_2 and prostacyclin from the airway epithelium. Knight et al. (Knight et al., 1995b; Knight et al., 1995a) determined that chronic *in vivo* treatment of patients displaying non-airways related inflammation with a non-steroidal anti-inflammatory compound (NSAID) eliminated an *in vitro* histamine-induced tachyphylaxis of the bronchial smooth muscle. When compared to normal patients who displayed tachyphylaxis in the presence of intact epithelium the endogenous levels of both metabolic products of prostacyclin, 6-keto-$PGF_{1\alpha}$, and PGE_2, were significantly lower in the NSAID-treated individuals. Addition of the H_2 antagonist, ranitidine, eliminated the histamine-stimulated elevation of prostacyclin in normal individuals but did not affect the increased release of PGE_2. This close association of histamine action on the airway epithelium and the upregulation of prostaglandin release suggests an intimate relationship between these two mediators and their regulation of airway

contractility and it is of interest to determine whether the *in vitro* responsiveness of the airways to NSAID treatment is maintained in the *in vivo* situation. Histamine does not appear to contribute to the late asthmatic response as it has been reported that neither urinary histamine metabolites nor bronchoalveolar lavage fluid histamine content correlated with the bronchial hyperresponsiveness observed in asthmatic patients (de Monchy et al., 1985) and histamine antagonists had no effect on this response.

Current Findings on Mechanism-Based Models of Airway Hyperresponsiveness

To elucidate the mechanisms of asthma, continued research into the pathogenesis, etiology, and mechanisms of this disease is required. Airway hyperresponsiveness is a characteristic of asthma which can be used to diagnose the disease (Dolovich et al., 1992) although the mechanism(s) of airway hyperresponsiveness are still not clearly understood. The elucidation of the mechanism of airway hyperresponsiveness is crucial to our understanding of asthma. A limiting factor for research on airway hyperresponsiveness is the practicality of the human subject as a model. This requires the use of animal models for additional information. Since asthma is a disease limited only to humans, an animal model of asthma must demonstrate some of the characteristics of asthma including airway inflammation, airway hyperresponsiveness, structural changes within the airway as seen in asthmatics, mucus secretion, edema and smooth muscle hypertrophy or hyperplasia. Various models have been used to mimic the asthmatic condition including allergen sensitization (Takishima et al., 1985),

occupational pollutants (Marek et al., 1996), physiologic stimuli (Mochizuki et al., 1999), infection (Dakhama et al., 1997) and atmospheric pollutants (Alpert et al., 1971; Holtzman et al., 1983a; Sielczak et al., 1983). All of these models reflect to a large extent the characteristics of asthma although each has its shortcomings. The findings from each model, their similarities and their differences to asthma are described below.

Allergens

Allergic asthma is developed spontaneously in humans to a wide range of allergens including house dust mite, ragweed, pollen, grass, animal dander, and mould (Dolovich et al., 1983). Development of allergen specific airway hyperresponsiveness begins with the initial exposure of an individual to the allergen producing an antigen specific response mechanism within the immune system. Subsequent exposure to that allergen, for which sensitization has now developed, triggers an exaggerated IgE-mediated inflammatory response within the lungs that is characterized by mast cell activation and subsequent degranulation (Terr, 1997). This, in turn, leads to release of pro-inflammatory mediators including: histamine (Dorsch et al., 1982; Phillips et al., 1990), platelet activating factor (PAF) (Hayes et al., 1992), and leukotrienes (Jonsson and Dahlen, 1994; Nagase et al., 1995). The localized release and accumulation of these pro-inflammatory mediators has been shown to cause edema (Cui et al., 1999), inflammatory cell recruitment (Hutson et al., 1988; Santing et al., 1994), airway luminal narrowing (Cui et al., 1999) and mucus secretion (Blyth et al., 1996). These characteristics of allergic asthma have been reproduced in animal models using ovalbumin (Elwood et al., 1991;

Ishida et al., 1989; Sanjar et al., 1990; Underwood et al., 1992), Ascaris suum (Chen et al., 1992; Gundel et al., 1989; Johnson and Stout, 1989; Underwood et al., 1992) and ragweed (Becker et al., 1989). These animal models demonstrate both *in vitro* and *in vivo* characteristics of allergic asthma.

Ovalbumin sensitization and challenge produces an airways eosinophilia (Elwood et al., 1991; Ishida et al., 1989) and an airway hyperresponsiveness to several bronchoconstrictors (Gieske and Baugh, 1992; Hessel et al., 1995; Lewis and Broadley, 1995). Several different mediators have been implicated in ovalbumin-induced AHR including PAF (Fukuda et al., 1990; Seeds et al., 1991; Sugasawa et al., 1991), leukotrienes (Tsunoda et al., 1991), tachykinins (Boichot et al., 1995), nitric oxide (Miura et al., 1996; Persson et al., 1993; Schuiling et al., 1998). The AHR associated with ovalbumin is closely linked to bronchial eosinophilia. Recently, the chemokine eotaxin has been identified in the BALF of ovalbumin-sensitized guinea pigs (Jose et al., 1994; Rothenberg et al., 1995). It is a potent eosinophil chemotactic mediator (Jose et al., 1994; Shinkai et al., 1999) that may play a critical role in the development of AHR in this model. There is some evidence showing that eosinophilia is not dependent on eotaxin (Yang et al., 1998) but its exact role remains to be confirmed. The *in vivo* finding in the allergen-induced model that elevated levels of inflammatory cells are found within the airway tissue is supported by histological evidence (Dunn et al., 1988). This model also exhibits epithelial cell desquamation and mucus plug formation (Blyth et al., 1996; Blythe et al., 1986; Kung et al., 1994; Underwood et al., 1995).

One element of asthma that has not been closely looked at is the development of airway smooth muscle hypertrophy or hyperplasia. There have been reports of elevated airway smooth muscle mass in the ovalbumin-sensitized guinea pig (Bai et al., 1995), cat (Padrid et al., 1995) and rat (Du et al., 1996; Sapienza et al., 1991) but the presence of this alteration in smooth muscle mass remains to be demonstrated in many other species.

Ascaris suum is a parasitic nematode commonly found in domestic pigs and is transmitted to humans as a result of handling pig manure. The larval stage of this nematode occurs in the lungs of the infected individual with a migration to the intestinal tract later in its lifecycle. It is this exposure to the larvae in the lungs that can lead to the allergen sensitization and subsequent airway hyperresponsiveness. Several animal species have been used to examine the pathogenesis of *Ascaris suum* infection including cynomolgus monkeys (Malo, 1989), rhesus monkeys (Pare et al., 1976), squirrel monkeys (Hamel et al., 1986), macaca monkeys (Richards et al., 1983), dogs (Krell, 1978), sheep (Chen et al., 1991), and guinea pigs (Cain et al., 1980). Airway hyperresponsiveness and the LAR have been observed in these models following antigen challenge. The mediators responsible for the LAR are variable between each species, as evidence for histamine as a predominant bronchoconstricting mediator has been found in cynomolgus monkeys (Malo, 1989); however, in dogs the predominant bronchoconstricting mediator appears to be a cyclooxygenase product which, when inhibited by pharmacological blockade, reveals a lesser histamine component (Richards et al., 1988). In contrast, the *in vitro* contractility of dog tracheal smooth muscle is attenuated by a cyclooxygenase product,

PGE_2 (McGrogan et al., 1998). In the sheep model of AHR it was determined that a large portion of the bronchoconstriction observed after antigen challenge originated from sensory nerves and that chemical inactivation of sensory nerves with capsaicin or receptor blockade with an NK_1 antagonist significantly attenuated the observed airway hyperresponsiveness (Reynolds et al., 1997). A more consistent finding is the elevation of leukotrienes in the airways following antigen challenge. Elevated levels of LTB_4 and LTC_4 have been found in the arterial blood of cynomolgus monkeys (Malo, 1989) and bronchoalveolar lavage fluid of sheep (Okayama et al., 1989) following *Ascaris suum* challenge. Further evidence comes from the use of receptor antagonists which clearly demonstrate that LTD_4 is partially responsible for mediating the LAR in both sheep (Soler et al., 1991) and monkeys (Turner et al., 1994). The temporal association of these elevated levels of leukotrienes indicates that they are associated primarily with the late asthmatic response.

The *Ascaris suum* model demonstrates species differences in inflammatory cell infiltrate. Neutrophilia has been seen in the bronchial lumen of guinea pigs following antigen provocation (Takishima et al., 1985), but in sheep sensitized to *Ascaris suum*, there was no observable change in the number of mast cells or eosinophils following challenge nor any change in the activation state of these cells (Chen et al., 1991). In dogs challenged with *Ascaris suum* the observed airway hyperresponsiveness was associated with airway neutrophilia, but not with an increase in pulmonary eosinophils following the challenge (Woolley et al., 1995). It is interesting to note that the authors did find that the

degree of airway hyperresponsiveness was correlated to the number of pulmonary eosinophils present prior to the antigen challenge (Woolley et al., 1995).

Occupational Pollutants

The recent attention to occupational health and safety in the workplace has led to investigations into the effect of several occupational pollutants on airway function. Many of these models are industry specific, such as animal excreta for animal husbandry workers (Taylor, 1998), acid anhydrides and isocyanates in chemical manufacturing (Fabbri et al., 1991a; Fabbri et al., 1991b; Fabbri and Mapp, 1991; Saetta et al., 1992), grains and flours in grain farming (Taylor, 1998) and moulds in microbiological scientists (Taylor, 1998). Tobacco smoke, both primary and secondary exposures (Daffonchio et al., 1992; Delay-Goyet and Lundberg, 1991; Omini et al., 1990), is also a major contributor to asthma. Of these, a discussion of the isocyanate and tobacco smoke models is included here.

The chemical family of isocyanates is used commercially in the polymerization of polyurethanes. The exothermic reactions vapourize the isocyanates and exposes manufacturers and users to toxic levels of toluene diisocyanate (TDI) and hexamethylene diisocyanate (HDI) which have been correlated with higher than normal incidences of asthma (Baur et al., 1994). The animal model of TDI-induced airway hyperreactivity has been studied extensively and like the models described above, demonstrates both an airway hyperresponsiveness and immune cell influx. One aspect of this model is the

inconsistency in the literature with respect to the etiology of the TDI-induced model. Several groups have demonstrated an IgE-mediated, mast cell-dependent mechanism whereby the airways become hyperresponsive to various bronchoconstrictors (Baur et al., 1994; Mullin et al., 1983; Scheerens et al., 1999). It has also been demonstrated that only a small portion of those animals sensitized to TDI develop an IgE-mediated hyperreactivity. The majority of the animals exhibited an IgE-independent, T-cell dependent airway hyperresponsiveness (Mapp et al., 1996; Satoh et al., 1995; Scheerens et al., 1996; Scheerens et al., 1999; Sugawara et al., 1993; Van Loveren et al., 1991; Van Loveren et al., 1996).

Tobacco smoke has been shown to increase the risk of asthma in both smokers and via passive smoking in children (Chilmonczyk et al., 1993). In studies using animal models, the primary mechanism by which tobacco smoke induces pulmonary dysfunction is the stimulation of tachykinin release by sensory neuropeptides within the lung (Daffonchio et al., 1992) (Daffonchio et al., 1990) (Delay-Goyet and Lundberg, 1991) (Dusser et al., 1995) (Hong et al., 1995) (Lee et al., 1995). This tachykinin-mediated airway hyperresponsiveness contributes to the early asthmatic response. There is some evidence that tobacco smoke can induce the LAR via production of eicosanoids (Hong et al., 1995; Lee and Hong, 1999) indicating that this model does display the characteristic dual-phase bronchoconstriction seen in asthmatics. The significance of tobacco smoke in asthma may come not from its action as a single pathogenic mechanism but as a

facilitator of lung epithelial damage and inflammation (Seymour et al., 1997; Sjostrand and Rylander, 1997).

Physiological Stimuli

There are several different physiological, or physical, challenges that provoke asthmatic attacks in individuals with otherwise normal lung function. The most common of these is exercise-induced asthma (EIA). The changes in respiratory function that occur during exercise include increased airway resistance and hyperinflation of the lungs (Anderson et al., 1975). Additional research has found that it is the increase in ventilation rate, and not the exercise itself, that induces asthma (Deal, Jr. et al., 1979) and has led to the use of isocapnic ventilation as a technique for assessing hyperventilation-induced asthma (HIA), a condition with characteristics similar to EIA. The mechanism by which HIA induces asthmatic attacks is the loss of water vapour from the respiratory surfaces (Anderson and Smith, 1991). A periciliary fluid lines the respiratory tract, covering the epithelial surface. The loss of water from this fluid produces a change in the osmolarity of the fluid that is believed to stimulate the release of pro-inflammatory chemical mediators from epithelial cells (Belcher et al., 1988). Central to the pathogenesis of hyperventilation-induced asthma and exercise-induced bronchoconstriction is the local release of leukotrienes (O'Byrne, 1997).

Hyperventilation-induced asthma is therapeutically controlled with sodium cromoglygate, β-agonists, leukotriene antagonists and antihistamines (Anderson et al.,

1975; O'Byrne et al., 1997) and can be used in the clinical setting for assessing therapeutic value of anti-asthma medication (Inman, 1999). Animal models of airway hyperresponsiveness for physiological stimuli have provided insight into the mechanisms by which changes in osmolarity of periciliary fluid regulate airway function. Although useful in the clinical assessment of asthma in patients, the airway hyperresponsiveness resulting from physiological stimuli is indicative of disease severity but is not a powerful model to investigate the underlying pathogenic mechanisms involved in asthma.

Infections

Viral models of airway hyperresponsiveness may be more relevant for investigations into the mechanism of asthma exacerbations as evidence suggests that viral infection may be a major contributor to periodic, acute exacerbations (Kent et al., 1993). Both airway hyperresponsiveness (Gern and Busse, 1999; Ouellette and Reed, 1965) and airways inflammation (Gern and Busse, 1999; Trigg et al., 1996) have been demonstrated in influenza-infected asthmatics and in murine (Yin and Lomax, 1986), rat (Mehta et al., 1997; Sorkness et al., 1994) and guinea pig (Hegele et al., 1993; Robinson et al., 1997) models of viral infection. The mechanism by which viral infection initiates airway hyperresponsiveness and inflammation is not clear but both changes in muscarinic receptor functionality (Sorkness et al., 1994) and epithelial damage (Robinson et al., 1997) have been implicated. Unique to the viral pathogenesis of airway hyperresponsiveness is the binding of viral proteins to intracellular adhesion molecule (ICAM)-1 (Hakonarson et al., 1998). Airway epithelial cells express ICAM-1 on their

luminal surface, which binds the viral antigen (Corne and Holgate, 1997). This binding initiates upregulation of both additional ICAM-1 receptors and vascular cell adhesion molecule (VCAM)-1 (Corne and Holgate, 1997). Both adhesion molecules facilitate immune cell translocation from the vasculature to the site of viral infection (Letts et al., 1994; Ohkawara et al., 1995; Van Oosten et al., 1995).

Support for the idea that viral infection contributes to exacerbations of asthma comes from several reports identifying that rhinovirus infection increases the airway hyperresponsiveness seen in ovalbumin-sensitized animals (Blyth et al., 1998; Robinson et al., 1997). These studies indicate that the development of the allergen-induced airway hyperresponsiveness mimics the pathological conditions of human asthma and infection with respiratory virus mimics the exacerbations of asthma both pathologically and physiologically. Additional research into the mechanism by which viral infection leads to exacerbations of asthma may prove beneficial in designing specific therapies to alleviate symptoms or eliminate viral infections.

Atmospheric Pollutants

The presence of these oxygen-based molecules is prevalent in industrialized nations with relatively high levels found in major urban centers (Jorres and Magnussen, 1998). The correlation found between mortality due to asthma and atmospheric pollution (Derriennic et al., 1989) has prompted investigators to closely examine the relationship between these pollutants and the pathogenesis of asthma.

Sulfur Dioxide

Sulfur dioxide (SO_2) is a by-product of fossil fuel combustion. Ambient air levels often reach above 100 parts per billion with peak recordings exceeding 200 parts per billion in many urban areas (Jorres and Magnussen, 1998). Exposure of humans to high levels of SO_2 has occurred both in industrial accidents (Harkonen et al., 1983) and chronically (Federspiel et al., 1980) with no direct evidence for SO_2-mediated alterations in lung function. In contrast, controlled clinical studies have demonstrated that sulfur dioxide-induced bronchoconstriction does occur in asthmatic individuals (Sheppard et al., 1980) although the presence of a pre-existing airway hyperresponsiveness appears to be a pre-requisite. In animal studies, acute SO_2 exposure produces a transient hyperresponsiveness to bronchoconstrictors (Lewis and Kirchner, 1984; Norris and Jackson, 1989) and a reduction in both pulmonary compliance and conductance (Atzori et al., 1992).

Ozone

Ozone (O_3) has been used in animal models both for studies of its toxicology in the general population (Balmes, 1993; Chitano et al., 1995; Peden, 1997) and for the asthma-like characteristics seen following ozone exposure (Gordon et al., 1984; Larsen, 1991; Menzel, 1984). Experimental models of ozone exposure display both airways inflammation (Holtzman et al., 1983a; Sun and Chung, 1997a) and AHR (Holtzman et al., 1983b; O'Byrne et al., 1984). The degree of AHR and inflammation are correlated to

ozone dose (time of exposure x concentration) rather than to the concentration or time of exposure itself (Gelzleichter et al., 1992). Both airway eosinophilia and neutrophilia are temporally correlated to *in vivo* airway hyperresponsiveness (Inoue et al., 2000; Vargas et al., 1998) but evidence for the causal effect of inflammatory cells on airway hyperresponsiveness remains to be established. In many instances, inhibition of either neutrophilia or eosinophilia (Li et al., 1992; O'Byrne et al., 1984) does not significantly affect the *in vivo* airway hyperresponsiveness. This dissociation of an inflammatory response and airway hyperresponsiveness suggests that there are direct effects of ozone on specific cell types including epithelium (Bhalla, 1999; Chitano et al., 1995; Hyde et al., 1992; Leikauf et al., 1995), nerves (Coleridge et al., 1993) or smooth muscle (Gordon et al., 1984; Jones et al., 1988b; Walters et al., 1986). Histological analyses have confirmed the presence of both eosinophilia and neutrophilia (Murlas and Roum, 1985) in the lung tissues following ozone exposure. Significant epithelial cell desquamation is also present following ozone exposure (Fabbri et al., 1984; Koto et al., 1995).

General Objective

Airway hyperresponsiveness observed in both humans and animal models is characterized by an increased responsiveness of airway smooth muscle to spasmogens and the associated inflammatory state. It is the purpose of this thesis to examine changes in the regulatory mediators controlling smooth muscle contractility. My objective was to determine the contribution, if any, of the bronchodilators NO and PGE_2 to modulation of airway hyperresponsiveness following ozone exposure. I used the conscious,

unrestrained guinea pig model of ozone-induced hyperresponsiveness to measure airway responsiveness at several timepoints after ozone exposure, with and without inhibition of NO and PGE_2 formation. I also examined the NOS activity in the lung tissue *in vitro*. I compared non-selective and selective (for the inducible form) COX inhibitors for their effects on ozone-induced hyperresponsiveness. I also examined contributions of thromboxane A_2, another product of cyclooxygenase, to ozone-induced airway hyperresponsiveness. Since the role of nitric oxide as a relaxant of smooth muscle, including airway smooth muscle, is well established and Salvemini et al. have demonstrated a synergistic interaction between cyclooxygenase and nitric oxide synthase both *in vitro* (Salvemini et al., 1993a; Salvemini et al., 1995a; Salvemini et al., 1996; Salvemini, 1997; Salvemini and Masferrer, 1996) (Salvemini et al., 1993a; Salvemini et al., 1995a) and *in vivo* (Salvemini et al., 1995b). Therefore, I also evaluated whether NO contributes to the modulation of airway hyperresponsiveness by eliciting changes in cyclooxygenase activity.

Specific Hypotheses

1. Ozone-induced histamine airway hyperresponsiveness is a time-dependent process in guinea-pigs.

2. Nitric oxide synthase, via release of nitric oxide, attenuates ozone-induced histamine airway hyperresponsiveness.

3. Cyclooxygenase products, specifically PGE_2 and PGI_2, attenuate ozone-induced histamine airway hyperresponsiveness.

4. Cyclooxygenase and nitric oxide synthase products act in combination to attenuate ozone-induced airway hyperresponsiveness.

2. Experimental Methods

Ozone Exposure and Measurement of Airway Hyperresponsiveness

To assess the changes in airway resistance resulting from ozone exposure, airway resistance, estimated using P_{enh}, was determined in response to histamine aerosol in animals exposed for thirty minutes to ambient air or ozone. The effect of ozone was examined at several different time points following the ozone exposure to identify time-dependent changes in airway responsiveness. The responses were examined in sham-treated animals, immediately following ozone and at two, four, eight and twenty four hours post-ozone exposure. Male guinea pigs (300-350 g, Charles River, St. Constant, Quebec) were exposed to 3.0 ± 0.3 ppm ozone or air (sham control) for 30 minutes in a Plexiglas chamber. Ozone was produced by a Welsbach High Voltage Discharge Ozone Gas Generator (California, USA) and ozone levels were monitored with a AFx UV Ozone analyzer, model IN-2000 (calibrated monthly) at a wavelength of 253.7 nm. Both sham-treated and 0 hour latency groups were immediately transferred to the Buxco Plexiglas whole body plethysmographs; all other groups were returned to cages supplied with water *ad libitum* for periods of 2, 4, 8 or 24 hours prior to commencement of the aerosol challenge. Aerosolization was accomplished using the Pulmo-Sonic nebulizer (DeVilbliss, Pennsylvania, USA) operating at 1.35 MHz and providing 1 cc of aerosol per minute with a particle size range between 1 and 3 µm. Animals were initially challenged with saline, followed by doubling concentrations of histamine from 2.5 to 320

μg/mL lasting 30 seconds for each concentration. Immediately following each concentration the maximum enhanced pause (P_{enh}) was recorded for a 15-minute period. Subsequent concentrations of histamine were administered only after P_{enh} had returned to baseline values. The maximum concentration for each animal was either 320 μg/mL or a lesser concentration producing a P_{enh} value greater than or equal to six. The Merck Frosst Animal Care Committee in accordance with the Canadian Council on Animal Care guidelines first approved all experimental protocols and procedures.

Description of Enhanced Pause (P_{enh})

Buxco Incorporated (Sharon, Connecticut, USA) has produced a whole body plethysmograph system capable of assessing changes in airway function by a closely related variable referred to as the enhanced pause or P_{enh}. Changes in pressure within the plethysmograph are detected by a transducer located on the animal chamber. Each cycle of respiration consists of an inspiratory and an expiratory phase, the first of which creates a negative pressure within the plethysmograph and the second of which creates a positive pressure. The entire waveform produced during a respiratory cycle closely resembles a sine wave with a maximum negative point (the peak inspiratory pressure or PIP) and a maximum positive point (the peak expiratory pressure or PEP). As is known from clinical data, the expiratory phase of the respiratory cycle is the phase of lung function most affected by bronchoconstriction. The determination of P_{enh} accounts for this with a value related to the time required for expiration. The expiratory phase is controlled by two components; telastic recoil of the lung tissue and the forced expiration

resulting from contraction of the respiratory muscles. In pathological diseases such as asthma, the physical characteristics of elastic recoil change and forced expiration increases to compensate for a loss in tissue elasticity. This is manifest as a decrease in the expiration time (Te) and an increase in the relaxation time (Tr). The value of P_{enh} itself is calculated by the formula:

$$P_{enh} = PEP/PIP \times ((Te-Tr)/Tr)$$

where PEP = peak expiratory pressure, PIP = peak inspiratory pressure, Te = expiratory time, Tr = relaxation time.

Measuring Histamine Airway Hyperresponsiveness

To quantify changes in airway responsiveness to histamine challenge, the mean P_{enh} values for each concentration in one particular treatment group were plotted semi-logarithmically and the points fitted to an exponential curve. The concentration of histamine that produces an elevation of P_{enh} to a value of five on the interpolated curve is termed the PC_5 value. This value of five was selected for its consistent position within the linear portion of each curve permitting, as an intermediate value, detection of either increases or decreases in P_{enh} under varying treatment conditions.

In Vivo Enzyme Inhibition

To determine the contribution of endogenous mediators to the modulation of the airway following ozone, selective enzyme inhibitors were administered prior to or during histamine provocation. Both the selective COX-2 inhibitor, DFU and the non-selective COX inhibitor, indomethacin, were injected i.p. 15 minutes prior to histamine challenge in a PEG:saline (60:40) solution. The thromboxane receptor agonist, U46619, was aerosolized in doubling concentrations to 10 μg/mL, the maximal tolerated concentration. The thromboxane receptor antagonist, SQ 29,548, was injected at a concentration of 1 mg/kg i.p. 15 minutes prior to aerosol provocation. The nitric oxide synthase inhibitor, L-NAME, at a concentration of 5120 μg/mL was aerosolized concurrently with histamine.

Lung Membrane Preparation

The assessment of NOS activity *in vitro* was conducted on lung tissues obtained from guinea pigs following ozone exposure. Following the treatment period, animals were euthanized with an i.p. injection of 65 mg sodium pentobarbital. The thoracic cavity was opened and the entire respiratory tract was excised and placed in ice-cold (4°C) sucrose-MOPS buffer (SMB). The main bronchi were separated from the surrounding lung tissue using a gentle scraping technique with the blunt end of a scalpel. This removed the lung tissue and left behind the lower order bronchi (1st through 4th generation) permitting study of the two separate tissue groups. Each preparation of bronchi or lung tissue consisted of pooled tissue from two separate animals. The tissue

was finely minced with scissors and homogenized using a Polytron (Brinkmann Instruments, New York, USA) homogenizer three times for seven seconds at 10,000 rpm. Each preparation was then centrifuged for ten minutes at 12,000xg. The pellet was discarded and the supernatant was filtered through gauze, aliquoted and stored at minus 20°C until required.

Bronchoalveolar Lavage

Following ozone treatment, guinea pigs were euthanized with 65 mg of i.p. sodium pentobarbital. The trachea was severed immediately caudal to the cricothyroid cartilage and 2x5 mL of phosphate buffered saline (PBS) was instilled via the trachea into the distal lung and then removed. The recovered suspension (approximately 7 mL) was centrifuged for 15 minutes at 1400 rpm. The supernatant was discarded and the pellet was resuspended in fresh PBS to yield a final cell concentration of 0.5 million cells per milliliter of solution. A 100 µL aliquot was added to each well of a dual chamber cytospin funnel and centrifuged at 500 rpm for 5 minutes. The slides were then stained using the Diff-Quik® staining procedure, which is a modification of the Wright-Giemsa method. Cells were differentially counted in four separate fields of view at 400x magnification under bright field illumination. The data for each treatment group were combined.

NOS Anion Exchange Chromatography

Tissues prepared according to the lung membrane preparation section, above, were used for the *in vitro* determination of NOS activity. Columns were prepared beginning with the addition of 2.00 ± 0.03 g of Bio-Rad AG 50W-X8 resin (200-400 mesh) to poly-prep columns. The resin was washed (in order) with 9 mL of 2 M NaOH, 9 mL of dH_2O, and 3 mL of SMB. Samples prepared were solubilized with 0.1% Triton-X 100. Reaction tubes were set up containing: 800-1000 nM ^{14}C-L-arginine (specific activity of 320 mCi/mmol), 1 mM β-NADPH (reduced form), 100 μM tetrahydrobiopterin, 100 μL of sample (with an approximate protein concentration of 1.0 mg/mL) and where required 100 μM L-NMMA, 100 μM EGTA (for calcium independent activity) or 1 mM $CaCl_2$ with 500 U calmodulin (for maximally stimulated NOS). The final volume of 200 μL was completed with dH_2O and the tubes were vortexed and incubated at 37°C for 15 minutes. The reaction was stopped with 1 mL of dH_2O containing 1 mM L-arginine and 1 mM L-citrulline. The resulting solution was added to the conditioned columns and allowed to drain. This was followed by 2 mL of dH_2O from which the effluent was collected and added to 13.5 mL of scintillation fluid. Samples were counted on a Beckman LS5000CE scintillation counter at 97% efficiency for beta decay. Final counts were corrected against values obtained in the presence of the NOS inhibitor L-NMMA and presented as L-NMMA-sensitive NOS activity.

Purification of [^{14}C]-L-Arginine

Due to non-enzymatic deterioration of the stock radiolabelled arginine, it was periodically re-purified prior to experimental use. Briefly, Dowex AG50W-X8 was converted to sodium form with excess NaOH and washed until effluent was neutral. The Dowex was transferred to a pipet and 200 μCi of [^{14}C]-L-arginine was loaded on the column. The column was rinsed with 3 x 2 mL of de-ionized water and the arginine was then eluted with 2 mL of 0.02 N NaOH and the effluent collected in a vial containing 0.05 mL of 1.0 M citric acid. The resulting solution of purified [^{14}C]-L-arginine was stored at 4°C until needed.

Contractility Studies

Guinea-pigs were sacrificed in a CO_2 chamber according to the Merck-Frosst Animal Care and Ethics Committee approved procedures. The trachea were removed from the guinea-pigs and placed in a modified Krebs' bicarbonate buffer containing (in mM): NaCl - 120, KCl - 4.7, $MgSO_4$ - 0.6, $CaCl_2$ - 2.5, KH_2PO_4 - 1.2, $NaHCO_3$ - 25 and glucose - 11 continuously gassed with 95% O_2/ 5% CO_2. Tracheae were cleaned of connective tissue and cut into rings between 2 to 4 mm in width. Rings were cut opposite the smooth muscle and joined with 6-0 silk to form chains of four open rings. The tracheal ring preparations were then suspended in a 20 mL bath containing 10mL of Krebs' bicarbonate buffer at 37°C, continuously gassed with 95% O_2/ 5% CO_2. A resting tension of 1 gram was applied and the tissue was allowed to equilibrate for 60 minutes during which time the tissues were washed every 3 minutes. Changes in active tension

were recorded using a force displacement transducer connected to a type R Dynograph (Beckman). Tissues were initially contracted with 80 mM KCl to provide an internal standard of tension generation between each tissue preparation.

Histamine Concentration-Response Curves

Tissues were pre-contracted with 115 μM histamine followed by a 60-minute washout period during which time the tissues were washed every 3 minutes. Response amplitudes are expressed as a percentage of the initial 80 mM KCl contraction.

Sodium Nitroprusside Concentration-Response Curves

Following the 115 μM histamine pre-contraction and 60 minute washout period the tissues were pre-contracted with 20 μM histamine (approximately 80% of maximum). Sodium nitroprusside (NaNP) was added cumulatively at 3-minute intervals and responses were expressed as a percentage of the initial 80 mM KCl contraction.

Effect of a PDE V Inhibitor on In Vitro Responsiveness

Following the 115 μM histamine pre-contraction and 60 minute washout period the tissues were pre-contracted with 20 μM histamine (approximately 80% of maximum). The phosphodiesterase V inhibitor, zaprinast (50 μM) was present in the bathing solution for the duration of the concentration-response curve. Sodium nitroprusside (NaNP) was added cumulatively at 3-minute intervals and responses were expressed as a percentage of the initial 80 mM KCl contraction.

Isolation of Peritoneal Macrophages

Peritoneal macrophages were isolated in order to provide a relatively pure source of inducible NOS protein from guinea pigs to serve as positive controls for the western blotting experiments. Guinea pigs were injected ip. with 100 μg/mL of LPS diluted in sterile lactated Ringers solution 24 hours prior to cell isolation. Guinea pigs were euthanized with carbon dioxide and 10 mL of Eagle MEM with Earle's salts (containing L-glutamine, penicillin, streptomycin and 10 U/mL heparin) was injected into the peritoneal cavity of each guinea pig and the abdomen was massaged for several seconds. The solution was aspirated and placed in a polypropylene tube. Exudate from 2 to 4 animals was combined and the suspension was centrifuged for 10 minutes at 250 x g at 4°C. The supernatant was discarded and the pellet resuspended in MEM with Earle's salts (L-glutamine, penicillin and streptomycin). Cell number was determined using a Coulter counter with a 500 times dilution. Cells were diluted to a final concentration of 8.3×10^5 cells/mL. Following dilution, 6mL was added to each well of a 6 well culture dish and incubated for 3 hours in a humidified 5% CO_2 chamber at 37°C. The medium was then aspirated and plates were washed 3 times with tissue culture medium. To maximize NOS induction 50 μg/mL of LPS was added to the culture medium and incubated for 24 hours. Macrophages were then removed from culture dishes with a rubber policeman and permeablized with 0.2% Triton X-100 and sonicated for 15 minutes. Cell lysates were frozen and stored at -20°C until required.

HPLC Measurement of Nitrates and Nitrites

The assay of nitrates and nitrites was developed to indirectly assess the production of NO by tracheal tissues. This information would serve to augment the pharmacological data obtained from the *in vitro* tissue bath experiments. Determination of tissue bath effluent nitrate and nitrite concentrations was performed using the technique described by Thayer and Huffaker (Thayer and Huffaker, 1980). Bathing solution was sampled (1.5 mL of 15 mL) and filtered through a Novo-Pak IC-H membrane previously conditioned to the Ag+ form to remove Cl- ions, responsible for strong interference with the separating column. Samples were loaded from a Waters WISP sample delivery system, model 712, into a Waters Partisil SAX column in an isocratically delivered 50 mM phosphate buffer, pH 3.0. Solvent delivery was accomplished with a Waters model 600 Multisolvent delivery system at a rate of 1mL per minute. Absorbance changes were measured at 210 nm with a Waters 486 absorbance detector. Nitrite eluted at 6.3 minutes and nitrate eluted at 9.7 minutes. Using potassium nitrate and potassium nitrite standards the lower limit of detection was determined to be 10 nmol for nitrite and 1 nmol for nitrate.

SDS-PAGE and Protein Detection by Western Blotting

The western blotting experiments were designed to support the *in vivo* pharmacological data concerning the changes in enzyme activity of COX and NOS.

Electrophoresis

Tissue homogenates were loaded on to 6% tris-glycine gels (for NOS immunoblotting) or 10% tris-glycine gels (for COX immunoblotting) with 10 wells and a thickness of 1.5 mm. Each blot contained SeeBlue Pre-stained standards (Helixx Technologies, Scarborough, ON) and positive controls for the protein being examined. Protein concentrations and lane designations are provided in each blot legend. The conditions for the electrophoresis were as follows: a constant voltage of 120 volts in a commercially available running buffer (Helixx Technologies) containing 250 mM Tris base, 2.0 M glycine, 35 mM SDS in deionized water, pH 8.3. Electrophoresis was complete when the dye front reached the bottom of the gel.

Protein Transfer

Following electrophoresis, proteins were transferred to nitrocellulose for one hour at a constant current of 1.5 Amps in a buffer containing 500 mM glycine and 75 mM Tris with 30% methanol in deionized water. The post-transfer nitrocellulose was stained with Ponceau S solution (0.1% Ponceau S in 5% acetic acid, Sigma, St. Louis, MO) to identify the presence of any protein bands remaining.

Protein Detection

The specific detection of proteins of interest was accomplished by immunoblotting with previously characterized selective antibodies. Nitrocellulose (NC) was first blocked with a 5% non-fat dried (NFD) milk solution in Tween-containing Tris-

buffered saline (TTBS) for one hour at room temperature. The NC was washed once for fifteen minutes and two additional times for five minutes each and then exposed to the primary antibody for one hour at room temperature. The antibody was diluted in the blocking solution and dilutions are defined for each blot in its corresponding legend. After one hour the NC was again washed for 1 x 15 minutes and 2 x 5 minutes and then exposed to the secondary antibody (α-rabbit for polyclonal and α-mouse for monoclonal antibodies) at a concentration of 1:2000 diluted in 1% NFD milk in TTBS. Following a one hour incubation at room temperature the NC was washed 1x15 minutes and 2x5 minutes in TTBS followed by 2x5 minutes in TBS. The detection solution (ECL, Amersham) was added to the NC as per the manufacturer's instructions for a period of one minute. Visualization of the protein bands was done with varying exposure times to autoradiographic film (Hyperfilm ECL, Amersham).

Statistical Analyses

In Vivo Data

P_{enh} values for each concentration of histamine were pooled in their respective group and sample means and standard errors were plotted using the SlideWrite (Advanced Graphics Software Inc., Carlsbad, CA) graphing program. Plots were semi-logarithmic and the data from each group were fitted to a best-fit exponential curve of the form $y=a_0+a_1*exp(-x/a_2)$. From each curve the concentration of histamine that produced a P_{enh} value of five (PC_5) could be determined. To determine differences between group

PC_5 values the concentration response curve for each individual animal was plotted, a curve interpolated and the resulting PC_5 values from each treatment condition pooled to determine a mean and standard error of the mean for the PC_5 values. Data from histamine alone, dexamethasone pre-treatment and histamine with L-NAME at either 2560 μg/mL or 5120 μg/mL were pooled and a multivariate analysis was performed using the Minitab (Minitab Inc., State College, PA) statistical software program. Comparisons between each treatment group for a particular latency period following ozone were performed using a One-way ANOVA with Dunnett's test. The control group is defined as those animals challenged only with histamine.

In Vitro Data

The production of citrulline by NOS was quantified, as described above (pg. 66) and data from each treatment condition were pooled to produce group means and standard error of the means. Statistically significant differences between sham-treated tissues and the ozone latency periods were determined using unpaired t-tests for each treatment group (basal, EGTA and calcium/calmodulin). A p value of less than 0.05 was considered statistically significant.

3. RESULTS

Characterization of the Ozone-Induced Airway Hyperresponsiveness Model

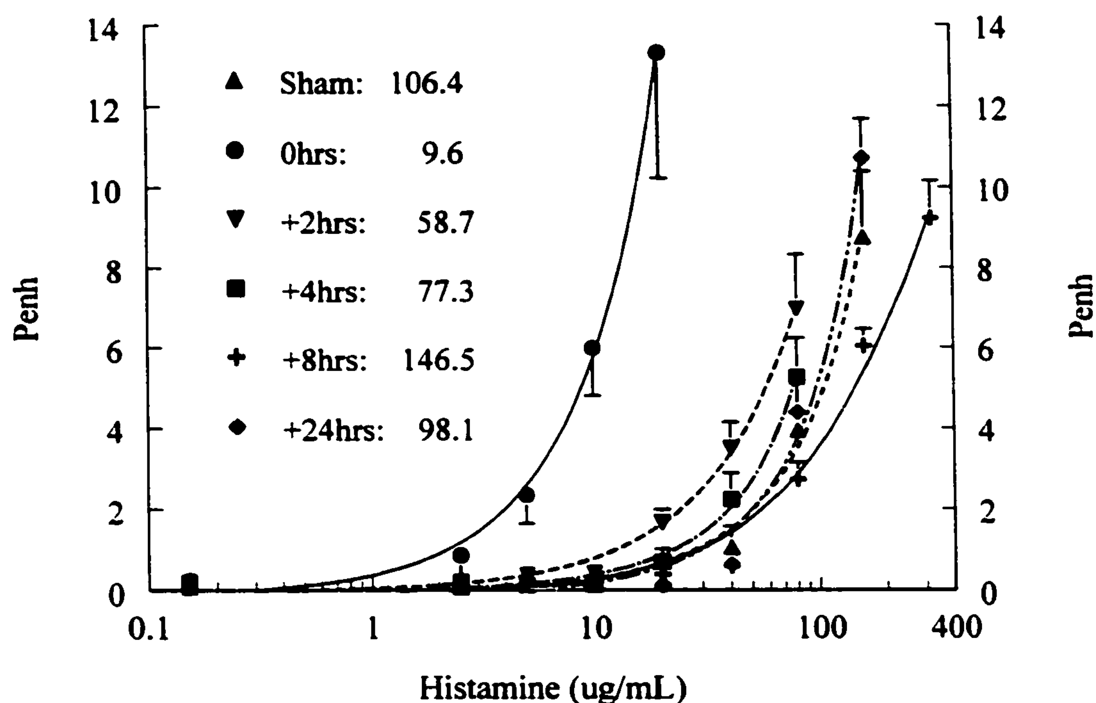
Time course of Ozone Effects on Histamine Airway Responsiveness

Inhaled ozone caused immediate and transient histamine airway hyperresponsiveness (Figure 3). The histamine PC_5 in the sham group was 106.4 ± 11.2 μg/mL and this was significantly reduced to 9.6 ± 0.8 μg/mL immediately following ozone (p value < 0.05). The degree of the shift was partially attenuated at 2 hours, with a histamine PC_5 of 58.7 ± 4.0 μg/mL and reduced further at 4 hours, with a histamine PC_5 of 77.3 ± 5.4 μg/mL. The histamine PC_5 value of 146.5 ± 17.2 μg/mL at 8 hours was significantly greater than that of the sham-treated group. At 24 hours the histamine PC_5 value was 98.1 ± 10.9 μg/mL, which was similar to that of the sham-treated group.

Changes in Inflammatory Cells in BAL following Ozone

In untreated animals the BAL consisted predominately of monocytes with the remaining cells being eosinophils (Table 1). Neutrophils were not detected in sham-treated airways. The monocyte population decreased significantly immediately following ozone (reduced from 191.4 ± 4.2 cells/mL x 10^{-3} to 115.6 ± 34.8 cells/mL x 10^{-3}, $p < 0.05$). The monocyte cell population did not change significantly from this level at all other time points examined.

Figure 3: In vivo responses to aerosolized histamine in sham- and ozone-treated guinea pigs.



Airway responsiveness (as measured by enhanced Pause, or P_{enh}) to aerosolized histamine following ozone exposure as measured by whole body plethysmography (WBP). Concentration-response curves are shown without (▲) or following a 30 minute exposure to ozone (3 ppm) at 0 hours (•), 2 hours (▼), 4 hours (■), 8 hours (+), and 24 hours (◆). Histamine PC_5 values are displayed in the legend. Data are shown as mean ± s.e.mean, n=5 to 8 animals per curve.

Table 1: Profile of inflammatory cell infiltrates as detected by bronchoalveolar lavage.

	Cell Number/mL x 10^{-3}			
	Monocytes	*Macrophages*	*Eosinophils*	*PMNs*
Control	191.4 ± 4.2	N.D.	28.6 ± 1.4	N.D.
0 hrs	115.6 ± 34.8	156.4 ± 12.6	68.0 ± 23.1	N.D.
2 hrs	134.8 ± 30.1	161.7 ± 22.6	73.2 ± 7.4	15.4 ± 1.4
4 hrs	88.1 ± 12.8	183.8 ± 44.8	84.3 ± 11.9	26.8 ± 4.8
8 hrs	122.4 ± 28.7	92.8 ± 53.4	133.6 ± 42.3	22.3 ± 5.1
24 hrs	160.4 ± 18.5	119.4 ± 25.5	74.6 ± 1.4	18.7 ± 3.2

Data are presented as mean ± SD, n=4 for each group. N.D.: Cell type was not detectable in bronchoalveolar lavage fluid.

Macrophages were evident immediately following ozone (156.4 ± 12.6 cells/mL x 10^{-3}, $p < 0.05$ when compared to sham controls) and remained at this level beyond four hours post-ozone. At eight hours, the macrophage population decreased significantly ($p < 0.05$) to 92.8 ± 23.4 cells/mL x 10^{-3} and did not change significantly from this level (119.4 ± 25.5 cells/mL x 10^{-3}) in the twenty-four hour group.

Eosinophils showed a significant increase in cell number immediately following ozone (68.0 ± 23.1 cells/mL x 10^{-3} versus 28.6 ± 1.4 cells/mL x 10^{-3} for control, $p < 0.05$) and did not change significantly from this elevated level. Neutrophils were undetectable until two hours post-ozone and were only 2% of the total cell population (15.4 ± 1.4 cells/mL x 10^{-3}). A significant increase in neutrophils was observed at four hours (26.8 ± 4.8 cells/mL x 10^{-3}, $p < 0.05$) and remained elevated at eight (22.3 ± 5.1 cells/mL x 10^{-3}) and twenty four hours (18.7 ± 3.2 cells/mL x 10^{-3}) post-ozone. Pre-treatment of the animals with dexamethasone (3mg/kg i.p.) did not alter the inflammatory cell response following ozone exposure (Table 2).

Effects of Dexamethasone on Ozone-Induced Airway Hyperresponsiveness

Dexamethasone had no effect on the histamine concentration-response curve in sham-treated animals (histamine PC_5 of 123.9 ± 11.7 µg/mL, Figure 4), but attenuated the ozone-induced changes in histamine PC_5 concentration. The histamine PC_5 concentration was 9.6 ± 0.8 µg/mL immediately following ozone in the absence of dexamethasone and

109.6 ± 9.7 μg/mL when dexamethasone treatment preceded ozone exposure ($p < 0.05$). The dexamethasone pre-treated 2 hour post-ozone group showed significant airway hyperresponsiveness, with a histamine PC_5 value of 52.4 ± 5.8 μg/mL ($p < 0.05$), but this was eliminated by 4 hours with a histamine PC_5 value of 217.0 ± 21.4 μg/mL. The dexamethasone pre-treated 8 hour post ozone group had a histamine PC_5 value of 201.4 ± 19.7 μg/mL and still demonstrated histamine hyporeactivity similar to that observed in the absence of dexamethasone. At 24 hours post ozone, the histamine PC_5 value was 123.1 ± 11.1 μg/mL following dexamethasone pre-treatment and was not significantly different from the histamine PC_5 value of 98.1 ± 10.9 μg/mL observed in the absence of dexamethasone.

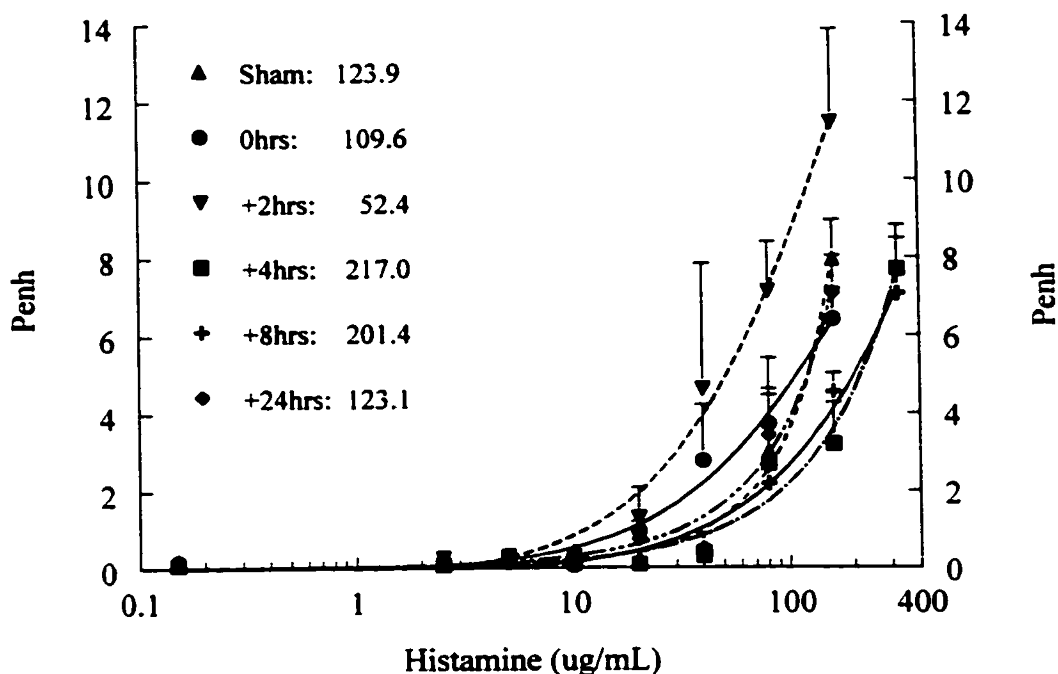
The dexamethasone-sensitive inhibition of histamine-induced airway hyperresponsiveness was not associated with an inhibition of the inflammatory cell infiltrate. The presence of both macrophages and neutrophils increased following ozone (133.7 and 1.4 cells/mL $x10^{-3}$, respectively). Both macrophages and neutrophils reached equally maximal concentrations in both untreated and dexamethasone treated animals. Eosinophils reached marginally greater concentrations in untreated (133.6 ± 42.3) animals when compared to dexamethasone treated (106.8 ± 27.1) animals.

Table 2: Profile of inflammatory cell infiltrates as detected by bronchoalveolar lavage following Dexamethasone (3 mg/kg).

	Cell Number/mL x 10^{-3}			
	Monocytes	*Macrophages*	*Eosinophils*	*PMNs*
Control	215.3 ± 8.7	N.D.	32.4 ± 1.8	N.D.
0 hrs	122.9 ± 30.4	133.7 ± 9.2	55.2 ± 19.6	1.4 ± 0.2
2 hrs	156.9 ± 26.6	143.8 ± 17.8	80.6 ± 10.2	12.2 ± 1.3
4 hrs	119.7 ± 19.2	154.3 ± 41.2	79.8 ± 9.1	33.9 ± 6.9
8 hrs	141.6 ± 31.5	101.8 ± 35.1	95.3 ± 24.5	27.8 ± 5.6
24 hrs	148.0 ± 23.5	104.4 ± 26.5	106.8 ± 27.1	22.0 ± 4.2

Data are presented as mean ± SD, n=4 for each group N.D.: Cell type was not detectable in bronchoalveolar lavage fluid.

Figure 4: Effect of Dexamethasone (3 mg/kg) pre-treatment on airway responsiveness to histamine.



Airway responsiveness (as measured by enhanced Pause, or P_{enh}) to aerosolized histamine following ozone exposure as measured by whole body plethysmography (WBP). Animals were treated with dexamethasone (3 mg/kg i.p.) 15 minutes prior to ozone exposure. Concentration-response curves are shown without (▲) or following a 30 minute exposure to ozone (3 ppm) at 0 hours (•), 2 hours (▼), 4 hours (▪), 8 hours (+), and 24 hours (◆). Histamine PC_5 values are displayed in the legend. Data are shown as mean ± s.e.mean, n=5 to 8 animals per curve.

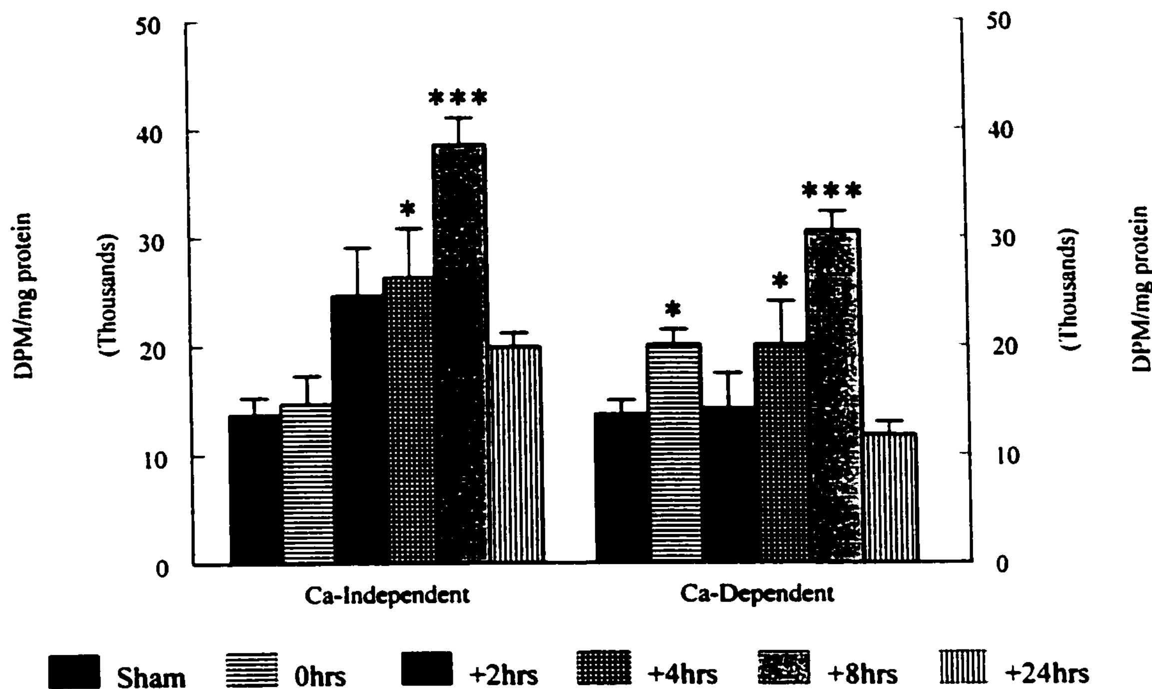
Nitric Oxide and Nitric Oxide Synthase

Biochemical Analysis of NOS Activity after Inhaled Ozone

Bronchi

Determination of nitric oxide synthase activity in guinea pig bronchial homogenates was conducted to identify changes in enzyme activity at various time points following ozone exposure. The results are shown in figure 5. In the sham group (solid bars) there was both a calcium-dependent, L-NAME sensitive, (13,760 ± 1,186 dpm/mg protein) and calcium-independent, L-NAME insensitive, (13,787 ± 1,457 dpm/mg protein) conversion of arginine to citrulline. Immediately following ozone there was a significant increase in cNOS activity (20,018 ± 1,393 dpm/mg protein, $p < 0.05$ compared to the sham group) with no significant difference in the calcium-independent activity (14,655 ± 2,601 dpm/mg protein). There was an increase in the calcium-independent activity (24,632 ± 4,455 dpm/mg protein) at two hours although this change was not statistically significant. Calcium-dependent NOS activity decreased to the level observed in the sham-treated group (14,126 ± 3,289 dpm/mg protein). Four hours following ozone, NOS enzymatic activity for both calcium-dependent (20,023 ± 4,019 dpm/mg protein) and calcium-independent (26,310 ± 4,534 dpm/mg protein) isoforms increased significantly ($p < 0.05$ compared to respective sham group). The enzyme activities increased further at eight hours post-ozone, with a calcium-dependent activity of 30,612 ± 1,800 dpm/mg protein and a calcium-independent

Figure 5: Enzymatic conversion of radiolabelled arginine to citrulline in guinea pig



bronchi following ozone exposure.

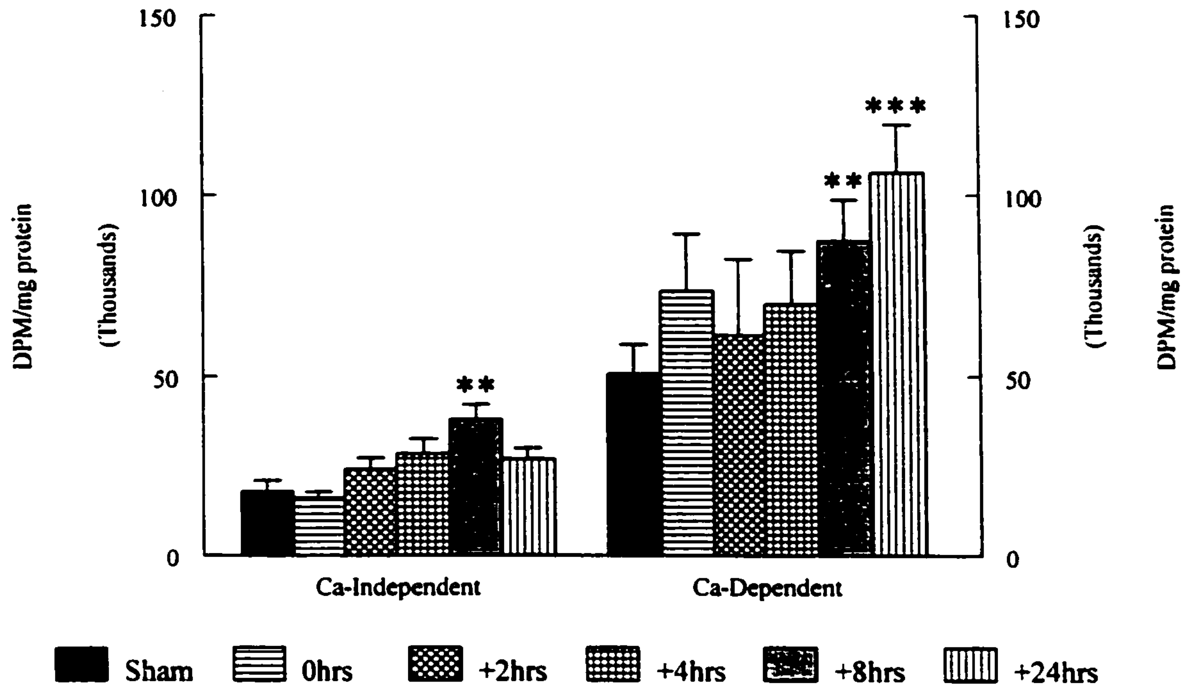
Effect of ozone exposure on NOS enzyme activity in guinea pig bronchial microsomes as measured by conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline. Microsomes were prepared by separation of upper bronchi (1st to 4th order) and homogenization in sucrose-MOPS buffer (see Methods section for details). The microsomal preparation was incubated for 30 minutes at 37°C, then transferred to an anion exchange column from which [^{3}H]-L-citrulline was eluted. Radioactivity was determined by a beta counter and the results reported as disintegrations per minute (DPM) per milligram of protein. Data are presented as mean ± s.e.mean, n=8, *=$p<.05$, ***=$p<.001$.

activity of 38,549 ± 2,476 dpm/mg protein. Both values were significantly higher than their respective controls ($p < 0.005$). By twenty four hours the level of enzyme activity returned to values not significantly different from baseline values for both calcium-dependent (11,781 ± 1,222 dpm/mg protein) and calcium-independent (19,858 ± 1,301 dpm/mg protein) isoforms.

Lung Tissue

In the sham group (solid bars), there was both a calcium-dependent (50,661 ± 8,007 dpm/mg protein) and calcium-independent (18,001 ± 3,005 dpm/mg protein) conversion of arginine to citrulline. Immediately following ozone, there was a non-significant increase in cNOS activity (73,497 ± 15,756 dpm/mg protein; Figure 6) and no change in the calcium-independent activity (15,917 ± 1,876 dpm/mg protein). The calcium-independent activity (24,102 ± 3,191 dpm/mg protein) increased, but not significantly, at two hours. The only significant changes in enzyme activity levels occurred at eight hours post-ozone, when both the calcium-dependent activity of 87,318 ± 11,406 dpm/mg protein and the calcium-independent activity of 37,926 ± 4,173 dpm/mg protein were significantly increased ($p < 0.01$). By twenty-four hours the level of enzyme activity returned toward baseline values for the calcium-independent (26,931 ± 3,063 dpm/mg protein) isoform. At twenty-four hours, the calcium-dependent activity reached the highest levels measured in the lung tissue (106,384 ± 13,353 dpm/mg protein) and was significantly ($p < 0.05$) higher than the sham-treated group.

Figure 6: Enzymatic conversion of radiolabelled arginine to citrulline in guinea pig lung tissue following ozone.



Effect of ozone exposure on NOS enzyme activity in guinea pig parenchymal microsomes as measured by conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline. Microsomes were prepared by removal of parenchyma from upper bronchi and homogenized in a sucrose-MOPS buffer (see Methods section for details). The microsomal preparation was incubated for 30 minutes at 37°C, then transferred to an anion exchange column from which [^{3}H]-L-citrulline was eluted. Radioactivity was determined by a beta counter and the results reported as disintegrations per minute (DPM). Data are presented as mean ± s.e.mean, n=8, *=p<.05, ***=p<.001.

In Vitro Contractility Studies and Pharmacological Manipulation

Histamine-Induced Contraction

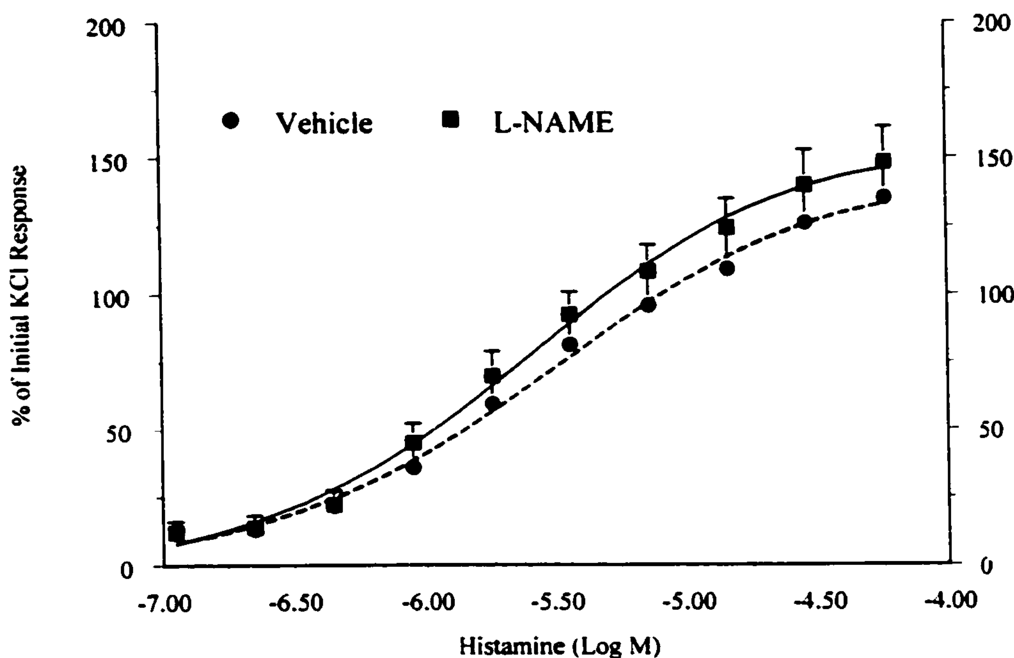
Effect of NOS Inhibitors

The contribution that endogenous NO has on regulating tracheal smooth muscle contractility was evaluated using two different nitric oxide synthase inhibitors in epithelium intact preparations of guinea pig trachea. The arginine analogue, L-NAME, at a concentration of 100 μM, marginally shifted the concentration-response curves to the left with an elevated maximal tension generation in both the presence and absence of the cyclooxygenase inhibitor, indomethacin (Figures 7 and 8). Similar findings were obtained with another analogue of arginine, L-NOARG (Figure 9). Indomethacin did not significantly alter histamine responsiveness in the presence of NOS inhibition (Figure 10).

In Vitro Induction of NOS in Guinea Pig Trachea

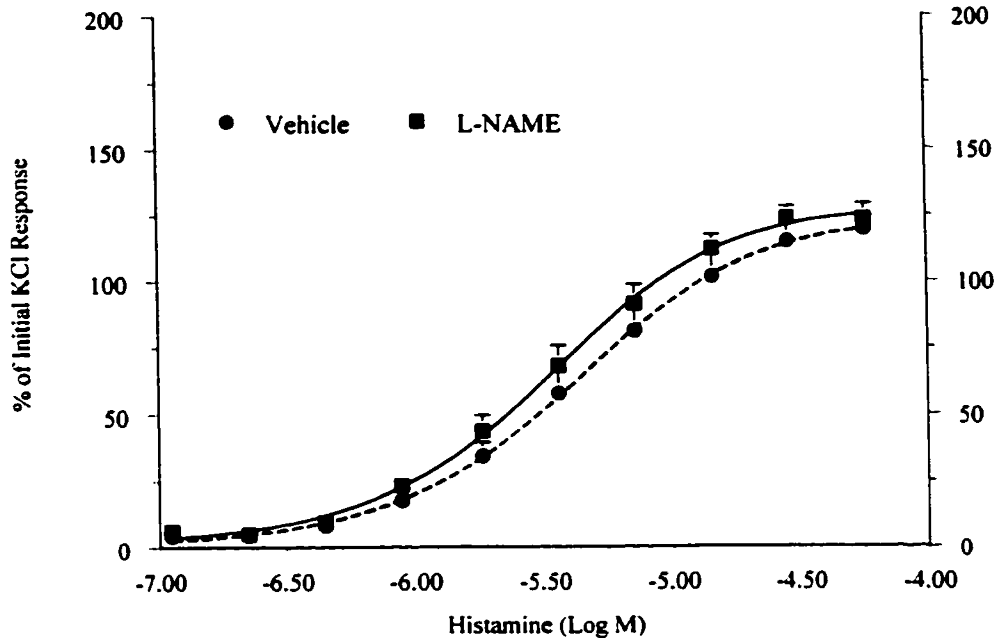
To examine the effect of NOS upregulation in the trachea, a combination of three inflammatory cytokines (interleukin-1β, tumour necrosis factor-α and interferon-γ, referred to as cytomix) were combined with bacterial lipopolysaccharide (LPS) to induce *in vitro* NOS upregulation. This cytomix/LPS mixture was added to the in vitro tracheal preparations while under resting tension for 30 minutes.

Figure 7: Effect of NO synthase inhibitor in the absence of COX inhibition on guinea pig trachea in the presence of epithelium.



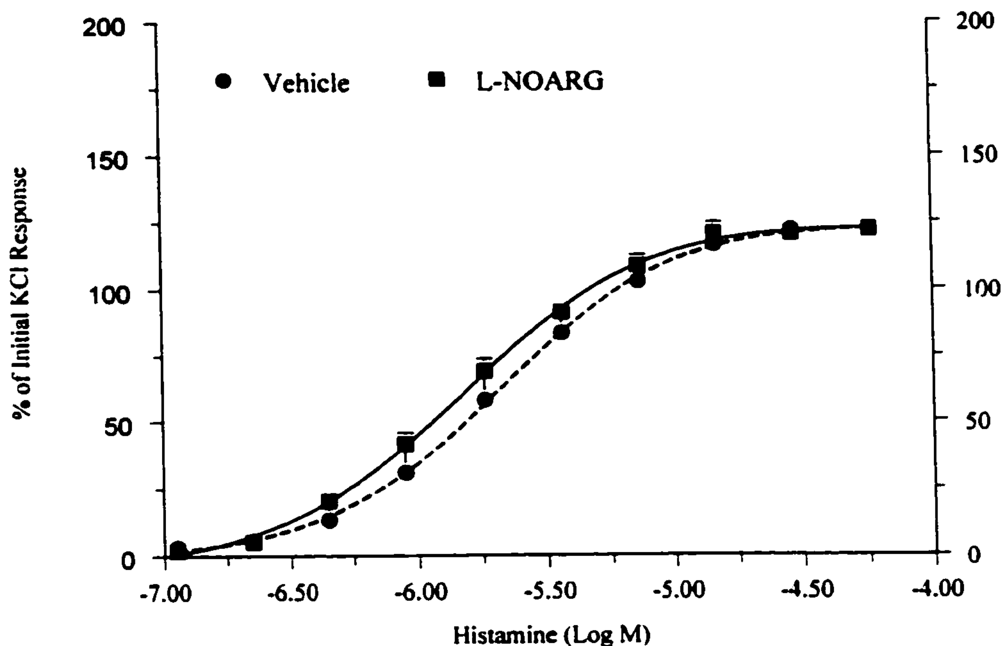
Histamine concentration-response curves for guinea pig tracheal rings in the absence (•) and presence (■) of the NOS inhibitor, L-NAME (100 μM). Untreated (no ozone exposure) guinea pig tracheas were excised and cut into 3 mm open rings. A tracheal smooth muscle chain of 4 open rings tied with surgical suture was placed in a tissue bath containing Krebs solution at 37°C. Tracheal chains were pre-contracted with potassium chloride (KCl) to determine the baseline contractile response of each preparation. Data are shown as mean ± s.e.mean, n=5 for each curve.

Figure 8: Effect of NO synthase inhibitor in the presence of COX inhibition on guinea pig trachea in the presence of epithelium.



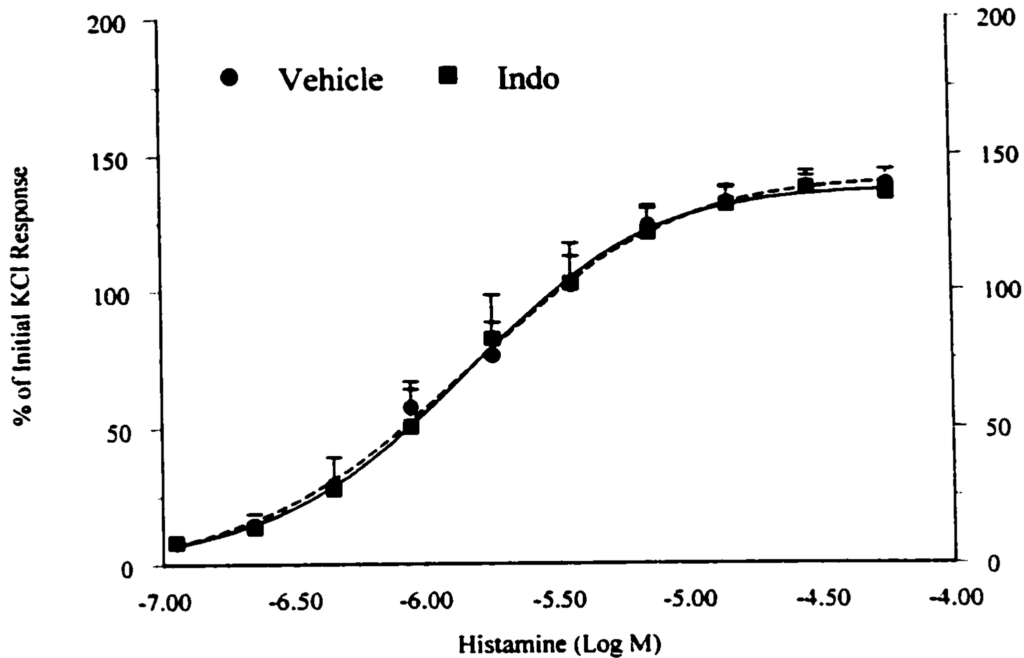
Histamine concentration-response curves for guinea pig tracheal rings in the absence (●) and presence (■) of the NOS inhibitor, L-NAME (100 μM) with a COX inhibitor, indomethacin (0.5 μg/mL), present in the bathing medium. Untreated (no ozone exposure) guinea pig tracheas were excised and cut into 3 mm open rings. A tracheal smooth muscle chain of 4 open rings tied with surgical suture was placed in a tissue bath containing Krebs solution at 37°C. Tracheal chains were pre-contracted with potassium chloride (KCl) to determine the baseline contractile response of each preparation. Data are shown as mean ± s.e.mean, n=5 for each curve.

Figure 9: Effect of a NO Synthase inhibitor (L-NOARG, 100 μM) on histamine responsiveness in the guinea pig trachea in vitro.



Histamine concentration-response curves for guinea pig tracheal rings in the absence (•) and presence (■) of the NOS inhibitor, L-NOARG (100 μM). Untreated (no ozone exposure) guinea pig tracheas were excised and cut into 3 mm open rings. A tracheal smooth muscle chain of 4 open rings tied with surgical suture was placed in a tissue bath containing Krebs solution at 37°C. Tracheal chains were pre-contracted with potassium chloride (KCl) to determine the baseline contractile response of each preparation. Data are shown as mean ± s.e.mean, n=5 for each curve.

Figure 10: Effect of COX inhibition on histamine responsiveness of guinea pig trachea in the presence of NOS inhibition.



Histamine concentration-response curves for guinea pig tracheal rings in the absence (•) and presence (■) of the COX inhibitor, indomethacin (0.5 µg/mL). Untreated (no ozone exposure) guinea pig tracheas were excised and cut into 3 mm open rings. A tracheal smooth muscle chain of 4 open rings tied with surgical suture was placed in a tissue bath containing Krebs solution at 37°C. Tracheal chains were pre-contracted with potassium chloride (KCl) to determine the baseline contractile response of each preparation. Data are shown as mean ± s.e.mean, n=5 for each curve.

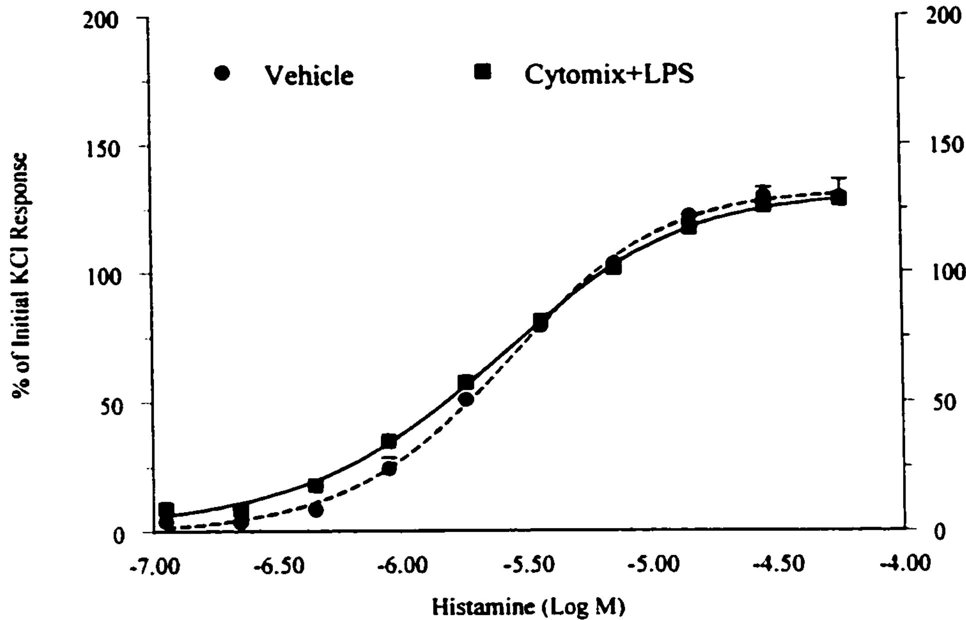
There was no discernible difference in histamine-stimulated smooth muscle contraction between the untreated tracheal tissue and that exposed to the cytomix preparation (Figure 11), possibly because exposure time was insufficient for enzyme upregulation.

Nitric Oxide Stimulated Relaxation

Effect of COX Inhibition

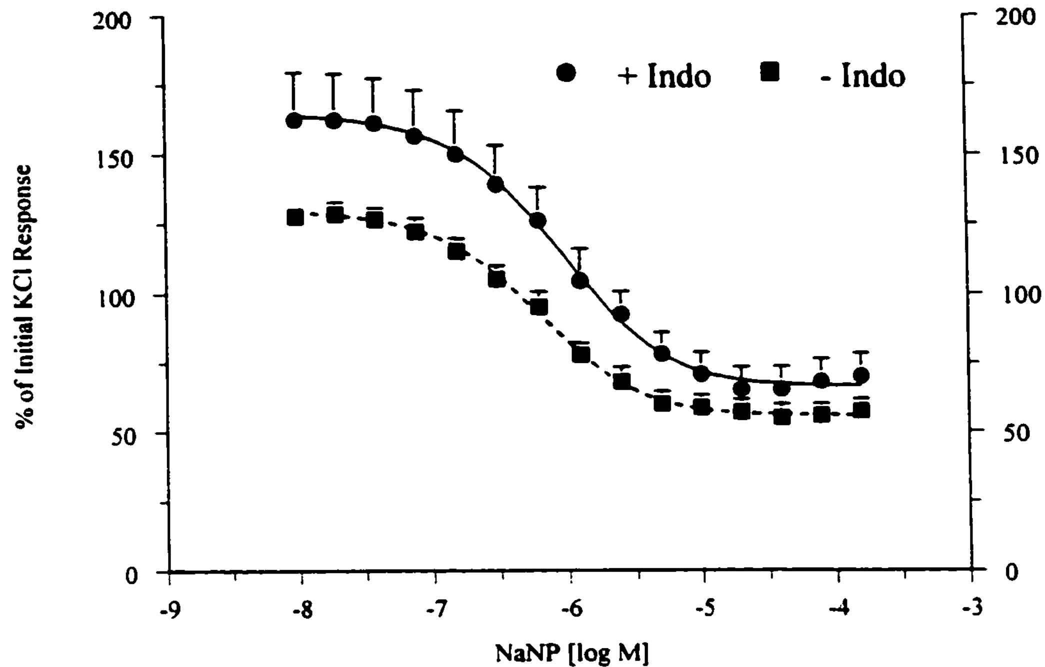
The response of tracheal rings to exogenous NO liberating compounds was determined in preparations pre-contracted with 20 μM histamine in the absence or presence of the COX inhibitor indomethacin (Figure 12). COX inhibition attenuated the response to lower concentrations of histamine and with these tissues pre-contracted with a relatively low concentration of histamine, 20 μM, indomethacin did attenuate the histamine pre-contraction, but this change did not reach statistical significance. The tracheal rings were concentration-dependently relaxed by sodium nitroprusside. The COX inhibited tissues showed a reduced response to the sodium nitroprusside when compared to the vehicle-treated tissue. Of particular note is the similar, maximally attainable, percentage relaxation of approximately 50% of the tissue responses to potassium chloride. There is no statistically significant decrease in muscle tension for the final five concentrations of sodium nitroprusside.

Figure 11: Effect of in vitro exposure of guinea pig tracheal rings to cytomix and LPS.



Histamine concentration-response curves for guinea pig tracheal rings in the absence (•) and presence (■) of LPS and cytomix (IFN-γ, TNF-α and IL-1β). LPS + cytomix was added to the bathing medium 30 minutes prior to the concentration-response curves. Untreated (no ozone exposure) guinea pig tracheas were excised and cut into 3 mm open rings. A tracheal smooth muscle chain of 4 open rings tied with surgical suture was placed in a tissue bath containing Krebs solution at 37°C. Tracheal chains were pre-contracted with potassium chloride (KCl) to determine the baseline contractile response of each preparation. Data are shown as mean ± s.e.mean, n=5 for each curve. All tissues were incubated with indomethacin (0.5 μg/mL).

Figure 12: Effect of COX inhibition on the guinea pig tracheal responsiveness to exogenous NO.



Sodium nitroprusside (NaNP) concentration-response curves for guinea pig tracheal rings in the absence (•) and presence (■) of indomethacin (0.5 µg/mL). Untreated (no ozone exposure) guinea pig tracheas were excised and cut into 3 mm open rings. A tracheal smooth muscle chain of 4 open rings tied with surgical suture was placed in a tissue bath containing Krebs solution at 37°C. Tracheal chains were pre-contracted with potassium chloride (KCl) to determine the baseline contractile response of each preparation. Data are shown as mean ± s.e.mean, n=5 for each curve.

Effect of NOS Inhibition

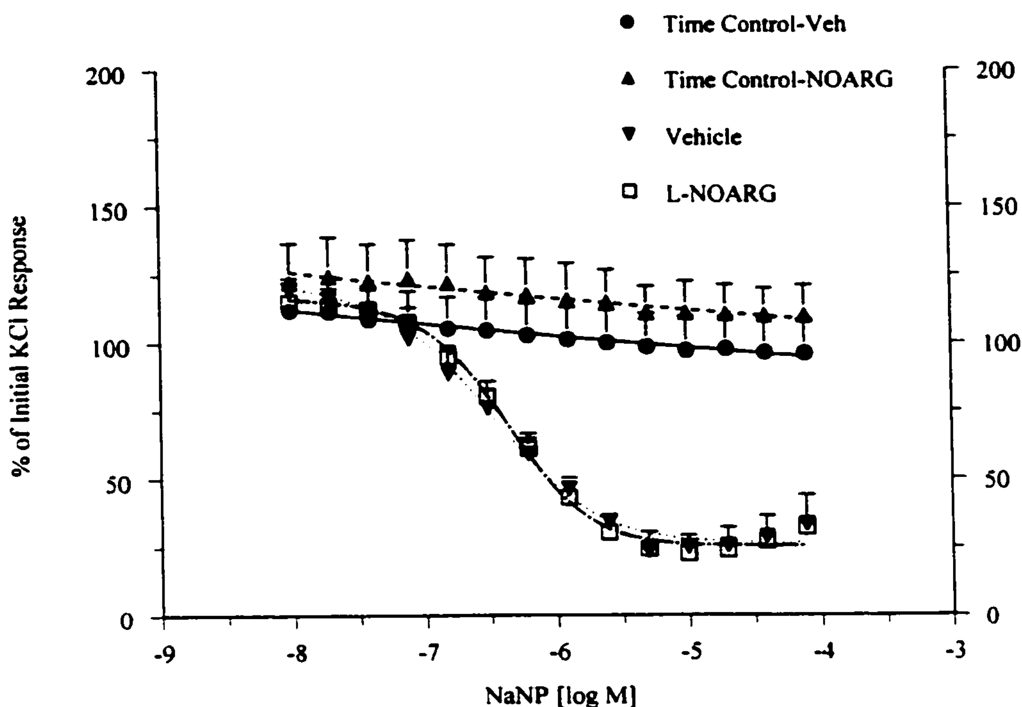
It was necessary to demonstrate in this model that the inhibition of NOS by selective inhibitors acted to inhibit NO production but did not alter the downstream pathway for smooth muscle relaxation. The presence of the NOS inhibitor L-NOARG alone increased the resting basal tension in the tracheal strips by approximately 10% (not statistically significant) when compared to control. In tissue strips relaxed with increasing concentrations of sodium nitroprusside, there was no detectable difference between control tissues and those treated with L-NOARG (Figure 13). It should be noted that a tachyphylaxis to sodium nitroprusside occurred after 35 minutes both in the presence and absence of the nitric oxide synthase inhibitor. All experiments were conducted in the presence of 0.5 μg/mL indomethacin.

Effect of PDE V Inhibition

The effect of the phosphodiesterase V inhibitor zaprinast was examined in the tissue strips. In the presence of 50 μM zaprinast the concentration-response curve to sodium nitroprusside was shifted significantly leftward, with the IC_{50} value decreasing by a log unit (log M IC_{50}: -6.6) when compared to the vehicle control (log M IC_{50}: -5.6). The final tension attained was 55% of that obtained with vehicle alone and there was no apparent tachyphylaxis after 35 minutes. The presence of the vehicle solution did augment the initial tissue response to histamine (log M IC_{50}: -5.3) although the relaxation

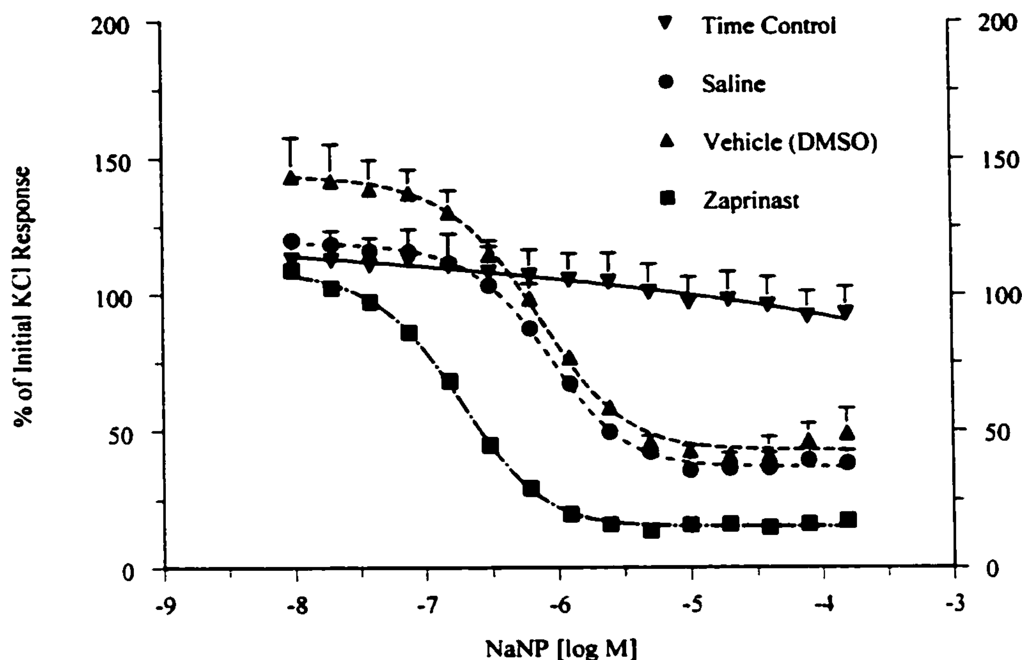
response closely mimicked that of the saline control (log M IC_{50}: -5.6). The source of this difference in initial responsiveness is not clear. There was a slight loss of tone when the tissues were left untreated after the initial histamine prime (time control) and did not exceed 14% of the initial tone (Figure 14).

Figure 13: Effect of the NOS inhibitor, L-NOARG, on NaNP induced relaxation in guinea pig trachea.



Sodium nitroprusside (NaNP) concentration-response curves for guinea pig tracheal rings in the absence (▼) and presence (□) of L-NOARG (100 μM). Tracheal chains were incubated in the absence of NaNP to assess the effect of time on the smooth muscle contractility in both the absence (•) and presence (▲) of L-NOARG (100 μM). Untreated (no ozone exposure) guinea pig tracheas were excised and cut into 3 mm open rings. A tracheal smooth muscle chain of 4 open rings tied with surgical suture was placed in a tissue bath containing Krebs solution at 37°C. Tracheal chains were pre-contracted with potassium chloride (KCl) to determine the baseline contractile response of each preparation. Data are shown as mean ± s.e.mean, n=5 for each curve.

Figure 14: Responses of guinea pig trachea with epithelium to a PDE V inhibitor following NaNP exposure.



Sodium nitroprusside (NaNP) concentration-response curves for guinea pig tracheal rings in the absence (●) and presence (■) of zaprinast (50 μM). Tracheal chains were incubated in the absence of zaprinast to assess the effect of time on the smooth muscle contractility (▼). The vehicle (DMSO) used for zaprinast was also added separately to determine the effect it may have on smooth muscle contractility (▲). Untreated (no ozone exposure) guinea pig tracheas were excised and cut into 3 mm open rings. A tracheal smooth muscle chain of 4 open rings tied with surgical suture was placed in a tissue bath containing Krebs solution at 37°C. Tracheal chains were pre-contracted with potassium chloride (KCl) to determine the baseline contractile response of each preparation. Data are shown as mean ± s.e.mean, n=5 for each curve.

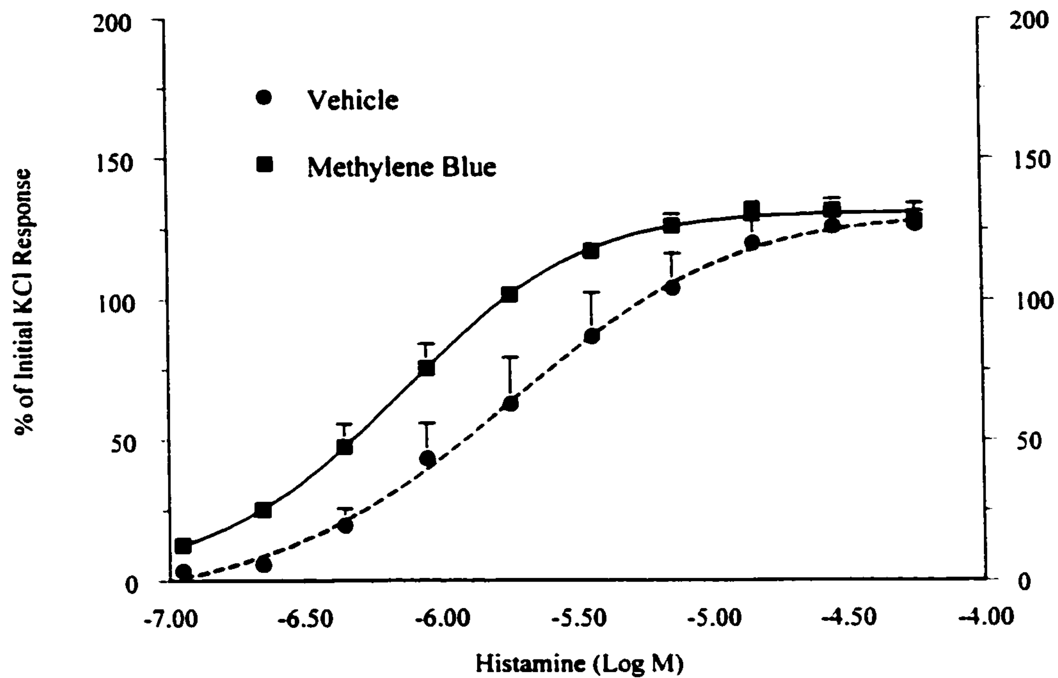
Effect of Guanylyl Cyclase Inhibition

The contribution of the guanylyl cyclase pathway to histamine stimulation of guinea pig trachea with epithelium was identified using the inhibitor methylene blue. As is shown in figure 15, the presence of methylene blue, 10 μM, significantly ($p < 0.05$) augmented the tracheal strip responsiveness to histamine resulting in a ten-fold leftward shift of the concentration-response curve. The response to histamine in the presence of methylene blue was not changed at high concentrations of histamine and in fact the tracheal strips reached a maximum tension generation of approximately 125% of the potassium chloride response. The two curves differed in the concentration at which the maximum response was reached. In the presence of the guanylyl cyclase inhibitor, the maximum responsiveness was attained a log unit concentration lower than in the absence of the inhibitor.

Measurement of Nitrates and Nitrites by HPLC

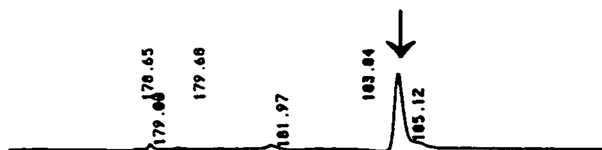
Sequential injection of 10 nM, 100 nM and 1 μM potassium nitrate in distilled water demonstrates a concentration proportional signal as shown in figure 16. The injection of distilled water shows the carryover that regularly followed injection of 1 μM potassium nitrate solution (Figure 17). Injection of the Henseleit-Krebs solution, used in the bathing medium for tissue bath experiments, is shown in figure 18. Due to the high salt content in the buffer, there was considerable interference with the retention time of the nitrate peak and therefore these studies could not be executed.

Figure 15: Responses of guinea pig trachea with epithelium to histamine in the presence of a guanylyl cyclase inhibitor (10 μM).

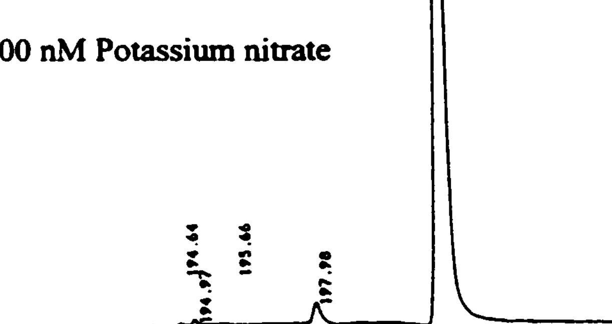


Histamine concentration-response curves for guinea pig tracheal rings in the absence (●) and presence (■) of the guanylyl cyclase inhibitor, methylene blue (100 μM). Untreated (no ozone exposure) guinea pig tracheas were excised and cut into 3 mm open rings. A tracheal smooth muscle chain of 4 open rings tied with surgical suture was placed in a tissue bath containing Krebs solution at 37°C. Tracheal chains were pre-contracted with potassium chloride (KCl) to determine the baseline contractile response of each preparation. Data are shown as mean ± s.e.mean, n=5 for each curve.

10 nM Potassium nitrate



100 nM Potassium nitrate



1 μM Potassium nitrate

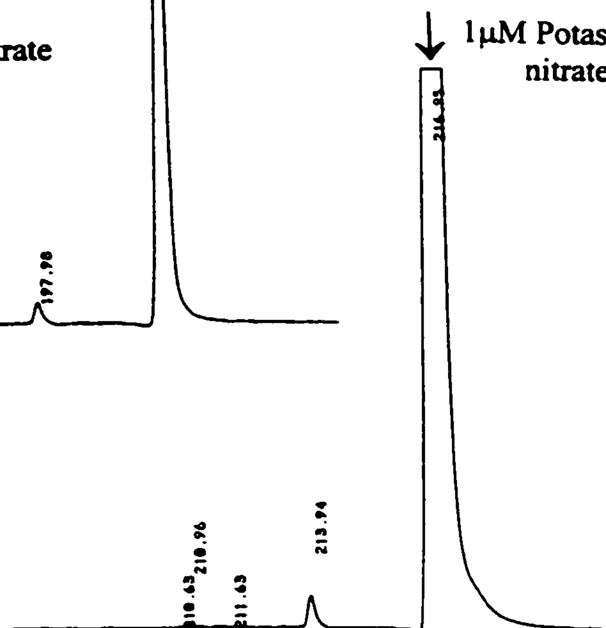


Figure 16: Chromatogram showing a nitrate standard curve.

Separate injections of potassium nitrate are shown above. Nitrate concentrations are, from top to bottom, 10 nM, 100 nM and 1 μM. The nitrate peak elutes at 9.7 minutes and is the right most peak on each trace, above.

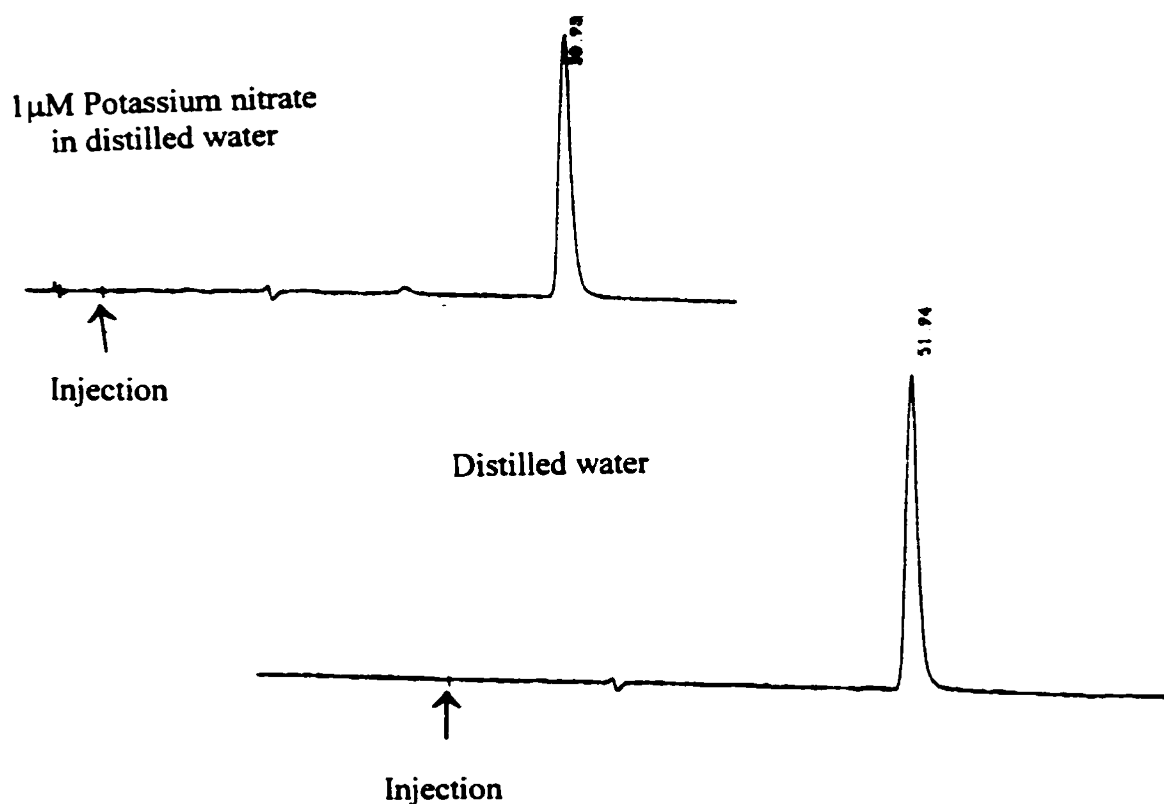


Figure 17: Chromatogram showing the nitrate elution profile and resulting carryover observed with a dH_2O injection immediately following the nitrate injection.

The HPLC tracings shown here show the injection of 1 μM of potassium nitrate (top) and the subsequent injection of distilled water (bottom). The peak in the bottom tracing is a result of excess nitrate remaining on the HPLC column (carryover).

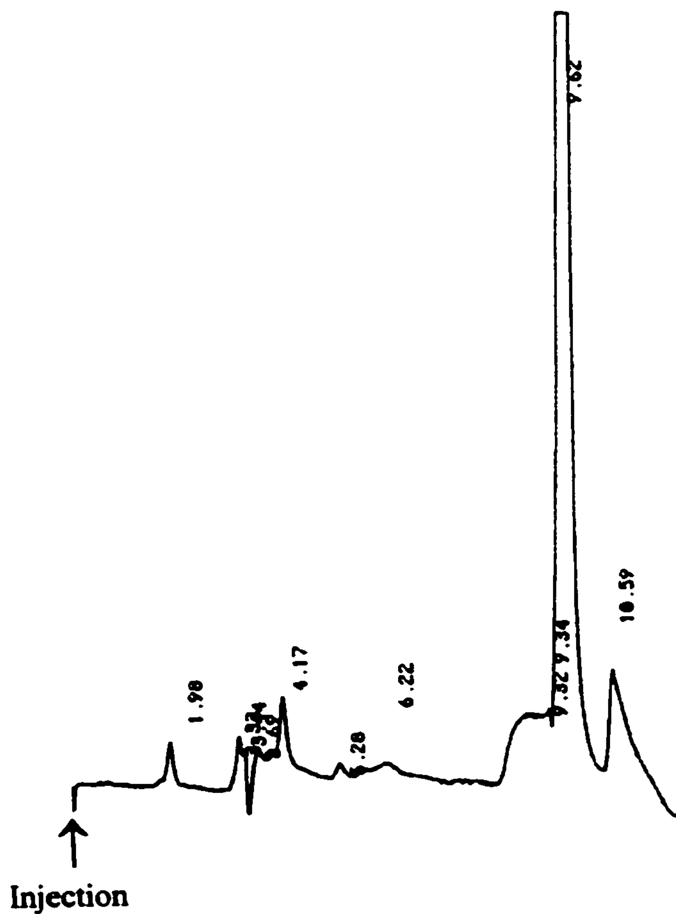


Figure 18: Chromatogram showing the elution profile of the Krebs' solution with multiple peaks.

The injection of the Krebs' solution, used for tissue bath experiments, produces many peaks. The high salt content of the Krebs' solution made separation of nitrate and nitrite extremely difficult.

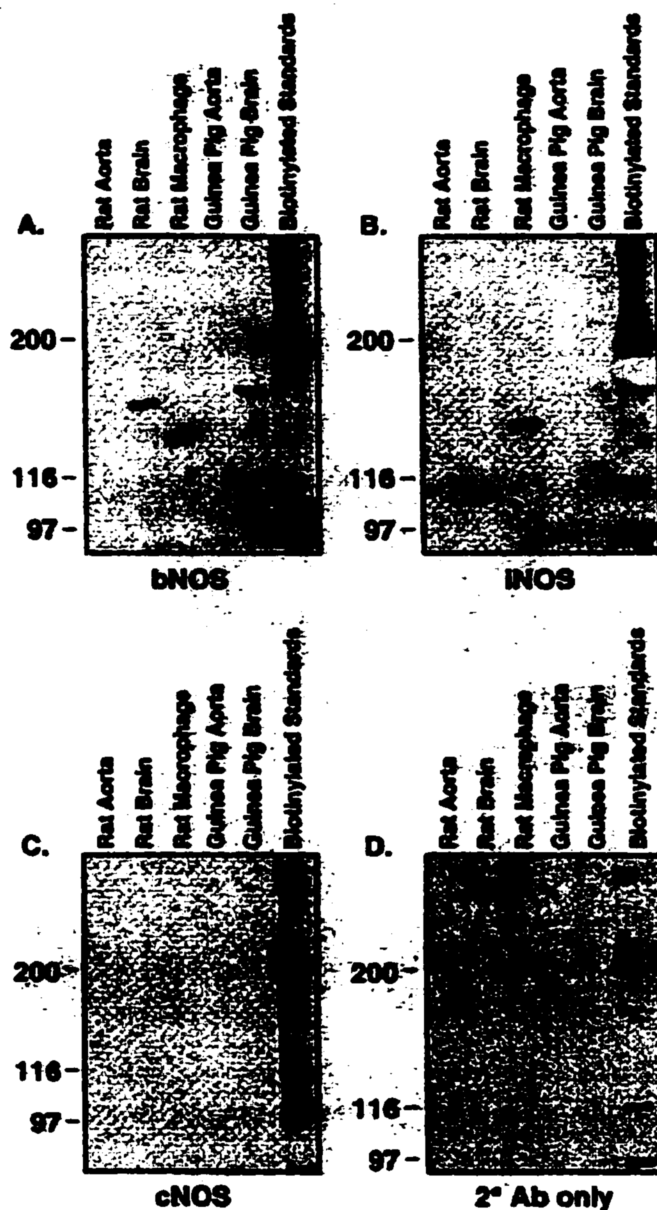


Figure 19: Protein expression of NOS isoforms in rat and guinea pig tissue preparations with monoclonal NOS antibodies.

Representative blots of each NOS isoform are shown above. Tissues were prepared as described in Experimental Methods. The neural isoform (A, bNOS) monoclonal antibody recognizes the brain preparations in both rat and guinea pig bNOS protein but also recognizes the iNOS protein in rat macrophages. The inducible isoform (B, iNOS) monoclonal antibody recognized the iNOS protein in the rat macrophage preparation. The endothelial isoform (C, eNOS) monoclonal antibody did not recognize any NOS isoform. Blot D shows the protein bands produced by the secondary antibody in the absence of protein specific primary antibody.

Immunochemical Detection of NOS

Some of our data suggested the presence of modulation of airway responsiveness by endogenous NO. Detection of the protein for nitric oxide synthase in lung and bronchial tissue using monoclonal antibodies for each isoform (neural, inducible and endothelial) is shown in figure 19. Tissues from both rats and guinea pigs were used for comparative purposes. In blot A, with the monoclonal antibody to the neural isoform, a single band is seen in rat brain (representing a neural source of NOS), rat macrophage (representing an inducible source of NOS), and guinea pig brain (neural or brain NOS). The bands for both the rat and guinea pig brain were at approximately 155 kDa and the band observed in the rat macrophage was at approximately 125 kDa. In blot B, with a monoclonal antibody to the inducible isoform of NOS, a single band was seen in the rat macrophage preparation with an approximate mass of 145 kDa. In blot C, with a monoclonal antibody to the endothelial isoform of NOS, no protein was detected. There was non-specific binding detected in the rat macrophage preparation at approximately 200 kDa, as shown in blot D. This non-specific binding was not observed in the presence of a primary antibody to any NOS isoform.

The same series of experiments were performed using a polyclonal antibody instead of a monoclonal antibody, the results of which are shown in figure 20. In blot A, with a polyclonal antibody to the neural isoform, a single band was observed in the guinea pig aorta (representative source of the endothelial isoform), guinea pig macrophage and rat macrophage. All three bands were approximately 185 kDa with the

positive control for NOS identified at approximately 155 kDa. In blot B, with a polyclonal antibody to the endothelial isoform, a faint single band was found in the guinea pig aorta with a mass of approximately 155 kDa. The signal obtained with the polyclonal antibody to the inducible isoform of NOS is shown in blot C. A single band was found in the guinea pig aorta, matching the band of approximately 155 kDa found in the positive control for NOS. In blot D, the absence of the primary antibody demonstrates the lack of non-specific binding with the secondary antibody.

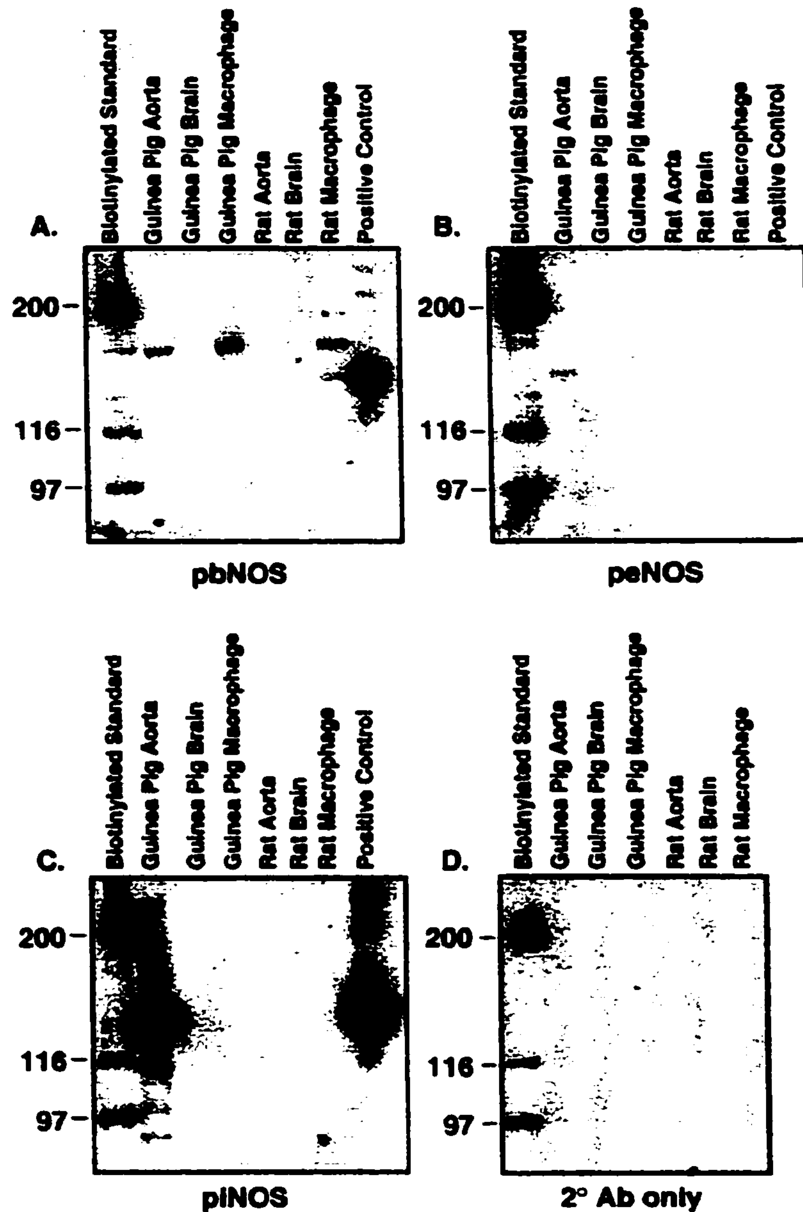


Figure 20: Protein expression of NOS isoforms in rat and guinea pig tissue preparations with polyclonal NOS antibodies.

Representative blots of each NOS isoform are shown above. Tissues were prepared as described in Experimental Methods. The neural isoform (A, pbNOS) polyclonal antibody failed to recognize either the rat or guinea pig bNOS protein. The inducible isoform (C, piNOS) polyclonal antibody recognized a protein similar in mass to NOS in the guinea pig aortic (endothelial) preparation. The endothelial isoform (B, peNOS) polyclonal antibody faintly recognized the guinea pig eNOS protein. Blot D shows the protein bands produced by the secondary antibody in the absence of protein specific primary antibody.

The results from two polyclonal antibodies directed against different epitopes than those described above are shown in figure 21. In blot A, the polyclonal antibody for the neural isoform shows a single band in both the rat and guinea pig brain preparations at the same mass of approximately 155 kDa as that of the positive control (commercially supplied, purified NOS). In blot B, with the polyclonal antibody to the inducible isoform, bands are seen in all preparations tested. Both the rat and guinea pig brain preparations show a faint band at approximately 155 kDa. The rat macrophage preparation has four distinct bands at approximately 200, 190, 145, and 105 kDa. The guinea pig macrophage preparation has one band at approximately 105 kDa.

Effects of L-NAME on Ozone-Induced Histamine Airway Hyperresponsiveness

Initial experiments were performed to identify the effect, if any, which aerosolized L-NAME would have on basal airway tone in sham-treated animals. For concentrations of L-NAME as high as 10.24 mg/mL, there was no observed effect on resting P_{enh} in the absence of histamine provocation (data not shown). When histamine was administered concomitantly with L-NAME at a concentration of 5.21 mg/mL, there was a decrease in the histamine PC_5 at both at 2 (histamine PC_5 of 18.7 ± 1.4 μg/mL, $p < 0.05$), 4 (histamine PC_5 of 29.7 ± 2.2 μg/mL, $p < 0.05$) and 8 (histamine PC_5 of 207.5 ± 19.6 μg/mL, $p < 0.05$) hours following ozone exposure when compared to sham-treated animals.

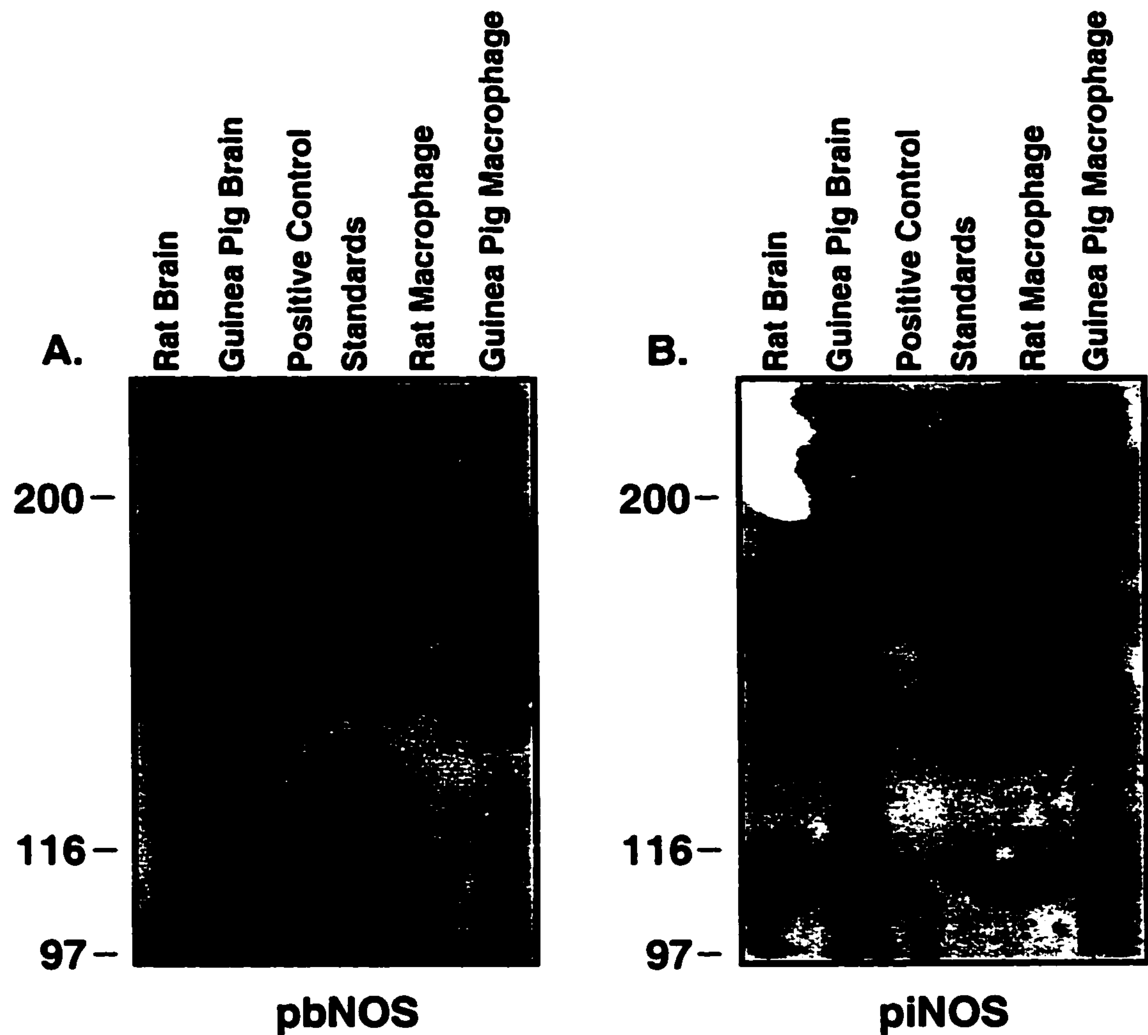


Figure 21: Protein expression of NOS isoforms in rat and guinea pig tissue preparations with polyclonal NOS antibodies.

Representative blots of each NOS isoform are shown above with different polyclonal antibodies than those in Figure 20. Tissues were prepared as described in Experimental Methods. The neural isoform (A, pbNOS) polyclonal antibody recognized both the rat and guinea pig bNOS protein. The inducible isoform (B, piNOS) polyclonal antibody recognized a protein similar in mass to NOS in the rat macrophage preparation but did not recognize any proteins in the guinea pig macrophage preparation.

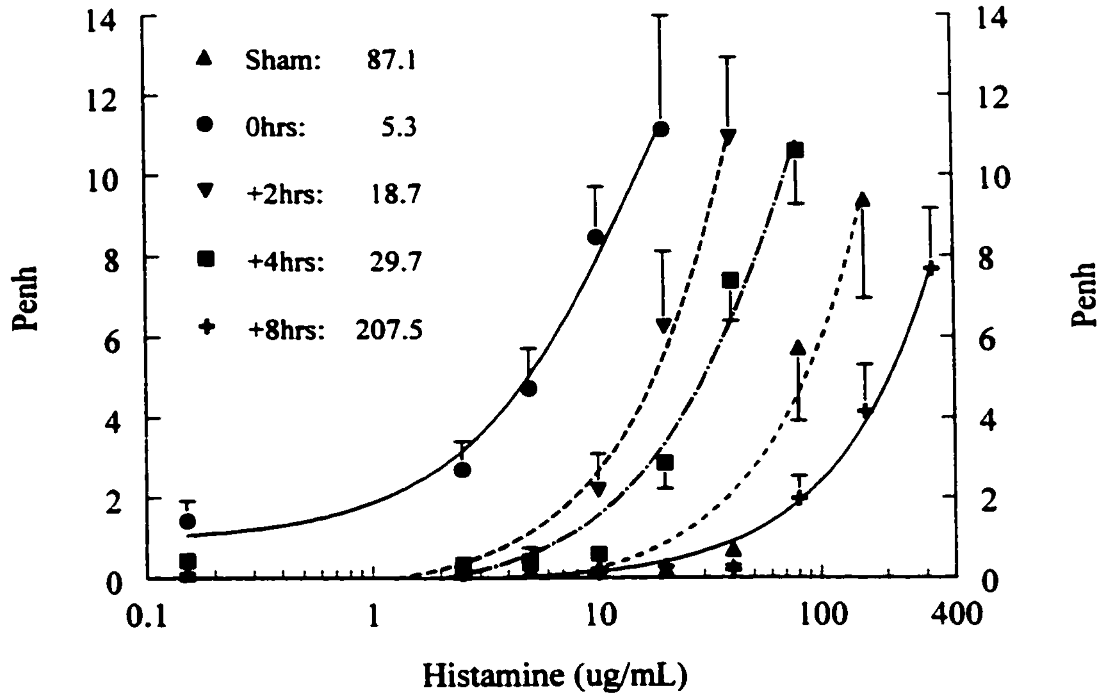
A small, but not statistically significant, decrease in the histamine PC_5 concentration was observed in the sham-treated group (histamine PC_5 of 87.1 ± 8.2 μg/mL). There was no change in the histamine PC_5 concentration immediately following ozone (histamine PC_5 of 5.3 ± 0.8 μg/mL) (Figure 22). A 50% decrease in the L-NAME concentration (2.56 mg/mL) eliminated any detectable *in vivo* effect on the histamine PC_5 concentration (Figure 23).

Cyclooxygenase Activity

Contractility Studies and Pharmacological Manipulation of Tracheal Preparations

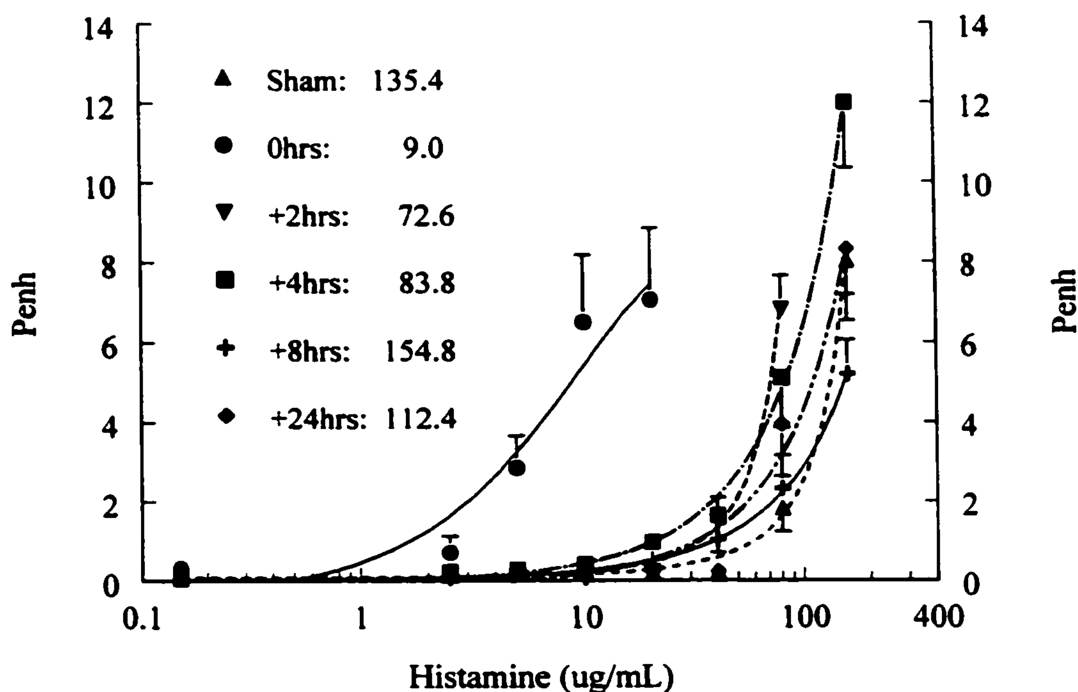
The effect of the COX inhibitor, indomethacin, on the *in vitro* contractility of guinea pig trachea is shown in figure 24. In the absence of the cyclooxygenase inhibitor the highest administered concentration of histamine produces a tension that is double that generated with the priming concentration of potassium chloride. Inhibition of COX produces a tissue that is hypersensitive to histamine when compared to the tension generation in the absence of indomethacin until the higher concentrations are administered where the tissue response reaches a plateau at just over one and a half times the priming concentration.

Figure 22: Effect of L-NAME (5120 μg/mL) in vivo treatment on airway responsiveness to histamine.



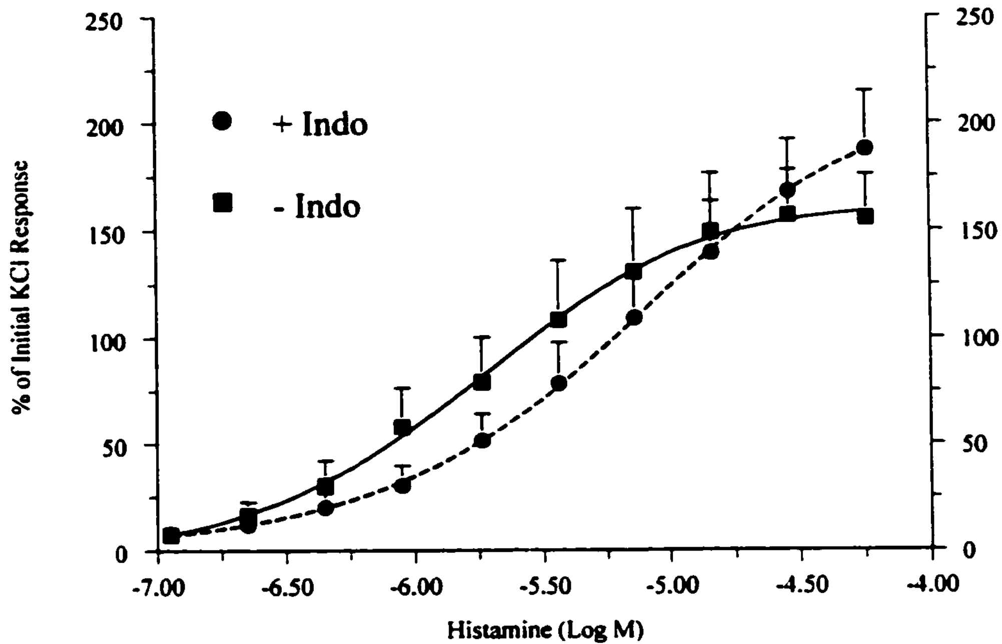
Airway responsiveness (as measured by enhanced Pause, or P_{enh}) to aerosolized histamine following ozone exposure as measured by whole body plethysmography (WBP). Animals were treated with aerosolized L-NAME (5120 μg/mL) concomitantly with histamine. Concentration-response curves are shown without (▲) or following a 30 minute exposure to ozone (3 ppm) at 0 hours (•), 2 hours (▼), 4 hours (■), and 8 hours (+). Histamine PC_5 values are displayed in the legend. Data are shown as mean ± s.e.mean, n=5 to 8 animals per curve.

Figure 23: Effect of L-NAME (2560 μg/mL) in vivo treatment on airway responsiveness to histamine.



Airway responsiveness (as measured by enhanced Pause, or P_{enh}) to aerosolized histamine following ozone exposure as measured by whole body plethysmography (WBP). Animals were treated with aerosolized L-NAME (2560 μg/mL) concomitantly with histamine. Concentration-response curves are shown without (▲) or following a 30 minute exposure to ozone (3 ppm) at 0 hours (•), 2 hours (▼), 4 hours (■),8 hours (+), and 24 hours (◆). Histamine PC_5 values are displayed in the legend. Data are shown as mean ± s.e.mean, n=5 to 8 animals per curve.

Figure 24: Effect of cyclooxygenase inhibitor indomethacin on guinea pig tracheal responses to histamine.



Histamine concentration-response curves for guinea pig tracheal rings in the absence (■) and presence (•) of indomethacin (0.5 μg/mL). Untreated (no ozone exposure) guinea pig tracheas were excised and cut into 3 mm open rings. A tracheal smooth muscle chain of 4 open rings tied with surgical suture was placed in a tissue bath containing Krebs solution at 37°C. Tracheal chains were pre-contracted with potassium chloride (KCl) to determine the baseline contractile response of each preparation. Data are shown as mean ± s.e.mean, n=5 for each curve.

Determination of PGE_2 by Radioimmunoassay in Lung Homogenates

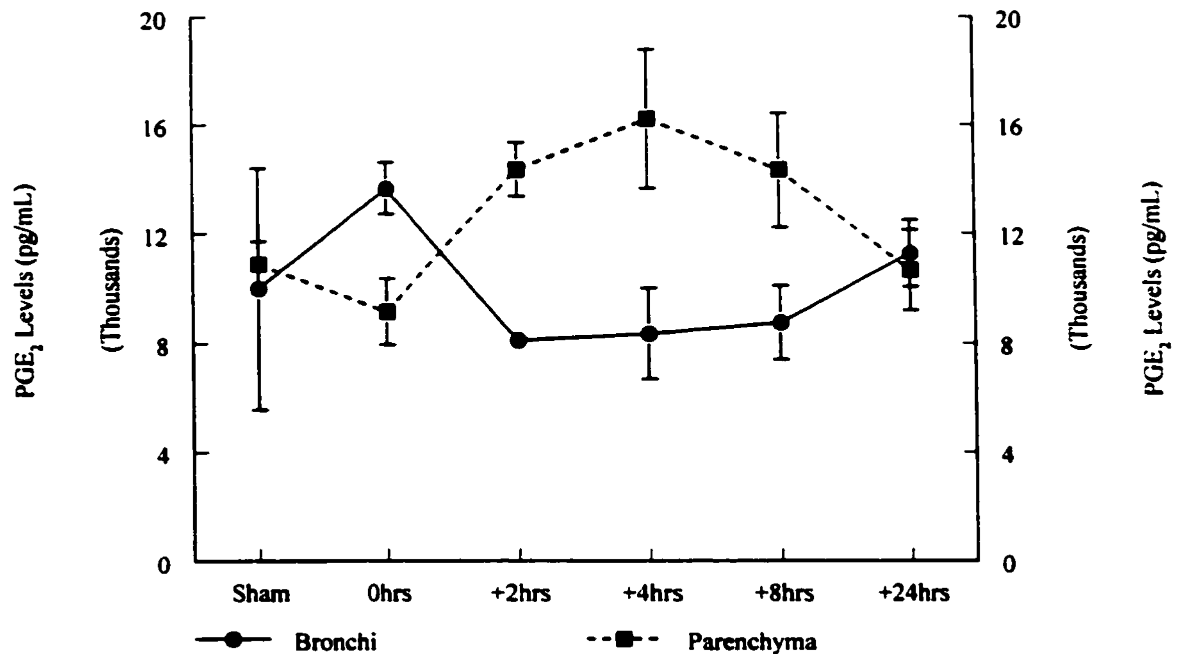
Measurements of the COX product PGE_2 were conducted to determine changes in the levels of this bronchodilatory mediator following ozone exposure. Initial experiments were conducted in guinea pig lung homogenates prepared in the absence of the COX inhibitor indomethacin and identified time-dependent changes in the level of PGE_2 within the tissue (changes not statistically signficant). When indomethacin was added to the homogenization buffer, the level of PGE_2 dropped below the detection limit of the enzyme immunoassay (400 fg/mL) indicating that the formation of the prostaglandins did not occur *in vivo* (Figure 25).

Immunochemical Detection of COX

Our results suggested that COX was participating in the regulation of airway responsiveness. Detection in both bronchial and lung tissue preparations of the inducible and constitutive isoforms of the cyclooxygenase protein is shown in figure 26. In blots A and B, with a polyclonal antibody to the constitutive isoform, a single distinct band at approximately 85 kDa was observed in both bronchial and lung preparations for all tissues examined. In blots C and D, with a polyclonal antibody to the inducible isoform, a typical double banding pattern for the inducible isoform of cyclooxygenase was observed in both the guinea pig macrophage preparation and the positive control for the inducible COX protein. The double banding pattern is also observed in all timepoints examined at a reduced intensity compared to the positive controls. The non-specific

signal is shown in figure 27, blots A and B, with a band clearly visible at approximately 64 kDa. This faint band is similar in intensity to that observed in figure 26.

Figure 25: Levels of PGE_2 in bronchial and lung tissue preparations from sham-treated and ozone-treated guinea pigs.



Concentration of PGE_2 in unexposed and ozone exposed bronchial (•) and parenchymal (■) homogenates. Tissue was homogenized, centrifuged and assayed for PGE_2 by radioimmunoassay. Data are presented as mean ± s.e.mean, n=4 for each data point.

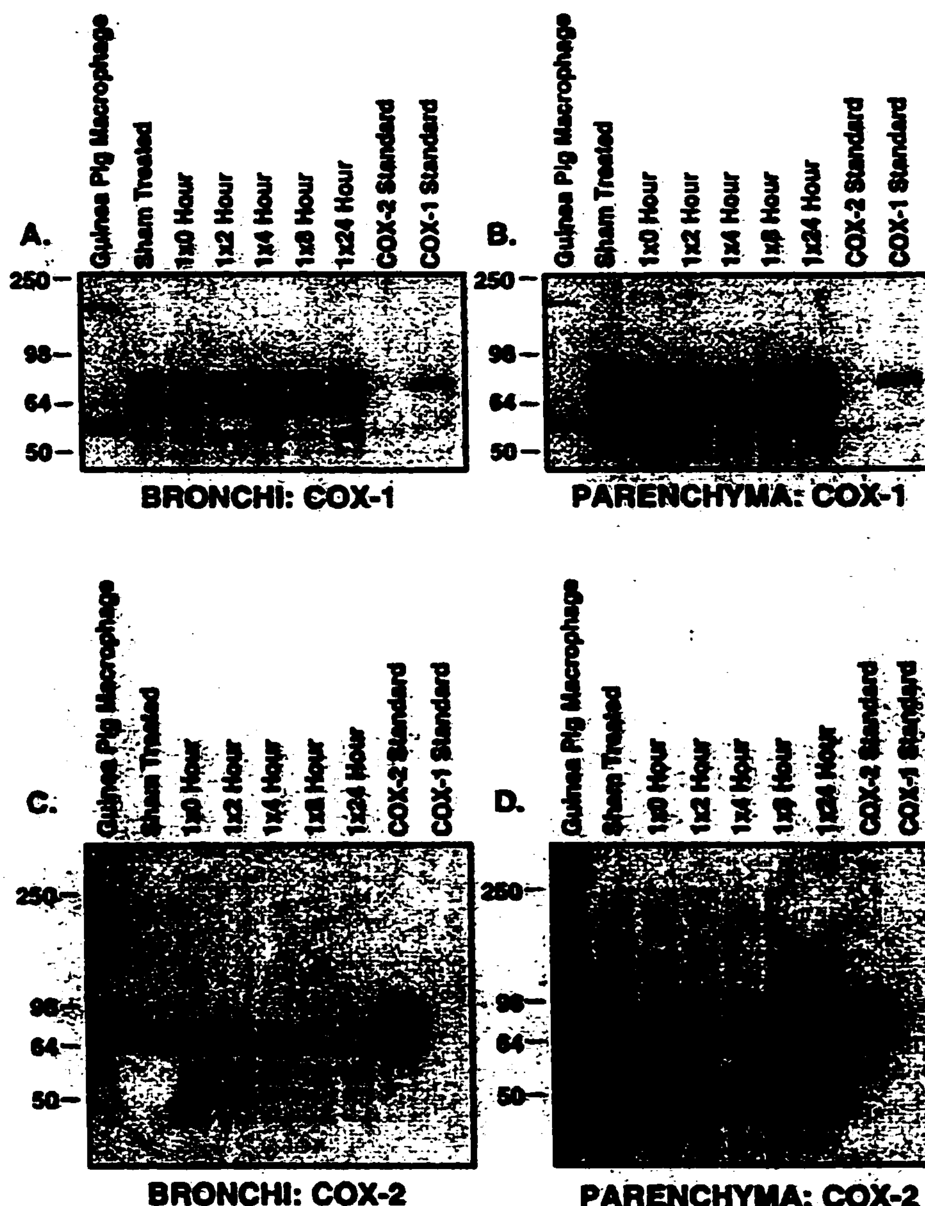


Figure 26: Protein expression of COX-1 and COX-2 isoforms in sham-treated and ozone-treated guinea pig bronchial and lung preparations using polyclonal antibodies selective for their respective proteins.

Representative blots for each COX isoform in bronchial and parenchymal preparations are shown above. Tissues were prepared as described in Experimental Methods. The constitutive isoform (COX-1, A and B) monoclonal antibody shows a similar banding pattern in both tissue preparations. There is a band of similar mass to COX-1 in each bronchial and parenchymal preparation tested. The inducible isoform (COX-2, C and D) monoclonal antibody recognized a protein similar in mass to COX-2 in each bronchial and parenchymal preparation tested. Commercially supplied protein standards are shown in the two right hand lanes.

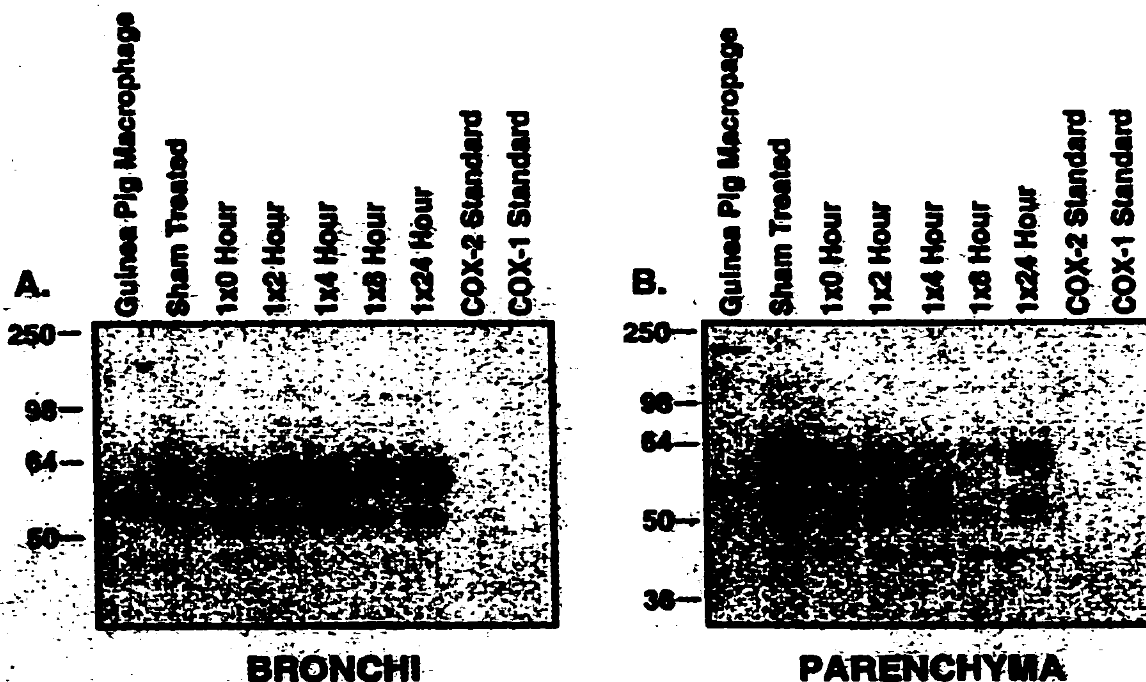


Figure 27: Non-specific protein expression of both COX-1 and COX-2 isoforms in the absence of a selective antibody in sham-treated and ozone-treated guinea pig bronchial and lung preparations.

Representative blots for the bronchial and parenchymal preparations are shown above. Tissues were prepared as described in Experimental Methods. The banding pattern observed in both the bronchial (A) and parenchymal (B) preparations is similar to that observed in Figure 26. The bands shown above result from non-specific binding of the secondary antibody to proteins in the tissue preparations. Commercially supplied protein standards are shown in the two right hand lanes.

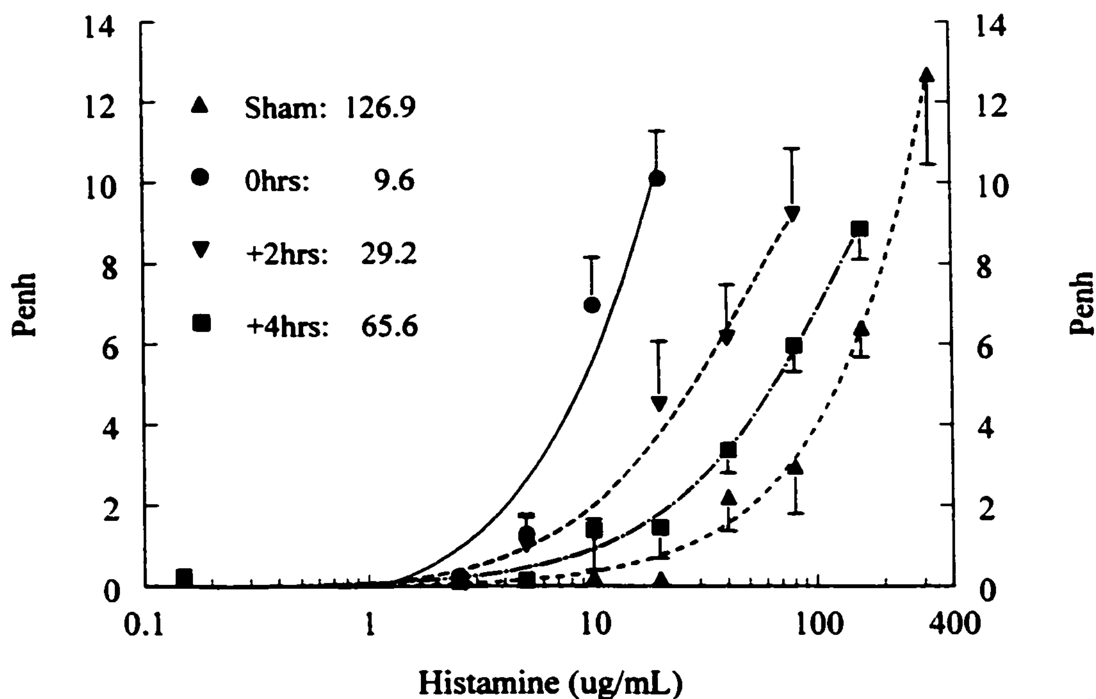
Effects of Indomethacin (10 mg/kg) on Ozone-Induced Airway Hyperresponsiveness

Pre-treatment of animals with the non-selective cyclooxygenase inhibitor indomethacin, 10mg/kg i.p., induced a mild hyporesponsiveness in the sham-exposed group (histamine PC_5 of 126.9 ± 14.7 μg/mL) and did not affect the ozone-induced histamine hyperresponsiveness immediately following ozone (histamine PC_5 of 9.6 ± 1.1 μg/mL). The indomethacin did increase the airway hyperresponsiveness at two hours following ozone, shifting the concentration-response curve leftward approximately two-fold to a histamine PC_5 value of 29.2 ± 3.1 μg/mL (p value < 0.05). There was no observable effect at four hours following ozone (histamine PC_5 of 65.6 ± 4.8 μg/mL). The eight hour and twenty four hour groups were not examined (Figure 28).

COX-2 Selective Inhibition - DFU (1 and 10 mg/kg)

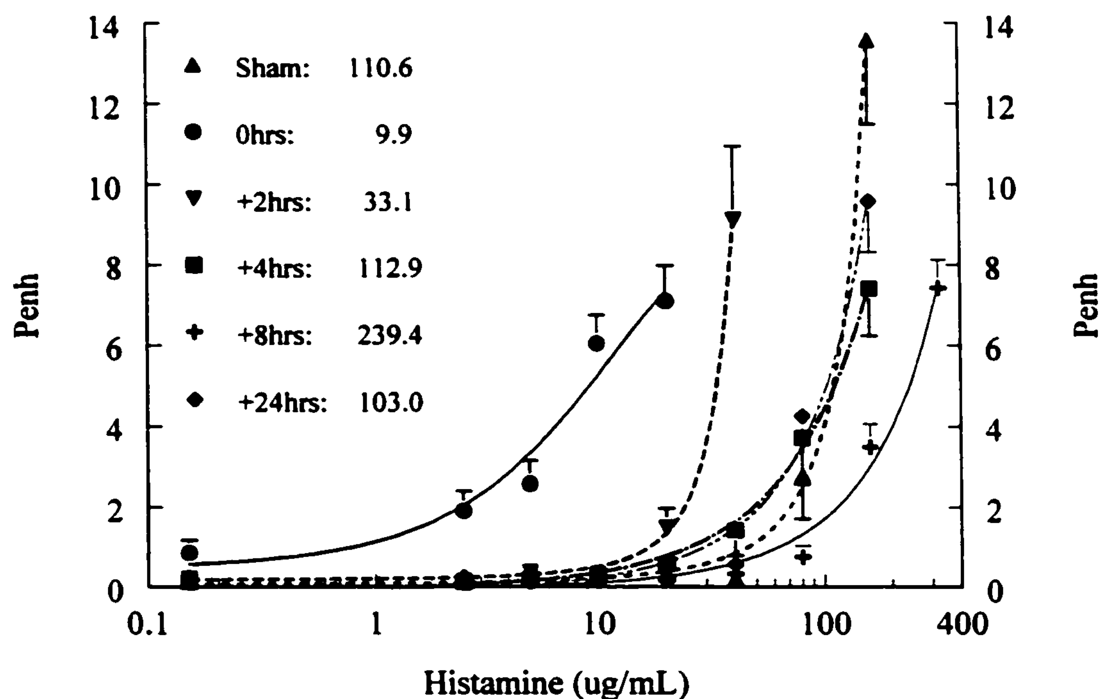
The cyclooxygenase-2 selective inhibitor DFU was administered in two different concentrations to identify the effects of varying concentrations on ozone-induced airway hyperresponsiveness. Animals were administered either 1 mg/kg i.p. or 10 mg/kg i.p. fifteen minutes prior to the histamine concentration-response curve. The results are shown in figures 29 and 30. Comparison of PC_5 values in the DFU-treated groups with those from the vehicle-treated groups shows no difference in either the sham-treated (histamine PC_5 of 110.6 ± 10.2 μg/mL for 1 mg/kg DFU group and 113.5 ± 10.4 μg/mL for 10 mg/kg DFU group) or immediately post ozone (histamine PC_5 of 9.9 ± 1.3 μg/mL for 1 mg/kg DFU group and 13.3 ± 2.1 μg/mL for 10 mg/kg DFU group) groups.

Figure 28: Effect of indomethacin (10 mg/kg) on guinea pig in vivo airway responses.



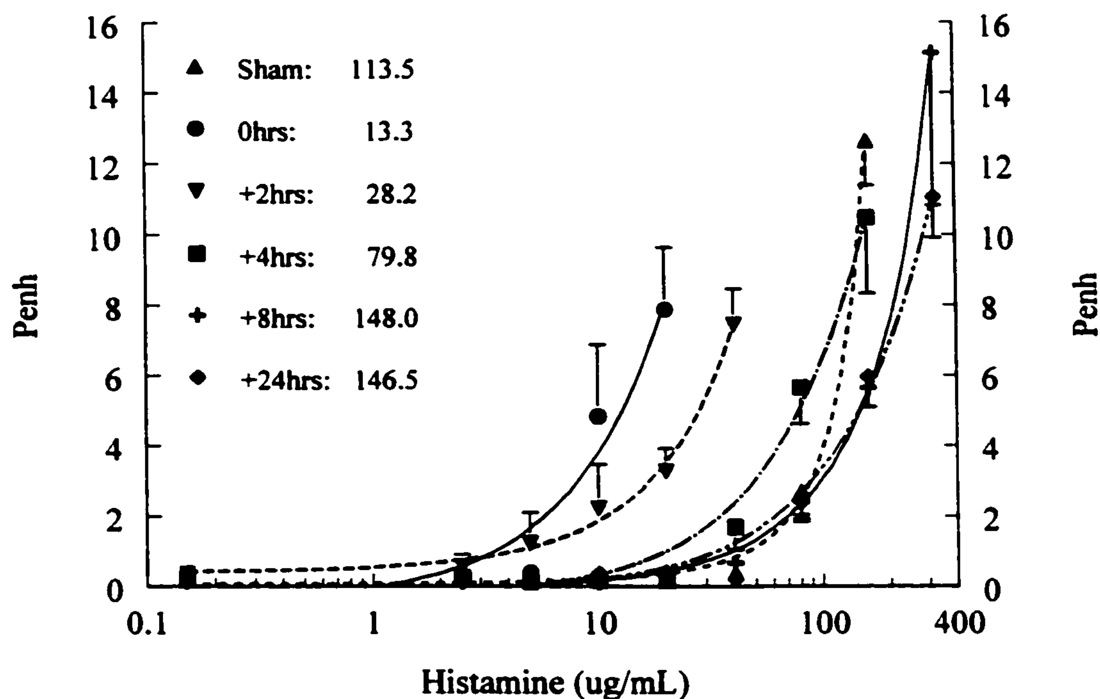
Airway responsiveness (as measured by enhanced Pause, or Penh) to aerosolized histamine following ozone exposure as measured by whole body plethysmography (WBP). Animals were treated with indomethacin (10 mg/kg i.p.) prior to histamine provocation. Concentration-response curves are shown without (▲) or following a 30 minute exposure to ozone (3 ppm) at 0 hours (●), 2 hours (▼), and 4 hours (■). Histamine PC_5 values are displayed in the legend. Data are shown as mean ± s.e.mean, n=5 to 8 animals per curve.

Figure 29: In vivo effect of DFU at 1mg/kg in the conscious guinea pig.



Airway responsiveness (as measured by enhanced Pause, or P_{enh}) to aerosolized histamine following ozone exposure as measured by whole body plethysmography (WBP). Animals were treated with DFU (1 mg/kg i.p.) prior to histamine provocation. Concentration-response curves are shown without (▲) or following a 30 minute exposure to ozone (3 ppm) at 0 hours (•), 2 hours (▼), 4 hours (■),8 hours (✚), and 24 hours (◆). Histamine PC_5 values are displayed in the legend. Data are shown as mean ± s.e.mean, n=5 to 8 animals per curve.

Figure 30: In vivo effect of DFU at 10 mg/kg in the conscious guinea pig.



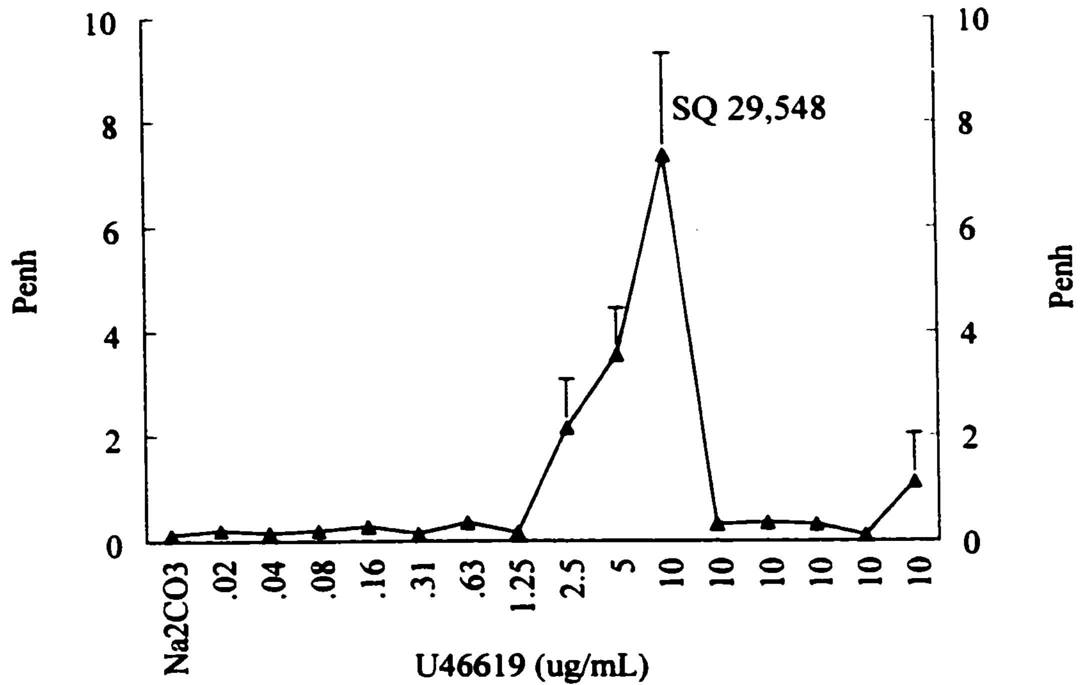
Airway responsiveness (as measured by enhanced Pause, or P_{enh}) to aerosolized histamine following ozone exposure as measured by whole body plethysmography (WBP). Animals were treated with DFU (10 mg/kg i.p.) prior to histamine provocation. Concentration-response curves are shown without (▲) or following a 30 minute exposure to ozone (3 ppm) at 0 hours (•), 2 hours (▼), 4 hours (■),8 hours (✚), and 24 hours (◆). Histamine PC_5 values are displayed in the legend. Data are shown as mean ± s.e.mean, n=5 to 8 animals per curve.

At two hours following ozone there was a two-fold leftward shift of the histamine concentration-response curve (histamine PC_5 of 33.1 ± 3.2 μg/mL in 1mg/kg DFU group and 28.2 ± 2.5 μg/mL in 10 mg/kg DFU group) in the DFU-treated animals. This leftward shift disappeared at four hours post ozone exposure, with a histamine PC_5 of 112.9 ± 10.7 μg/mL and 79.8 ± 6.6 μg/mL for the 1 mg/kg and 10 mg/kg DFU groups, respectively. The pre-treatment with DFU shifted the curves to the right at four, eight and twenty four hours following ozone. Of particular note is the fact that DFU at a concentration of 1 mg/kg showed a marked attenuation of the histamine concentration-response curve and also reduced the peak P_{enh} values when compared to vehicle-treated groups.

Effect of a thromboxane receptor agonist on airway function

The airway responsiveness to aerosol challenge with the thromboxane receptor agonist U46619 was examined in sham-treated guinea pigs. The aerosol challenge demonstrated a good concentration-response relationship with maximal tolerability reached at a concentration of 10 μg/mL of U46619 and a PC_5 value of 6.97 μg/mL. The inhibition of this maximal response was examined using the thromboxane receptor antagonist SQ 29,548 at a dose of 1 mg/kg administered i.p. The administration of the thromboxane receptor antagonist completely inhibited the response to the thromboxane agonist with an effect that lasted over 90 minutes (Figure 31).

Figure 31: Effect of U46619 on guinea pig airway reactivity and inhibition by SQ 29,548.

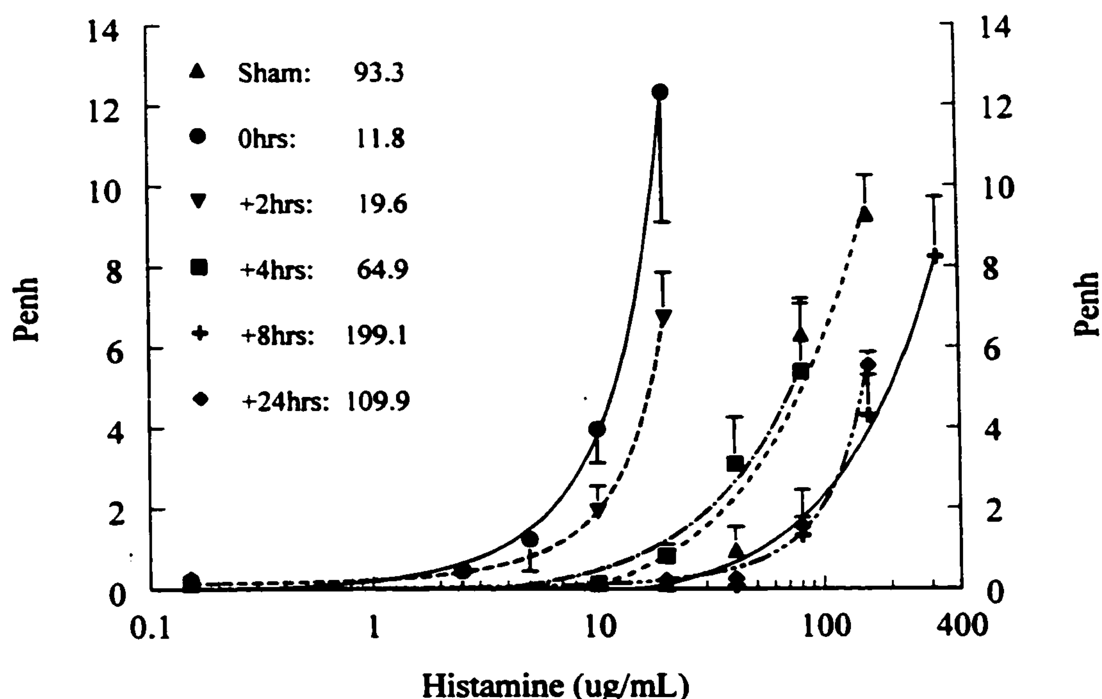


Airway responsiveness (as measured by enhanced Pause, or Penh) to doubling concentrations of aerosolized U46619 (▲) as measured by whole body plethysmography (WBP). Animals were treated with SQ 29,548 (1 mg/kg i.p.) following the concentration-response curve and re-challenged with the highest dose of U46619. Data are shown as mean ± s.e.mean, n= 8 animals.

Effect of thromboxane receptor antagonism on histamine-induced bronchospasm

To examine the role of thromboxane in ozone-induced airway hyperresponsiveness, the thromboxane receptor antagonist SQ 29,548 was administered i.p. to animals immediately following ozone exposure and 15 minutes prior to histamine provocation. The receptor antagonist did not alter the airway responses in sham-treated animals (histamine PC_5 of 93.3 ± 8.5 µg/mL) or those immediately following ozone exposure (histamine PC_5 of 11.8 ± 1.7 µg/mL). The PC_5 value was decreased three-fold in the two hour post-ozone group (histamine PC_5 of 19.6 ± 2.0 µg/mL) compared to vehicle control (histamine PC_5 of 58.7 ± 4.0 µg/mL) and the P_{enh} values were significantly elevated at each histamine concentration. The shift of the concentration-response curve disappeared at four hours post-ozone (histamine PC_5 of 64.9 ± 4.8 µg/mL). There was some attenuation of the histamine response at eight hours (histamine PC_5 of 199.1 ± 21.6 µg/mL), but no significant effect (histamine PC_5 of 109.9 ± 11.1 µg/mL) at twenty-four hours. The rightward shift of the histamine response at twenty four hours is similar to the shift observed at twenty four hours post ozone in the animals treated with the high concentration of DFU (histamine PC_5 of 146.5 ± 16.2 µg/mL; Figure 32).

Figure 32: Responses to histamine following treatment with a thromboxane receptor antagonist in the conscious guinea pig.

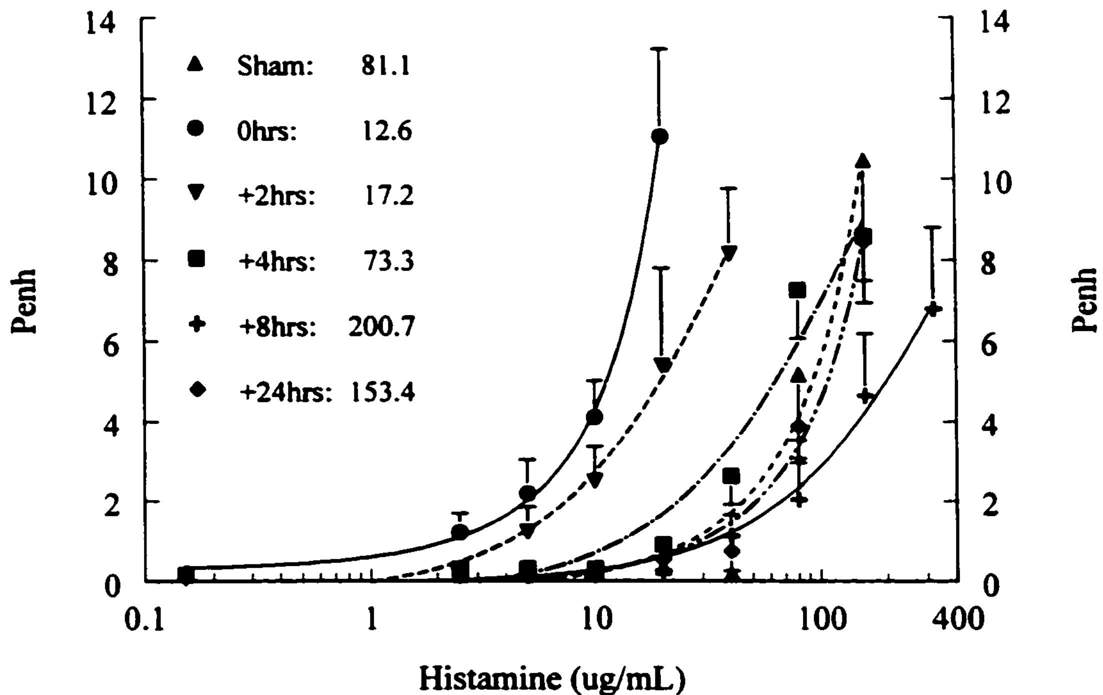


Airway responsiveness (as measured by enhanced Pause, or P_{enh}) to aerosolized histamine following ozone exposure as measured by whole body plethysmography (WBP). Animals were treated with SQ 29,548 (1 mg/kg i.p.) following ozone exposure and prior to the histamine concentration-response curve. Concentration-response curves are shown without (▲) or following a 30 minute exposure to ozone (3 ppm) at 0 hours (●), 2 hours (▼), 4 hours (■),8 hours (+), and 24 hours (◆). Histamine PC_5 values are displayed in the legend. Data are shown as mean ± s.e.mean, n=5 to 8 animals per curve.

Combined Inhibition of COX and NOS

The nitric oxide synthase inhibitor L-NAME (2650 μg/mL), in a concentration that had no effect on airway hyperresponsiveness, when used in combination with the COX-2 selective inhibitor DFU (10 mg/kg) produced no change in the histamine responsiveness in the sham-treated group (histamine PC_5 of 81.1 ± 7.8 μg/mL) or immediately following ozone exposure (histamine PC_5 of 12.6 ± 1.8 μg/mL) when compared to their control groups (histamine PC_5 values of 106.4 ± 11.2 μg/mL and 9.6 ± 0.8 μg/mL, respectively; Figure 33). At two hours there was a leftward shift of the concentration-response curve (histamine PC_5 of 17.2 ± 2.9 μg/mL), a significant ($p < 0.05$) increase in histamine responsiveness to that observed with L-NAME alone (histamine PC_5 of 72.6 ± 6.2 μg/mL). Four hours following ozone the combination of DFU and L-NAME (histamine PC_5 of 73.3 ± 5.8 μg/mL) increased the histamine responsiveness more than DFU alone (histamine PC_5 of 79.8 ± 6.6 μg/mL) or L-NAME alone (histamine PC_5 of 83.8 ± 7.7 μg/mL) but the shift was not statistically different ($p > 0.05$) for either group. At eight hours post-ozone exposure the combination of DFU (10 mg/kg) and L-NAME (2560 μg/mL) attenuated the response when compared with either DFU or L-NAME alone. The combined inhibition of COX-2 and NOS (histamine PC_5 of 153.4 ± 17.2 μg/mL) was significantly ($p < 0.05$) greater than that in the presence of either L-NAME (histamine PC_5 values of 112.4 ± 9.9 μg/mL) or DFU alone (histamine PC_5 value of 146.5 ± 16.2 μg/mL) at twenty four hours following ozone exposure.

Figure 33: Combined effect of a COX-2 (10 mg/kg) and a NOS (2560 μg/mL) inhibitor in the conscious guinea pig.



Airway responsiveness (as measured by enhanced Pause, or P_{enh}) to aerosolized histamine following ozone exposure as measured by whole body plethysmography (WBP). Animals were treated with both DFU (10 mg/kg i.p.) and aerosolized L-NAME (2560 μg/mL). Concentration-response curves are shown without (▲) or following a 30 minute exposure to ozone (3 ppm) at 0 hours (●), 2 hours (▼), 4 hours (■),8 hours (+), and 24 hours (◆). Histamine PC_5 values are displayed in the legend. Data are shown as mean ± s.e.mean, n=5 to 8 animals per curve.

Table 3: Summary table of provocation concentrations (PC_5) for in vivo whole body plethysmography.

	Untreated	*Dex*	*L-NAME (2560 µg/mL)*	*L-NAME (5120 µg/mL)*
Sham	106.4 ± 11.2	123.9 ± 11.7	135.4 ± 14.9	87.1 ± 8.2
0 hrs	9.6 ± 0.8*	109.6 ± 9.7	9.0 ± 0.7*	5.3 ± 0.8*
2 hrs	58.7 ± 4.0*	52.4 ± 5.8*	72.6 ± 6.2*	18.7 ± 1.4*
4 hrs	77.3 ± 5.4*	217.0 ± 21.4*	83.8 ± 7.7*	29.7 ± 2.2*
8 hrs	146.5 ± 17.2	201.4 ± 19.7	154.8 ± 18.4	207.5 ± 19.6*
24 hrs	98.1 ± 10.9	123.1 ± 11.1	112.4 ± 9.9	N.D.

	Indo (10mg/kg)	*DFU (1mg/kg)*	*DFU (10mg/kg)*	*DFU/ L-NAME*	*SQ-29,548 (1mg/kg)*
Sham	126.9 ± 14.7	110.6 ± 10.2	113.5 ± 10.4	81.1 ± 7.8	93.3 ± 8.5
0 hrs	9.6 ± 1.1*	9.9 ± 1.3*	13.3 ± 2.1*	12.6 ± 1.8*	11.8 ± 1.7*
2 hrs	29.2 ± 3.1*	33.1 ± 3.2*	28.2 ± 2.5*	17.2 ± 2.9*	19.6 ± 2.0*
4 hrs	65.6 ± 4.8*	112.9 ± 10.7	79.8 ± 6.6*	73.3 ± 5.8	64.9 ± 4.8*
8 hrs	N.D.	239.4 ± 21.6*	148.0 ± 17.3*	200.7± 20.9*	199.1 ± 21.6*
24 hrs	N.D.	103.0 ± 9.6	146.5 ± 16.2*	153.4 ± 17.2*	109.9 ± 10.5

Values are given as mean ± s.e. mean of the histamine concentration (µg/mL saline) required to reach an enhanced Pause (P_{enh}) value of 5 (PC_5). N.D. indicates that the PC_5 value for histamine was not determined. An asterisk indicates that the histamine PC_5 value is statistically significant ($p < 0.05$) when compared to its corresponding sham group.

4. DISCUSSION

The experiments described above aimed to determine the role of nitric oxide synthase and cyclooxygenase in the modulation of lung function. The key findings presented here are that nitric oxide synthase activity is increased following ozone exposure and that both nitric oxide synthase and cyclooxygenase modulate lung function through the production of bronchodilatory mediators. Using a sensitive nitric oxide synthase assay, I demonstrated that both calcium-dependent and calcium-independent NOS activity were significantly elevated following ozone treatment. Also, treatment with the non-isoform selective NOS inhibitor, L-NAME, further enhanced the sensitivity to histamine-induced bronchoconstriction *in vivo* following ozone. This observation combined with the finding of the elevated NOS activity *in vitro* suggests that NOS is upregulated following ozone exposure in the guinea pig and produces NO, which participates in the pathophysiological regulation of the airways.

Previous reports have demonstrated that COX products regulate ozone-induced airway function in guinea pigs (Lee and Murlas, 1985; Schelegle et al., 1987). I wanted to investigate it by assessing the contribution of the inducible isoform of COX (COX-2). The *in vivo* administration of a COX-2 selective antagonist produced a time-dependent increase in the histamine-induced bronchoconstriction following ozone exposure indicating that bronchodilatory eicosanoid production occurred as a result of COX-2 enzyme activity.

Ozone exposure resulted in a time-dependent airway hyperresponsiveness, which was maximal immediately following ozone exposure and returned to near baseline levels twenty four hours following the ozone exposure. The identification of this time dependence is important in understanding the contribution of both neural and inflammatory processes in the genesis of airway hyperresponsiveness. It was shown that macrophage, eosinophil and neutrophil cell counts were elevated in the bronchoalveolar lavage fluid following ozone exposure. The time course for this elevation in inflammatory cell infiltrate was unrelated to the histamine-induced airway hyperresponsiveness observed *in vivo*. This discussion will examine the following: i) major findings; ii) methodology; and iii) unusual or anomalous findings.

Major Findings

The findings reported in this thesis indicate that inhaled ozone caused a time dependent histamine airway hyperresponsiveness. It was also clear that the mechanisms underlying the airway hyperresponsiveness vary at the different time points observed. The use of a high concentration and a low concentration for both L-NAME (2560 ug/mL and 5120 ug/mL, a competitive, non-selective NOS inhibitor) and the competitive COX-2 selective inhibitor DFU (1 mg/kg and 10 mg/kg) provided additional information regarding the relative contributions made by both enzymes to the regulation of the airway

following ozone exposure and the changes in contribution of these enzymes to airways function over time.

Immediately following ozone exposure, the airways responded only to the high concentration of the NOS inhibitor and did not respond to either concentration of the COX-2 inhibitor. The effect observed in the presence of the high concentration of the NOS inhibitor is likely the result of the inhibition of the constitutive NOS isoform, which releases nitric oxide continuously to regulate basal airway tone (Belvisi et al., 1992; Belvisi et al., 1993). This confirms previous findings that constitutively produced nitric oxide regulates basal airway tone via a cGMP-dependent relaxation of airway smooth muscle (Jia et al., 1995; Sadeghi-Hashjin et al., 1996). The contribution of the basal NO production to regulation of the airway must be considered when interpreting data obtained from NOS inhibition studies.

It has been previously reported that, immediately following ozone exposure, the combination of the tachykinin NK_1 receptor antagonist CP 99996 and the tachykinin NK_2 receptor antagonist SR 48968 inhibited the airway histamine AHR by over 65%, when compared to animals treated with ozone alone (Masson et al., 1996). This tachykinin-mediated response is indicative of a sensory nerve-mediated response of the airways to ozone exposure (Koto et al., 1995; Savoie et al., 1995; Wu et al., 1997). The two predominant tachykinins, substance P (SP) and neurokinin A (NKA), have been localized to sensory nerves of the airways of several animal species (Geppetti et al., 1993; Hua et

al., 1985; Martling et al., 1990) and human airways (Belvisi et al., 1994; Martling et al., 1987) and are known to mediate airway hyperresponsiveness, through NK_1-mediated bronchoconstriction, following ozone exposure (Geppetti et al., 1993; Joos and Pauwels, 1993; Murlas et al., 1993) (Koto et al., 1995; Tepper et al., 1993). Based on these studies, and the findings presented here, it is clear that sensory nerves and the release of tachykinins predominantly mediate the airway hyperresponsiveness observed immediately following ozone. Neither NOS nor COX inhibition altered the histamine-induced airway hyperresponsiveness immediately following ozone exposure indicating that neither enzyme contributes, to a detectable level, to airway hyperresponsiveness at this time point. Dexamethasone, known to upregulate neural endopeptidase (NEP, the enzyme which degrades both SP and NKA (Graf et al., 1998; Lang and Murlas, 1992; Lang and Murlas, 1993; van der Velden et al., 1998)) by increased protein expression in bronchial epithelial cells, may have significantly reduced the activity of SP and NKA and inhibited the bronchoconstriction immediately following ozone.

Cyclooxygenase

This indomethacin-sensitive airway responsiveness observed in these studies are in agreement with the findings of several other groups who have demonstrated that cyclooxygenase products, including PGE_2 (Grodzinska et al., 1975; Knight et al., 1995a; Knight et al., 1995b), $PGF_{2\alpha}$ (Arakawa et al., 1993), and PGI_2 (Knight et al., 1995b), are involved in mediating airway responsiveness. In addition, indomethacin augments

airway reactivity to antigenic stimulation (Uhlig et al., 1996) and to the thromboxane receptor agonist U46619 (Wong et al., 1998) in guinea pigs.

The production and release of the bronchodilatory prostaglandins appears to be the result of COX-2 induction following ozone exposure. The acute inflammatory response following ozone elicits infiltration of macrophages, eosinophils and neutrophils. These cells are capable of releasing pro-inflammatory cytokines, including IL-1, interferon-γ and tumour necrosis factor-α, and stimulating the upregulation of COX-2 (Ermert et al., 1998; Ohara et al., 1998). The inducible cyclooxygenase enzyme is upregulated in several inflammatory conditions including inflammatory bowel disease (Singer et al., 1998), lupus nephritis (Tomasoni et al., 1998), congestive heart failure (Wong et al., 1998) and is induced by lipopolysaccharide (Matsumura et al., 1998; Uhlig et al., 1996) and inflammatory cytokines (Wong et al., 1998).

A bronchodilatory COX-2 component modulating ozone-induced airway hyperresponsiveness was evident following ozone exposure as the COX-2 inhibitor, DFU, augmented airway hyperresponsiveness. The two predominant bronchial smooth muscle inhibitory prostaglandins are PGE_2 and prostacyclin (PGI_2) (Abela and Daniel, 1995; Grodzinska et al., 1975; Wasserman et al., 1980) (Spannhake et al., 1980). In humans, PGE_2 is an inhibitor of bronchial smooth muscle contraction (Knight et al., 1995a) and in the dog (Daniel et al., 1992) and guinea pig (Grodzinska et al., 1975) both PGE_2 and PGI_2 inhibit bronchial smooth muscle contractility, although PGI_2 does not

protect against histamine-induced contraction in humans (Knight et al., 1995a). PGE_2 also inhibits acetylcholine release from cholinergic nerves via a mechanism thought to involve stimulation of pre-synaptic receptors (Serio and Daniel, 1989; Walters et al., 1984). Whether PGE_2, PGI_2 or a combination of the two is responsible for the bronchodilatory activity following ozone exposure is not clear.

The contribution of the bronchodilatory prostaglandin(s) to modulation of the airway following ozone exposure was not detectable at four hours. It is important to note that at the low concentration of the COX-2 inhibitor the airway responses to histamine are significantly inhibited whereas at the high concentration there is no statistically significant difference from the control group. One contributing factor to this finding may be that the low concentration (1 mg/kg) of DFU used in these studies is very close to the ED_{50} values of 0.95 mg/kg found for rat paw hyperalgesia and 1.1 mg/kg for rat paw oedema (Riendeau et al., 1997). The existence of two different isoforms of COX-2 with different enzyme inhibition constants would account for the observations obtained with DFU. A COX-2 isoform with a relatively low enzyme inhibitory constant, and producing a bronchoconstricting mediator would be inhibited by the low concentration of DFU and attenuate the airway response to histamine. A second COX-2 isoform with a relatively high enzyme inhibitory constant, and producing a bronchodilatory mediator would be inhibited only by the high concentration of DFU. The higher concentration of DFU would then, by inhibiting both COX-2 isoforms, result in airway responses similar to those in the absence of DFU. This is conjecture as there is currently no evidence to

support the idea of more than one COX-2 isoform. Additional studies are need to clarify the mechanism by which DFU inhibits COX-2.

Histological studies by Ermert et al. (1998) have identified that COX-2 is localized in macrophage- and mast-cell like cells, in vascular smooth muscle cells, airway smooth muscle cells (Belvisi et al., 1997) and in close proximity to the bronchial epithelium (Mitchell et al., 1994). The contribution of the epithelium to the release of bronchodilatory prostaglandins has been well characterized. Epithelial cells, when stimulated, are capable of producing significant amounts of PGE_2 and may be the source of the bronchodilatory prostaglandin produced following ozone (Knight et al., 1995b; McGrogan et al., 1998). Work by McGrogan et al. has demonstrated that allergen exposed canine trachea produces both PGE_2 and PGI_2 in the presence of an intact epithelium (McGrogan et al., 1998; McGrogan and Daniel, 1996).

In addition to the prostaglandins, thromboxane, acting through the G-protein coupled TP receptor, has been implicated as a modulator of airway hyperresponsiveness (Aizawa et al., 1985; Ciabattoni et al., 1993; Janssen et al., 1991). Our evidence that the COX-2 enzyme is upregulated following ozone exposure focussed attention on the contribution of thromboxane to the changes in histamine responsiveness. Studies conducted by Aizawa et al. (1985) have demonstrated that ozone induces a thromboxane-dependent airway hyperresponsiveness in dogs immediately following ozone exposure. In that particular study, the authors administered the thromboxane

synthase inhibitor prior to ozone exposure. For the protocol used in my study, the thromboxane receptor antagonist was administered post-ozone exposure and did not alter the airway responsiveness immediately following ozone exposure. The differences observed between the two studies may be attributed to the temporal difference of thromboxane antagonist administration and further investigation is required to elucidate the reason for the difference.

Jones et al. (1990) demonstrated that treatment with two different thromboxane antagonists did not inhibit airway hyperresponsiveness immediately following ozone exposure in dogs. In my studies, evidence for thromboxane acting as a bronchoconstricting mediator contributing to hyperresponsiveness was found eight hours after ozone exposure, where the thromboxane antagonist significantly reduces the histamine hyperresponsiveness at that time. In addition to the direct role of thromboxane on the bronchiolar smooth muscle, both thromboxane and U46619 have been shown to modulate, via TP receptor stimulation, cholinergic neurotransmission both pre-synaptically, by activation of acetylcholine release, and post-synaptically, via potentiation of the acetylcholine response (Abela and Daniel, 1995; Daniel and O'Byrne, 1991; Janssen and Daniel, 1991). The antagonism of the thromboxane receptor may, in fact, limit the bronchoconstricting effect of acetylcholine and thereby attenuate the histamine-induced bronchoconstriction.

The substrate for COX, arachidonic acid, is also used by the 5-lipoxygenase pathway in the synthesis of leukotrienes, hydroperoxyeicosatetraenoic acid (HPETE) and hydroxyeicosatetraenoic acid (HETE) (Brock et al., 1995; Jones et al., 1987; McFarlane et al., 1987; Wilborn et al., 1996). Since leukotrienes, specifically the cysteinyl leukotrienes LTC_4, LTD_4, and LTE_4, produce bronchoconstriction and contribute to the pathogenesis of airway hyperresponsiveness (Abraham et al., 1986; Inman, 1999; Turner et al., 1994), it is important to consider the effects of COX inhibition on this pathway. The 5-LO enzyme is a soluble cytosolic enzyme that, although active (Brock et al., 1995), does not produce leukotrienes until it translocates to the nuclear membrane (Brock et al., 1995; Peters-Golden and McNish, 1993; Rouzer and Kargman, 1988) where it associates with a 5-LO activating protein (FLAP) (Dixon et al., 1990).

Once active, the 5-LO/FLAP dimer produces its bronchoconstrictive mediators. The selective inhibition of COX-2 may shunt arachidonic acid to the 5-LO pathway. There is evidence from mouse peritoneal macrophages that this shunting of arachidonic acid occurs (Wightman and Dallob, 1990). In these cells, prostaglandin production suppresses phospholipase C activity. COX inhibition activates phospholipase C and subsequently elevates 5-LO activity and leukotriene biosynthesis (Wightman and Dallob, 1990). The extent to which this is the case within the guinea pig airway is unknown.

Nitric Oxide Synthase

Elevated levels of nitric oxide in exhaled air of asthmatics (Borland et al., 1993; Kharitonov et al., 1994; Kharitonov et al., 1995a) (Kharitonov et al., 1995b) suggest that nitric oxide production is elevated in asthmatic airways, as a result of elevated nitric oxide synthase gene transcription and protein expression. The induction of iNOS and NO production in various cell types has been confirmed by several groups (Akarasereenont et al., 1995; Corbett and McDaniel, 1995; Minc-Golomb and Schwartz, 1994; Punjabi et al., 1994; Willis et al., 1994). Nearly all cells, including smooth muscle cells (Bernhardt et al., 1991; Buga et al., 1989; Fleming et al., 1991b; Wood et al., 1990), epithelial cells (Goureau et al., 1992; Kobzik et al., 1993; Robbins et al., 1994), neurons of the central and peripheral nervous systems (Belvisi et al., 1993; Boeckxstaens et al., 1991; Lei et al., 1993; Li and Rand, 1990; Li and Rand, 1991; Mayer et al., 1990), neutrophils (McCall et al., 1992; Yui et al., 1991), macrophages (Lowenstein et al., 1992; Pendino et al., 1993; Tayeh and Marletta, 1989), and endothelial cells (Busse and Mulsch, 1990; Forstermann et al., 1991b; Mulsch and Busse, 1991) can respond by inducing iNOS when appropriately stimulated.

Biochemical measurements conducted in our study confirmed that an increase in both cNOS and iNOS activities occurred in the lung tissue following ozone exposure. The increase in NOS activity progressively increased up to eight hours following ozone exposure when the enzyme activity was significantly higher than all other time points. This maximal NOS activity coincides with an unexpected airway hyporesponsiveness

that was only observed at the eight-hour time point and was consistent across all treatment groups except the dexamethasone-treated group and the highest concentration of the COX-2 inhibitor. The hyporesponsiveness does coincide with a decrease in the number of macrophages in the bronchoalveolar lavage fluid although this correlation is not observed at twenty-four hours following ozone exposure. The mechanism by which ozone exposure elicits an airway hyporesponsiveness at eight hours is unclear. It is of note however, that this airway hyporesponsiveness developed in the presence of a NOS inhibitor, indicating that NO is not mediating this hyporesponsiveness.

Although NO is capable of toxic damage through lipid peroxidation (Chen and Qu, 1997) or peroxynitrite formation (Buttery et al., 1996; Lorch et al., 2000; Zhu et al., 1998), there is no observable deleterious *in vivo* effect of the elevated NOS activity on airway function in these studies. The potential for NOS mediated toxic damage is probably obscured as ozone itself is a highly reactive, toxic substance which can cause direct epithelial cell damage (Adamson et al., 1999; Bhalla et al., 1999). NO also reacts non-enzymatically with arachidonic acid to form isoprostanes, a family of prostaglandin-like compounds with bioactive properties (Bernareggi et al., 1998; Okazawa et al., 1997a) which also serve as markers of lipid peroxidation (Montuschi et al., 1998). The combination of ozone, nitric oxide-mediated lipid peroxidation and isoprostane formation is likely to contribute to airway hyperresponsiveness following ozone. For this reason, eliminating NO production through NOS inhibition alone would not ameliorate toxic damage or airway hyperresponsiveness.

The non-enzymatic, free radical catalyzed (caused by ozone or excess NO) formation of isoprostanes from arachidonic acid may contribute to the pathophysiological mechanism of ozone-induced airway hyperresponsiveness. Two well characterized isoprostanes, 8-iso PGE_2 and 8-iso $PGF_{2\alpha}$, show species variations with regard to their action on airway smooth muscle. In canine airways isoprostanes elicit bronchodilation (Janssen et al., 2000), yet in both humans (Janssen et al., 2000; Kawikova et al., 1996) and guinea pigs (Kawikova et al., 1996) isoprostanes elicit bronchoconstriction. Isoprostanes stimulate the secondary generation of thromboxane (Okazawa et al., 1997b) and are antagonized by thromboxane receptor antagonists (Kawikova et al., 1996) in the guinea pig lung. Thromboxane antagonism appeared to produc a dual response in this study. At two hours post ozone, a decrease in the histamine PC_5 was observed with the thromboxane antagonist SQ-29,548. At eight hours post ozone, SQ-29,548 produced a decrease in the histamine PC_5 value. Since isoprostanes elicit bronchoconstriction in the guinea pig, via thromboxane generation, the thromboxane antagonist must be expected to shift the concentration-response curve to the right, increasing the PC_5 value. This was observed at eight hours post ozone and may indicate that isoprostanes are present. Since the isoprostanes are produced non-enzymatically, it would seem likely that these compounds would be formed immediately after ozone exposure and not eight hours later. If isoprostanes are not present until eight hours, the indication is that the thromboxane antagonist is inhibiting thromboxane generated by a mechanism other than isoprostanes.

Nitric oxide mediated airway hyperresponsiveness may result not from an excess NO production, but a lack of NO production. In an allergen-induced guinea pig model, a deficiency of the NOS substrate, L-arginine, results in airway hyperresponsiveness (Boer et al., 1999). Although not examined in the current studies, a substrate deficiency does not appear to be the mechanism by which ozone elicits airway hyperresponsiveness as a L-NAME-dependent increase in histamine-induced AHR was observed following ozone exposure. The contribution of a NOS substrate deficiency to airway hyperresponsiveness remains unclear, as allergen-induced airway hyperresponsiveness in humans is not mediated by a L-arginine deficiency (Taylor et al., 1998) although different species may differ in requiring L-arginine versus recycling L-citrulline to supply substrate.

The *in vivo* administration of a NOS inhibitor augmented the histamine-induced airway responsiveness following ozone exposure. Although considerable evidence supports the selective inhibition of NOS by L-NAME, these experiments did not investigate the responses to its inactive enantiomer, D-NAME. It is, therefore, possible that L-NAME acted via a mechanism other than the competitive inhibition of NOS. The finding that L-NAME shifts the histamine concentration-response curve to the left lends support to the hypothesis that nitric oxide synthase activity modulates airway responsiveness following ozone exposure.

Interactions between NOS and COX

Previous work has demonstrated that nitric oxide enhances prostaglandin production *in vitro* (Salvemini et al., 1995c; Salvemini et al., 1995a; Salvemini, 1997; Salvemini and Masferrer, 1996; Swierkosz et al., 1995). What needs clarification is the effect that this interaction has in the regulation of airway function and if this interaction is physiologically significant. There is *in vitro* evidence that nitric oxide synthase mediates an enhancement of cyclooxygenase activity. The objective was to demonstrate that this interaction occurred *in vivo*.

There was an augmentation of the histamine responsiveness in the sham-treated group by the combined inhibition of COX-2 and NOS when compared to the inhibition of either enzyme independently. This effect is not observed in any ozone treatment group. Since ozone upregulates COX-2, physiologically large quantities of eicosanoids may be produced, such that any action of nitric oxide on the COX enzyme was negligible. Studies examining the cellular localization of the two enzymes in this model would provide important information regarding the likelihood that NOS and COX-2 interact to regulate the airways.

Possible Mechanisms of Ozone-Induced Airway Hyperresponsiveness

The changes in histamine airway responsiveness observed over time might be the result of a combination of factors, including changes in the inflammatory state of the airways and the state of the epithelial barrier. The presence of inflammation following

ozone exposure in guinea pigs has been established (Murlas and Roum, 1985; Sun and Chung, 1997a) and was confirmed in this study. The current studies examined, in greater detail, the temporal change in the profile of the inflammatory cells present in the lungs following ozone exposure. Although macrophages and eosinophils were elevated throughout the twenty-four hour period and neutrophils were present two hours post-ozone, there was no correlation between the inflammatory cells present in the bronchoalveolar lavage fluid and the *in vivo* airway hyperresponsiveness. The activation state of the inflammatory cells, as well as their presence, may be a critical factor in determining whether these cells participate directly in the modulation of the airways.

It is also possible that the damage to the intact epithelial barrier augments the sensitivity of the bronchial smooth muscle to histamine provocation. As has been previously shown by several groups, ozone causes epithelial cell damage, leading to general loss of surface epithelial cells (Boatman et al., 1974; Easton and Murphy, 1967; Fedan et al., 2000; Nikula and Wilson, 1990; Pinkerton et al., 1993). Following a two-hour ozone exposure, Matsubara et al. (1995) demonstrated that the epithelial layer sustained variable epithelial denudation lasting up to five hours after ozone exposure. It was also shown that this damage was completely repaired by forty-eight hours. Associated with this physical damage to the epithelial layer, a methacholine-induced airway hyperresponsiveness was observed at five hours post-ozone exposure and airway responsiveness returned to baseline values at twenty-four hours (Matsubara et al., 1995).

A possible consequence of this epithelial cell layer damage is the exposure of the afferent sensory nerve fibres present at the sub-epithelial surface (Coleridge et al., 1993; Schelegle et al., 1993) to the airways lumen. It is possible that the sub-epithelial tissue, which includes a population of sensory nerve cells, is now exposed to exogenous stimuli and the histamine aerosol is capable of directly stimulating the sensory nerve fibres and eliciting a neuropeptide driven bronchoconstriction. It is also possible that histamine penetrates the epithelial barrier in higher concentrations and causes bronchoconstriction via direct stimulation of histamine receptors on the airway smooth muscle (Matsumoto et al., 1986).

Scientific Basis of Methodology

NOS Activity using Ion Exchange Chromatography

To address the difficulty of quantifying the production of nitric oxide *in vitro*, an assay was developed using a radiolabelled substrate, L-arginine (Palmer and Moncada, 1989). Stoichiometric studies have determined that L-arginine is converted by nitric oxide synthase to its product, L-citrulline and nitric oxide in a 1:1 ratio (Iyengar et al., 1987). By inference, the production of radiolabelled citrulline from radiolabelled arginine is an accurate measure of nitric oxide production. This assay provides a robust, reliable and repeatable measurement of nitric oxide synthase activity and has been used in several publications (Klatt et al., 1994) and previously by the author (Kostka et al., 1993). A major concern when interpreting the data from this assay is the potential recycling of citrulline to arginine by the arginosuccinate synthase/arginosuccinate lyase

cycle, which may result in measured enzyme activities that are lower than the actual activity. If the citrulline to arginine recycling system is active in the presence of a large pool of radiolabelled L-arginine there is the potential for the radiolabelled product, L-citrulline to be recycled back into radiolabelled arginine. This could result in two possibilities: i) a small pool of the radiolabelled L-arginine is continuously cycled to citrulline and back to arginine resulting in only a fraction of the citrulline that was produced being detected and the enzyme activity would therefore be underestimated or ii) although not necessarily limited to a small portion of the original radiolabelled L-arginine pool, the recycling of radiolabelled citrulline to arginine will also result in a lower than actual amount of radiolabelled citrulline remaining upon completion of the assay and the NOS activity would therefore be underestimated. If sufficient exogenous L-arginine is added to ensure a sufficient supply of arginine then the citrulline to arginine recycling system may not contribute in any significant way to the measurement of NOS activity.

Protein Expression of NOS and COX by Western Blotting

The specific conditions used to separate the NOS and COX proteins have been previously published (Charette et al., 1995; Ermert et al., 1998; McKanna et al., 1998; Nagasaki et al., 1996) and were reproduced in the current studies. Protein detection was conducted using four different antibodies to the neural NOS protein, two different antibodies to the endothelial NOS protein and four different antibodies to the inducible NOS protein. Each antibody was tested at least fifteen times in an attempt to obtain clear, conclusive NOS protein bands. Variations in antibody, both primary and secondary,

incubation times; wash buffer ionic strength; parameters for electrophoresis and radiographic film exposure times failed to provide a conclusive signal for the NOS protein. Technical errors were not a source of problem as concurrent detection of NOS proteins in rat tissues provided clear, conclusive NOS protein bands for each isoform. With no confirmed cross reactivity of these antibodies with the guinea pig NOS proteins it is likely that the primary antibodies used in these studies do not recognize the NOS protein from this species. Species selective forms of the primary antibodies for the NOS isoforms are necessary in order to successfully identify the NOS proteins in the guinea pig lung.

Lung Function Measurement with Whole Body Plethysmography

Accurate evaluation of data from whole body plethysmography (WBP) for assessment of airway hyperresponsiveness requires that changes in pressure within the plethysmograph be measured. Of concern with this technique is whether this barometric change is indicative of airway function, and more specifically of airway resistance.

Airway resistance is the relationship between transairway pressure and airflow and is represented by the mathematical equation: Airway Resistance (R_{aw}) = Transairway Pressure/Airflow. The calculation of airway resistance requires the measurement of three variables: alveolar pressure, atmospheric pressure and instantaneous airflow (Comroe et al., 1962). The technique of WBP used in these studies has been previously established (Hamelmann et al., 1997) to measure all three variables measured accurately. Both

atmospheric pressure and instantaneous airflow are measured simultaneously by a pneumotachograph attached to the side of the main chamber of the plethysmograph. Above this main chamber is the reference chamber with a pressure transducer affixed across the two chambers. The alveolar pressure is determined by changes in pressure between the two chambers. A bias flow of O_2/CO_2 through the main chamber ensures adequate oxygen for the test animal and has a neutral effect on the volume changes of the main chamber.

The inspiratory phase of the respiratory cycle is characterized by a negative pressure relative to the reference chamber. The maximum flow rate of air into the lungs of the test animal, and therefore the highest flow rate obtained during any one respiratory cycle, is defined as the Peak Inspiratory Pressure (PIP). The Peak Expiratory Pressure (PEP) is the maximum flow rate of air during the exhalation phase of the respiratory cycle. Using these two variables and the time for the exhalation phase, the dimensionless value P_{enh} is calculated to quantify the changes in atmospheric pressure, airflow and alveolar pressure as a measure of airway resistance (R_{aw}).

In vivo comparisons between the WBP technique and those currently accepted by the scientific community are necessary to demonstrate the reliability of data using WBP. Using the technique of measuring intrapleural pressure changes ($\triangle P$) in anaesthetized animals simultaneously with WBP, Hamelmann et al. (1997) demonstrated that P_{enh} and $\triangle P$ are directly correlated. In the same study, the authors also demonstrated an *in vivo*

correlation between pulmonary resistance (R_L) and P_{enh}. Pulmonary resistance was measured using anaesthetized, tracheotomized, ventilated mice.

In using the WBP technique for measuring airway resistance, an additional source of resistance is added, that of the upper airways. The nasal passages are of particular importance in the case of guinea pigs as they are predominately nasal breathers. It has been demonstrated in mice (Hamelmann et al., 1997) that following aerosol bronchoconstrictor challenge the upper airways do not significantly contribute to changes in airway resistance. In rats, there is clear evidence that the upper airways do contribute to changes in airway resistance (Bellofiore et al., 1987). It is not clear from our experiments that the changes in airway resistance developed in the upper airways, lower airways or both.

Nitric Oxide Synthase and the Lung

Contractility of Guinea Pig Trachea *In Vitro*

The purpose of these experiments was to demonstrate the *in vitro* regulation of guinea pig tracheal smooth muscle by the nitric oxide synthase/guanylyl cyclase pathway. Previous experiments have shown pharmacologically that this pathway regulates tracheal smooth muscle (Sadeghi-Hashjin et al., 1996). It was the intent of these experiments to reproduce these published results and, additionally, measure the production of nitric oxide oxidative metabolites. The contractility studies confirmed that tracheal smooth muscle contraction by histamine *in vitro* is modulated by nitric oxide synthase. Inhibition

of NOS activity by L-NAME or L-NOARG enhanced histamine-stimulated contraction of the tracheal smooth muscle, in agreement with previous findings (Yan et al., 1994). The guinea pig tracheal smooth muscle relaxed concentration-dependently in response to a nitric oxide donor. The mechanism by which NO mediated this relaxation was dependent upon the activity of guanylyl cyclase, as demonstrated by inhibition by methylene blue, and the relaxation occurs through a PDE V-sensitive signal. Taken together, these results support the findings that tracheal smooth muscle relaxation is modulated by nitric oxide acting via the guanylyl cyclase pathway to elevate cGMP levels and downregulated by hydrolysis of cGMP by PDE V (Thusu et al., 1995; Undem et al., 1994; Williams and Parsons, 1995).

Production of Nitric Oxide Metabolites by Guinea Pig Trachea In Vitro

The well-established mechanism of airway smooth muscle regulation by nitric oxide is based primarily on indirect evidence obtained from pharmacological manipulation of the NO-guanylyl cyclase system (Jia et al., 1995; Mollace et al., 1991; Sadeghi-Hashjin et al., 1996; Thusu et al., 1995; Zhan et al., 1999). I used a chromatographic assay of two physiological metabolites of nitric oxide, nitrate and nitrite, to present additional, direct evidence of the production of nitric oxide by airway smooth muscle. The half life of nitric oxide in physiological conditions is 10 to 30 seconds (Beckman and Crow, 1993; Squadrito and Pryor, 1998), so it is extremely difficult to determine directly the amount of nitric oxide produced in an aqueous environment. The development of a nitric oxide detecting probe, has made

measurements of NO in cell culture media achievable, but in multicellular tissues, the probability of NO reacting with oxygen species and producing reactive oxygen metabolites is high (Squadrito and Pryor, 1998). For this reason, I measured the two most stable and common oxygen metabolites of nitric oxide. A spectrophotometric method for assaying nitrite has been developed but its limit of sensitivity is insufficient for the picomolar levels expected in the tissue bath experiments (Kalff et al., 2000; Salvemini et al., 1993b). This method of nitrite detection does not provide quantitative data and cannot measure nitrates directly. An additional step is required to convert nitrate back into nitrite. A previously published report (Thayer and Huffaker, 1980) detailed the HPLC assay of both nitrate and nitrite which could be detected at low picomolar concentrations (40 pmol and 120 pmol, respectively). Standards of both potassium nitrate and potassium nitrite were injected in a concentration range down to 1 pmol to 10 μmol to determine the lower limit of detection. The results showed that nitrate could be measured at a concentration of 50 pmol and nitrite at approximately 200 pmol and established the lower limit of quantitation for the HPLC assay for my studies.

To ensure that the assay maintained its sensitivity in the presence of the bathing medium, the same nitrate and nitrite standard curves were prepared in the physiological salt solution used for the contractility studies. Samples were pre-treated with silver sulphate to remove any chloride ions and filtered to remove particles larger than 0.2 μm. The additional ions of the Krebs' solution added several peaks close to the elution peak of nitrate although none of these peaks interfered with the nitrate elution profile.

Following the analysis of the nitrate standard prepared in the Krebs' solution, serial injections of distilled water produced a single interfering peak at approximately the same elution point as the nitrate standard. The source of this interference peak could not be determined and limited the HPLC assay detection of nitrate to 5 µmol. This elevated lower limit of quantitation was too high for detection of nitrates in the organ bath bathing medium and confounded the quantitative measurement of changes in nitrate production.

Since nitrate is the final and most stable by-product of oxygen metabolism with nitric oxide, it is this concentration that is particularly critical in evaluating nitric oxide production. With the solution to the interfering peak elusive and nitric oxide metabolite concentrations possibly well below detection limits, the use of the HPLC assay for these metabolites could not be validated.

Alternative methods to measure nitrates and nitrites include capillary ion analysis and HPLC measurement of radiolabelled arginine and citrulline. The technology of capillary ion analysis may provide the means necessary to measure quantitatively the changes in both nitric oxide metabolites, but its cost was too prohibitive to use in this study. The third alternative technique available is the use of the HPLC system to measure the conversion of radiolabelled arginine to citrulline (Robertson et al., 1993). This system would merely provide similar data to that obtained from the ion exchange chromatography and for the most part be redundant in this study. It would also require a HPLC system entirely dedicated to radioactive materials and acquisition of a beta-decay

detector, which were not feasible for this particular use. Therefore, the indirect measurement of nitric oxide via its oxidative products, nitrate and nitrite, was not pursued further.

Anomalous or Inconclusive Findings

NOS Cellular Localization and Expression

Determination of the expression of nitric oxide synthase in the lung tissues, changes in its expression and its cellular localization of the enzyme were major goals of this study. For this reason, both protein detection experiments and histological experiments were undertaken to answer these questions. The detection of the protein was dependent upon isoform selectivity and cross species reactivity of the primary antibody used. First I determined whether the isoform selective antibodies recognized the protein present in this system. For the NOS antibodies it was also necessary to determine whether the antibodies, raised against rat proteins, were able to cross-react with the corresponding guinea pig proteins. The manufacturers had not tested the cross reactivity of the antibodies to the guinea pig isoforms.

To ensure that any lack of detection was due to the lack of antibody specificity and not procedural errors, tissues from both the guinea pig and rat were used to assess selectivity of recognition. By using tissues expressing predominately one isoform of NOS, the selectivity of the antibodies for a particular isoform could be determined with relative certainty. LPS-stimulated peritoneal macrophages were used as a source of the

inducible NOS protein, vascular preparations were used to identify endothelial NOS and brain tissue was used as a source of the neural isoform. Three of four different antibodies to the neural isoform (results from only 3 are shown here), chosen for their various and differing epitopes, recognized both the rat and guinea pig brain tissue with a single band at approximately 155 kDa. One polyclonal antibody against the neural isoform detected protein bands for NOS in the endothelial and inducible preparations, but not the neural preparation.

Two antibodies for the endothelial isoform did not show strong immunoreactivity to proteins from the rat or guinea pig tissues. This may have resulted from a low endothelial cell to vascular tissue ratio, such that the percentage of cells containing the eNOS isoform was too low to be detected by Western blotting techniques. Preliminary work completed using endothelial cell culture did demonstrate that the eNOS antibodies recognized the endothelial isoform.

For the inducible NOS isoform, there were four different commercially available antibodies, all of which recognized the rat isoform of the iNOS protein, but none of which bound the guinea pig isoform. This lack of recognition in guinea pig tissues proved to be a major difficulty in testing the hypotheses set out in this thesis. If the expression of nitric oxide synthase was altered in this model of airway hyperresponsiveness, we expected that this increase would originate primarily from the upregulation of the inducible isoform in response to ozone. This assumption is based on

many reports confirming the ability of inflammatory cell influx into the airways to induce calcium-independent nitric oxide synthase transcription (Currie et al., 2000; Geller et al., 1993; Kwon and George, 1999; Saleh et al., 1998; Tsuji et al., 1995) and confirming the finding in this study that inducible cyclooxygenase enzyme is upregulated *in vivo* following ozone. Testing this assumption required that the antibody directed against the inducible isoform of nitric oxide synthase was both species and isoform selective. Since not one of the four antibodies examined met this requirement, the attempt to identify both changes in iNOS expression and its cellular localization failed.

Inflammatory Cell Responses following Ozone

The post-ozone bronchoalveolar eosinophilia and subsequent neutrophilia identified in my *in vivo* studies confirm the findings of several other investigators (Holtzman et al., 1983a; Longphre et al., 1999; Murlas and Roum, 1985; Pino et al., 1992; Schultheis and Bassett, 1991). They showed that the *in vivo* ozone model of AHR demonstrates the inflammatory response characteristic of asthma. Administration of corticosteroids prior to ozone exposure has previously been shown to inhibit both airway hyperresponsiveness and immune cell infiltration (Salmon et al., 1998; Stevens et al., 1995). Although inhibition of the airway responsiveness was clearly demonstrated by dexamethasone in my study, there was no inhibition of the inflammatory response. Clearly, the concentration of dexamethasone administered was sufficient to inhibit the airway responses and therefore was not a factor contributing to the persistence of the airway inflammation. A possible contributing factor was the finding by both Merck

Frosst Animal Husbandry staff and the supplier (Charles River) that in several cases the guinea-pigs obtained from Charles River have a pre-existing lung inflammation of unknown etiology. These animals demonstrated an elevated basal eosinophil count within the lungs. These cells, if activated, may have been capable of secreting pro-inflammatory cytokines and promoting a typical inflammatory reaction following ozone despite the pre-administration of dexamethasone. Further investigation regarding any pre-existing inflammatory state in these animals is necessary to confirm this theory.

Functional In Vitro *Studies*

The *in vitro* assessment of the guinea pig trachea was undertaken to identify whether this tissue was modulated by cyclooxygenase and nitric oxide synthase. Neither the *in vitro* nor *in vivo* sensitivity to cyclooxygenase inhibition was statistically different from control for sham-treated animals. The inhibition of NOS did not alter the histamine responsiveness *in vitro*. The combined inhibition of both COX and NOS also had no effect on either of the *in vitro* responses to histamine. In tissues that have not been exposed to ozone, the evidence presented here suggests that neither COX nor NOS plays a significant role in regulating airway function. This finding directly contradicts that of Yan et al. (1994) who demonstrated that L-NAME augmented the *in vitro* guinea pig tracheal responses to histamine; an effect that was reduced in the absence of the epithelium. In both sets of studies, the same concentration of inhibitor and a similar dose range of histamine were used. The authors of the Yan et al. study utilized a tracheal

strip preparation which differs from the tracheal chains used in this study. There is also a difference in the method of conducting the histamine concentration-response curves. This study used a parallel design in which some tracheal preparations were treated with L-NAME while others received only vehicle. Yan et al. utilized a series design in which the tracheal strips were contracted with a cumulative histamine concentrations, rinsed, treated with L-NAME and then contracted with cumulative histamine concentrations again. It is possible that the higher concentrations of histamine used for the first concentration-response curve in the Yan et al. study elicited a proportionally greater release of NO and the subsequent addition of L-NAME produced a greater shift in the concentration response curve than that observed in these studies.

The guinea pig tracheal preparations were examined for their capacity to respond to nitric oxide donating compounds. The sensitivity of the tracheal preparations to NO donating compounds indicated that the upper airways relax in the presence of NO and may therefore be modulated by nitric oxide synthase. The concomitant inhibition of cyclooxygenase revealed a cyclooxygenase-dependent modulation of the tracheal resting tone. The decrease in histamine responsiveness suggests the presence of a bronchoconstricting COX product. Inhibition of both phosphodiesterase V and guanylyl cyclase provided further support for the ability of the tracheal preparations to respond to nitric oxide through production of its second messenger, cyclic guanosine monophosphate (cGMP).

Attempts to induce the expression of nitric oxide synthase in the *in vitro* tracheal preparations using a combination of LPS, interferon-γ and tumour necrosis factor-α (cytomix) were not successful. The sensitivity of the histamine airway responsiveness to dexamethasone pre-treatment indicates that enzyme induction is necessary for the development of ozone-induced airway hyperresponsiveness. Dexamethasone inhibits the expression of iNOS in vascular tissue (Hom et al., 1995), epithelial cells (Punjabi et al., 1994) and in lung tissue following ozone exposure (Haddad et al., 1995). The reported effectiveness of the cytomix treatment (Gutierrez et al., 1995) was not reproducible for the *in vitro* tissue bath studies. The current studies confirmed the LPS-stimulated induction of iNOS in a macrophage preparation *in vitro*. The reason for successful induction of iNOS in the macrophage cell culture and the failure of iNOS induction in the tracheal tissue bath is not clear. The underlying mechanism contributing to this was not investigated further.

Nitric Oxide Synthase Protein Expression

An important goal in this study was to determine the expression of nitric oxide synthase and cyclooxygenase in the lung tissues and to detect changes in their expression. For this reason, both protein detection experiments and immunohistochemical experiments were undertaken. The detection of the proteins was dependent upon isoform selectivity and cross species reactivity. The initial study objective was to determine whether the commercially available isoform selective antibodies were selective indeed in this system.

Cyclooxygenase Protein Expression

The constitutive isoform of COX (COX-1) was expressed in all tissues examined and the intensity of the expression was not altered by the exposure of the animals to ozone. The detection of the COX-2 protein proved to be far more difficult as there was a non-specific signal at the same molecular weight as the COX-2 protein.

The lack of specificity of the antibodies for the inducible isoforms of both nitric oxide synthase and cyclooxygenase prevented the detection of these proteins through Western blotting or immunochemical detection. The development of antibodies with a greater specificity for the guinea pig isoforms of iNOS and COX-2 will provide the necessary tools for detecting changes in expression and cellular localization of these proteins in the lung tissue and their changes following ozone exposure.

Overall Summary

Airway responses to histamine in conscious guinea pigs following ozone exposure were initially ten-fold more sensitive and progressively declined until eight hours post-ozone, at which time an airway hyporesponsiveness was observed. The airway responses to histamine were similar to baseline values at twenty-four hours. The airway responses to histamine were dexamethasone-sensitive, suggesting that both NOS and COX-2 enzyme induction was important in the generation of the hyperresponsiveness. *In vivo* inhibition of either NOS or COX-2 augmented the histamine-induced airway

responsiveness following ozone exposure. Both cyclooxygenase and nitric oxide synthase activities were upregulated following ozone and contributed to the bronchodilatory regulation of the airways in a dexamethasone-sensitive manner.

As an acute phase model of airway hyperresponsiveness the ozone exposed guinea pig demonstrates several characteristics of human asthma including inflammation and airway hyperresponsiveness. Following ozone exposure there is an increase in the production of inhibitory prostaglandins and nitric oxide synthase activity which functionally antagonizes histamine-induced bronchoconstriction. The *in vivo* activities of COX-2 and NOS are time dependent and interrelated. The concurrent inhibition of both enzymes produced greater histamine responsiveness than the inhibition of the individual NOS and COX-2 enzymes. The relevance of the airway responses immediately following ozone is not clear as neither COX-2 nor NOS products participate in modulation of the airways. In human asthma, NO release is elevated (Adisesh et al., 1998; Hamid et al., 1993; Jatakanon et al., 1998; Massaro et al., 1995) and cyclooxygenase inhibition has varied effects (Finnerty and Holgate, 1990; Kirby et al., 1989; Shimizu et al., 1995; Shimizu et al., 1997; Wilson et al., 1994). The presence of both COX and NOS activity at and beyond two hours post-ozone exposure presents a physiological state more closely related to human asthma. The use of the time point immediately following ozone may not provide useful information when comparing results in an animal model to asthma. Nitric oxide synthase does not significantly contribute to *in vivo* toxic tissue damage but does regulate basal airway tone in pre- and

post-ozone-treated guinea pigs. The relevance of this to human asthma is not clear as ozone itself is a highly reactive molecule that can cause tissue damage independently of nitric oxide-mediated lipid peroxidation.

Clinical Implications

Research continues to examine the underlying mechanisms involved in the development of asthma, with a goal of providing better and more specific treatments for the disease, with an end of developing treatments that will reverse tissue damage or even eliminate the disease. The use of steroids has been effective in controlling the symptoms of asthmatics for many years, but poor patient compliance is a problem (Celano et al., 1998; Navarro, 1996). Leukotriene antagonists have provided a second treatment modality for asthmatics. These antagonists have been shown to provide good control of asthmatic attacks in several asthma sub-populations but are found to be effective in only 50 to 75% of asthmatic patients who take the leukotriene antagonists. Each currently available treatment, although effective, does not significantly reverse the pathology of asthma.

This gap in the current treatment modalities highlights the incomplete understanding of the pathogenesis of asthma. The recent attention received by nitric oxide and its bronchodilatory properties has led to the use of inhaled NO in some clinical cases of respiratory distress (Dobyns et al., 1999; Martinez et al., 1999), particularly in infants (Ream et al., 1999), although the survival benefit remains unclear. The results

described in this thesis support the notion that nitric oxide synthase activity is upregulated in a model of airway hyperresponsiveness and participates in attenuating this hyperresponsiveness. The degree to which this is the case in the asthmatic lung is not clear. It is possible that low levels of modulatory nitric oxide produced in the airways are responsible for regulation of normal airway function. However, upregulation of NOS activity following ozone exposure may have an effect which is predominately toxic in nature, overwhelming any positive effect that NO may have on bronchodilation. With support in the literature for the toxic properties of NO, the next step is to demonstrate *in vivo* that elevated levels of NO production have a detrimental effect on airway function and do not result in any physiologically significant bronchodilation.

Future Work

There are several key aspects of airway regulation that were partially addressed in this thesis and require additional attention in order to provide a clearer and more comprehensive picture of airway regulation in the guinea pig ozone-induced airway hyperresponsiveness model.

An important detail that requires clarification in this particular model is the lack of dexamethasone-sensitive airway inflammation following ozone exposure. As described above, the inflammatory response, as assessed by inflammatory cell influx, following ozone in dexamethasone-treated animals is not consistent with the current literature, which has clearly shown a dexamethasone-sensitive inhibition of both airway

hyperresponsiveness and airways inflammation in several models of airway hyperresponsiveness. An assessment of guinea pigs from an alternative source may clarify that the pre-existing eosinophilia observed in the animals used for these experiments was responsible for the lack of sensitivity of the inflammatory response to dexamethasone.

An important tool for the further characterization of this model would be guinea pig specific antibodies for all three isoforms of nitric oxide synthase and the two isoforms of cyclooxygenase. By developing specific antibodies, both protein expression studies and cellular localization studies could be carried out. This, combined with studies of COX-2 and NOS mRNA expression, would be useful in demonstrating any change in enzyme expression following ozone and whether this change in expression is time-dependent. If so, is the time-dependence correlating with the inflammatory response or with the measured increase in activity of the NOS enzyme. Alternatively, use of highly selective e, i and nNOS inhibitors would contribute to analysis of their roles. Cellular localization studies would add to the concept of COX/NOS interaction by providing evidence of whether these two enzymes are within proximity of each other such that an interaction is possible. Additional information would come from identifying if specific cell types which do not normally express NOS or COX enzymes upregulate one or both of these enzymes following ozone. This information would further the understanding of the pathophysiological changes that occur in this model of airway hyperresponsiveness.

One of the greatest difficulties in the study of asthma is the lack of an animal model that mimics the human disease of asthma in all aspects, particularly its chronicity. Several laboratories have begun to develop combination models of airway hyperresponsiveness which utilize two or more inducers of airway hyperresponsiveness (e.g. ozone and allergen) which display characteristics that more closely resemble those of asthma (Gilmour, 1995; Sun et al., 1997; Sun and Chung, 1997b; Tsai et al., 1998). Final development and confirmation of the validity of this type of model has proved to be elusive, but when it is developed it will provide significant information that was either previously unavailable or it will confirm that already known from other models currently in use.

I have shown that nitric oxide synthase activity is increased following ozone exposure and that both nitric oxide synthase and cyclooxygenase modulate lung function through the production of bronchodilatory mediators. Using a sensitive nitric oxide synthase assay, I demonstrated that both calcium-dependent and calcium-independent NOS activity were significantly elevated following ozone treatment. Also, treatment with the non-isoform selective NOS inhibitor, L-NAME, further enhanced the sensitivity to histamine-induced bronchoconstriction *in vivo* following ozone. This observation combined with the finding of the elevated NOS activity *in vitro* suggests that NOS is upregulated following ozone exposure in the guinea pig and produces NO, which participates in the pathophysiological regulation of the airways. The *in vivo* administration of a COX-2 selective antagonist produced a time-dependent increase in

the histamine-induced bronchoconstriction following ozone exposure indicating that bronchodilatory eicosanoid production occurred as a result of COX-2 enzyme activity. Ozone exposure resulted in a time-dependent airway hyperresponsiveness, which was maximal immediately following ozone exposure and returned to near baseline levels twenty four hours following the ozone exposure. The identification of this time dependence is important in understanding the contribution of both neural and inflammatory processes in the genesis of airway hyperresponsiveness. It was shown that macrophage, eosinophil and neutrophil cell counts were elevated in the bronchoalveolar lavage fluid following ozone exposure. The time course for this elevation in inflammatory cell infiltrate was unrelated to the histamine-induced airway hyperresponsiveness observed *in vivo*.

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