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NITRIC OXIDE AND AIRWAY SMOOTH MUSCLE RESPONSIVENESS

BY

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To my parents, husband Sheng

and daughter Roro

ABSTRACT

Airway hyperresponsiveness to bronchoconstrictors has been found in asthma and related to the severity of the disease. The factors which result in hyperresponsiveness are not completely understood. A possible mechanism is an imbalance between endogenous bronchoconstrictors and dilators. NO is known to relax tracheal smooth muscle by activating soluble guanylate cyclase and increasing the level of intracellular cyclic guanosine monophosphate (GMP). The first hypothesis tested was that the NO-cyclic GMP-relaxant pathway is involved in the regulation of airway responsiveness. Inhibition of endogenous nitric oxide by N^{ω}-nitro-L-arginine (L-NNA) significantly increased airway responsiveness to inhaled methacholine in normoresponsive Lewis rats but less so in hyperresponsiveness Fisher rats. In addition, carbachol increased cyclic GMP levels in tracheal tissues from both strains; this cyclic GMP accumulation in tracheal tissues was also less in Fisher than in Lewis rats and abolished by L-NNA in both strains, indicating that it was mediated by a NO-dependent mechanism. These results suggest that endogenous NO plays a role in regulation of airway responsiveness and contributes to the strain-related difference in airway responsiveness in rats. To investigate the NO-cyclic GMP-relaxant pathway in rat airway, the effect of sodium nitroprusside (SNP, a NO donor) on airway responsiveness to a cholinergic agonist was measured in hyperresponsive Fisher rats and compared with the less responsive Lewis strain. Fisher rats were resistant to SNP as evidenced by less relaxation of carbachol contracted tracheal rings by SNP and less cyclic GMP accumulation induced by SNP in cultured

airway smooth muscle cells in Fisher rats compared with Lewis rats, indicating an impaired response to SNP in Fisher airways.

NO is known to be synthesized from L-arginine in a reaction catalyzed by NO synthase (NOS). Liver cytochrome P450 also catalyzes the oxidative cleavage of C=N bonds of compounds containing a -C(NH_2)NOH function, producing NO in vitro. We hypothesized that the biosynthesis of NO in airway smooth muscle cells could result from P450 enzymes acting on appropriate substrates. NO can be synthesized in a number of lung cell types. However, to date, no constitutive form of NOS activity has been found in airway smooth muscle cells. We next examined the possibility that airway smooth muscle itself might be able to synthesize NO. Formamidoxime, a compound containing the -C(NH_2)NOH function, was found to produce NO in cultured airway smooth muscle cells. As well, formamidoxime relaxed pre-contracted trachealis and cyclic GMP accumulation in airway smooth muscle cells in culture. These effects were inhibited by P450 inhibitors but not by NOS inhibitors. Thus, an L-arginine-independent pathway for production of NO was demonstrable in airway smooth muscle cells. This NO production was catalyzed by P450 but not by NOS.

In conclusion, my studies have demonstrated an important role for endogenous NO production in determining the airway responsiveness of normal rats to inhaled cholinergic agonists. This mechanism contributes to strain-related differences in airway responsiveness in the rat.

RÉSUMÉ

L'hyperréactivité bronchique est présente dans l'asthme et est reliée à la sévérité de la maladie. Les facteurs qui contribuent au développement de l'hyperréactivité bronchique sont encore mal connus. L'existence d'un débalancement entre les substances endogènes responsables de la bronchoconstriction et de la bronchodilatation représente un mécanisme potentiel contribuant à l'hyperréactivité bronchique. L'oxide nitrique (NO) favorise la relaxation du muscle lisse trachéal par l'activation de la forme soluble de la guanylate cyclase et en augmentant le niveau intracellulaire de guanosine monophosphate cyclique (cGMP). Notre première hypothèse de recherche était que la voie NO- cGMP soit impliquée dans la régulation de l'hyperréactivite bronchique. L'inhibition de l'oxide nitrique endogène par l'administration de N^{ω}-nitro-L-arginine (L-NNA) a augmenté de façon significative la réactivité bronchique à la méthacholine inhalée chez les rats avec réactivité normale de type Lewis et également chez les rats avec hyperréactivité de type Fisher, mais à un degré moindre chez ces derniers. De plus, l'administration de carbachol a augmenté les taux de cGMP mesurés dans les tissus trachéaux chez les deux types de rats; l'accumulation de cGMP dans les tissus trachéaux était moindre chez les rats Fisher que chez les rats Lewis et était abolie par l'administration de L-NNA chez les deux types de rats, suggérant la participation de NO. Ces résultats suggèrent que le NO endogène participe à la régulation de l'hyperréactivité bronchique et contribue aux différences quant au degré de la réactivité bronchique caractérisant les deux types de rats. Afin d'investiguer la participation de la voie NO-cGMP dans le développement de la réactivité bronchique chez le rat, nous avons étudié l'effet du nitroprussiate de sodium (SNP, un donneur de

NO) sur la réactivité bronchique cholinergique chez les rats avec hyperréactivité Fisher en comparaison avec les rats avec réactivité normale Lewis. L'administration de SNP a provoqué un degré moindre de relaxation des anneaux trachéaux préalablement contractés par du carbachol ainsi qu'une accumulation moindre de cGMP dans les cellules musculaires lisses trachéales en culture chez les rats Fisher en comparaison avec les rats Lewis, témoignant une réponse altérée au SNP des voies aériennes des rats Fisher.

NO provient de la catalyse de la L-arginine par le NO synthase (NOS). La production de NO in vitro provient également de la rupture des ponts C=N de composés contenant des éléments -$C(NH_2)NOH$ par l'enzyme cytochrome P450. Nous avons emis l'hypothèse que la biosynthèse de NO dans les cellules musculaires lisses bronchiques puisse provenir de l'action des enzymes P450. Plusieurs types de cellules d'origine pulmonaire peuvent synthétiser le NO. Par contre, il n'existe aucune évidence d'activité de l'enzyme NOS par les cellules musculaires lisses trachéales. Nous avons vérifié la possibilité que les cellules musculaires lisses trachéales soient capable de synthétiser le NO. Nous avons trouvé que les cellules musculaires lisses trachéales en culture produisent NO en présence de formamidoxime, un composé contenant des éléments -$C(NH_2)NOH$. De plus, le formamidoxime a induit la relaxation du muscle lisse trachéal préalablement contracté et l'accumulation de cGMP dans les cellules musculaires lisses trachéales en culture. Ces effets sont inhibés par des inhibiteurs du P450 et non par des inhibiteurs de NOS. Ainsi, nous avons pu démontrer la présence d'une voie indépendante de la L-arginine pour la production de NO dans les cellules musculaires lisses trachéales. Cette production de NO est catalysée par le P450 et non par le NOS.

En conclusion, ces études ont démontré un rôle important de la production endogène de NO comme facteur déterminant de la réactivité bronchique aux agents cholinergiques inhalés chez les rats normaux. Ces méchanimes peuvent contribuer aux différences de réactivité bronchique caractérisant divers types de rats.

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I offer my final thanks to my husband who is also my best friend, my parents who share many of my duties as a mother, and my sweet daughter who fills my heart with happiness. From them, I always receive love, encouragement, support and understanding. This is for them!

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PREFACE

This thesis is composed of five chapters. Chapter 1 is a general review of the literature

which provides background information pertaining to the thesis. Chapter 2, 3, and 4 are original manuscripts which have been submitted for publication, and are complete with their own Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgement and References. This thesis format has been selected with the approval of the Department of Medicine and in accordance with the Guidelines for Thesis Preparation of the Faculty of Graduate Studies and Research, McGill University (revised September 1994). As required by those regulations, the text of the section for manuscript-based structure in the guidelines is reproduced in full below.

> "Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.
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> If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.
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> The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.
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> Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.
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> In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who

contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis."

Chapter 2 (Endogenous nitric oxide contributes to strain-related differences in airway responsiveness in rats. Y. Jia, L. Xu and J.G. Martin) has been submitted to the Journal of Applied Physiology and is in revision; Chapter 3 (Airways of a Hyperresponsive Rat Strain Show Decreased Relaxant Responses to Sodium Nitroprusside. Y. Jia, L. Xu, S. Heisler and J.G. Martin) has been accepted by American Journal of Physiology and is in press; Chapter 4 (Nitric oxide can be synthesized by tracheal smooth muscle cells. Y. Jia, S. Heisler and J. G. Martin) has been submitted to the American Journal of Physiology. Chapter 5 contains conclusions and claims for originality.

In accordance with the Thesis Preparation Guidelines, the relative contributions of the co-authors are noted here briefly. For all projects, overall supervision was provided by Dr. James G. Martin. Dr. Seymour Heisler also supervised studies contained in chapter 3 and 4. The measurement of lung resistance in vivo was conducted by Ms. Lijing Xu, a technician working under Dr. Martin's supervision.

Chapter 1

Introduction

1.1 Nitric Oxide and the Airway

1.1.1 History of the Oxides of Nitrogen[1]

An endogenous production of nitrate was first found in 1916 through dietary studies which showed that excreted nitrate exceeded the ingested nitrate. (184). Since excess nitrate was thought to be produced by intestinal microorganisms and had little to do with mammalian biology, this finding did not draw much research interest for decades until Green and his colleagues found that nitrate was synthesised mainly outside the intestine (100). This finding provided the impetus for renewed interest in the study of nitric oxide (NO) in mammalian biology.

In the meantime, another important finding was made in the area of cardiovascular physiology. Furchgott and Zawadzki proved that removal of the endothelium inhibited the relaxant effect of acetylcholine and even induced a contraction in isolated arterial strips. Thus the endothelium was essential for acetylcholine induced vasodilation (90). The vasodilatory effect was thought to be induced by a substance in endothelium which was released by the action of acetylcholine. Furchgott named the substance endothelium-derived relaxing factor (EDRF). Afterwards, other vasoactive

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Nitric oxide, in the presence of oxygen, can be converted into a group of chemicals named nitrogen oxides. Some of the biological functions and toxicity of nitric oxide may be induced by nitrogen oxides other than NO. The term "nitric oxide" is used in this thesis just for simplicity. However, the specific nitrogen oxides are mentioned in text when necessary.

substances such as histamine, serotonin, bradykinin, substance P and thrombin were also found to be able to cause release of EDRF from endothelial cells and induce vasodilation (89).

The chemical nature of EDRF was discovered in 1987. EDRF was first found to be very similar to NO in its biological properties (122). Soon afterwards, Moncada and his colleagues proved that the biological function of EDRF was related to NO release (205). They also found an L-arginine-dependent NO production in endothelium-dependent relaxation (207). Their findings were strong evidence that EDRF was NO.

Since then, a great deal of research interest has been focused on NO. A variety of potential biological functions of NO have been identified including smooth muscle relaxation, platelet inhibition, neurotransmission, tumour cell lysis, bacterial killing and stimulation of hormonal release.

The biology of NO in airways has been studied only in the last few years although nitrovasodilators such as nitroglycerine, have been recognized as a possible treatment of bronchospasm for more than a century (204). Nitrovasodilators have been found to induce airway smooth muscle relaxation with concomitant elevations in cyclic GMP, an NO mediated second messenger (142,143). Inhaled NO gas has also been shown to decrease airway responsiveness to methacholine (MCh) in both guinea pigs (67) and human subjects (116). Inhalation of NO exerts a bronchodilatory effect in bronchial asthma as well (116). Endogenous production of NO in airways has been found by measuring NO in expired air (104,276). Roles for endogenous NO in the airways, including regulation of airway responsiveness to bronchoconstrictors, have also been

suggested using NO synthase (NOS) inhibitors (197). These findings give new insights into the biology of NO in normal and asthma airways.

1.1.2 Production of Endogenous Nitric Oxide in the Airway.

Nitric oxide is synthesized from L-arginine in two steps: from L-arginine to N-hydroxy-L-arginine and from N-hydroxy-L-arginine to NO and L-citrulline (268). Both steps are catalyzed by NOS. A number of NOS isoforms have been purified from different tissues, and several more have been cloned and, in some cases, functionally expressed. All of the isoforms require NADPH, tetrahydrobiopterin, FAD and FMN as cofactors. In general, NOS are divided into two major groups: a constitutive, Ca^{2+}/calmodulin-dependent type which can be activated by mediators such as bradykinin, acetylcholine, calcium ionophore, histamine, leukotrienes, and platelet activating factor (33,156,196,206), and an inducible isoform that is induced at the transcriptional level by cytokines, lipopolysaccharide (LPS) and endotoxin (72,115,266), and typically does not show a dependence on Ca^{2+}/calmodulin. Based on the physical and biochemical characteristics of the purified enzymes, NOS can also be classified into three isoforms (250,267): NOS-I is a constitutive Ca^{2+}/calmodulin-dependent enzyme found in the soluble fraction of brain, NOS-II is a inducible isoenzyme in macrophages and NOS-III is a constitutive enzyme located in the particulate fraction of endothelial cells. Molecular cloning studies indicate that these isoforms are the products of distinct genes (182).

NO can be generated by the lung through activation of various isoenzymes of

NOS. NOS has been detected in a number of cell types in the lung including macrophages(396,397,2928), neutrophils (232), mast cells (30), nonadrenergic noncholinergic inhibitory neurons (25,38), fibroblasts (137), vascular smooth muscle cells (39), pulmonary arterial and venous endothelial cells (64,123), and airway epithelial cells (46,152,249). Using immunocytochemical and histochemical techniques, Kobzik et al (152) observed marked immunostaining of constitutive NOS in lung nerves and endothelium in rat and human, and in rat airway epithelium. Immunohistochemical studies also demonstrated the expression of inducible NOS in alveolar macrophages from inflamed rat lung tissue (152) and human subjects with bronchiectasis and acute bronchopneumonia (275). Inducible NOS was also expressed in epithelial cells in both human bronchi and normal rat trachea. (152,234). NO production was detected in LPS-induced rat alveolar macrophages (136). NOS activity has been reported in bovine bronchial epithelial cells as well (235).

With widespread expression of NOS, the respiratory system produces endogenous NO, which can be detected in exhaled air from humans and animals (104,162). In normal human subjects, NO levels during a single exhalation start to rise simultaneously with the increase in CO_2, indicating that the major formation of NO occurs in the respiratory bronchioles but not anatomic dead space (217). However, the nasal airways have also been suggested to be the origin of NO in exhaled air in normal human subjects (5). Exercise and hyperventilation at rest increase production of NO into exhaled air, suggesting that hyperventilation is a stimulator of NO production (217). In women, expired NO increases during the menstrual cycle (290). NO in exhaled air is also

increased in some inflammatory respiratory diseases such as asthma and cystic fibrosis (5,95,147,218), as well as in allergen-induced airway obstruction in guinea pigs (215), but is undetectable in the expired air of ventilated patients (191). Clearly, NO has a variety of biological functions in normal and diseased lung.

1.1.3 Biological Functions of Nitric Oxide in the Lung

In mammalian physiology, NO, by activating soluble guanylate cyclase and increasing intracellular cyclic GMP, has a variety of biological functions including modulation of vascular tone to regulate blood flow and blood pressure. It also plays a role in central and peripheral neurotransmission and is involved in host defence mechanisms, where it may account for non-specific immunity (185). In the airway, nitrovasodilators, S-nitrosothiols and NO gas induce smooth muscle relaxation. In addition, endogenously produced NO has been found to play an important role in the regulation of airway smooth muscle tone and responsiveness to bronchoconstrictors as well as in other physiological functions in the airways, as described below in detail.

1.1.3.1 NO Relaxes Airway Smooth Muscle

In many species such as dog, guinea pig and human, NO has been demonstrated to relax airway smooth muscle constricted with MCh, histamine, or leukotriene D_4

(96,131,295). Inhaled NO decreases baseline pulmonary resistance and MCh-induced bronchoconstriction in guinea pigs (67). Inhalation of NO also decreases the airway response to MCh provocation in human subjects and induces a weak bronchodilation in asthmatic patients but has no effect on volume-corrected specific airway conductance in normal subjects and patients with chronic obstructive pulmonary disease (116).

Dissolved NO gas was approximately 1 log less potent in mediating the relaxation of bovine trachealis compared with endothelium-denuded bovine pulmonary artery (37). In guinea pig airways, the relaxant effect of S-nitrosothiols (RS-NO, IC_{50}=1-20 μM), relatively stable NO adducts that form readily under physiological conditions from the reaction of NO with reduced thiols (201) was less potent than isoproterenol (IC_{50}=0.016 μM) and more potent than theophylline (IC_{50}=74μM) (131). The potency of NO in relaxing airway smooth muscle varies considerably with the oxidation state of the molecule, as well as with the species, airway generation, and epithelial integrity of the airway. In guinea pigs and human airways, S-nitrosothiols are 2 logs more potent than NO_2^-. The 50% inhibitory concentrations (IC_{50}) of RS-NO were between 1 to 10 μM in guinea pig trachealis while in human airways, IC_{50} of RS-NO was 10 to 70 μM (96,131). Proximal airways had larger relaxations to nitroglycerin and nitroprusside, NO donors, than did distal airways (102). NO when applied inside the tracheal tube relaxed guinea pig trachea more potently in epithelium-denuded than in epithelium-intact preparations (190). The barrier function of epithelium and/or endogenous NO production in epithelium may account for this observation.

The mechanisms of NO induced airway smooth muscle relaxation are believed to

involve the activation of soluble guanylate cyclase and an increase of intracellular cyclic GMP, which in turn activates a cyclic GMP-dependent protein kinase (81,83) to induce relaxation of the airway smooth muscle. This proposed mechanism has been studied using nitrovasodilators, which are believed to release NO (75,282). Several studies have demonstrated significant correlations between cyclic GMP accumulation and relaxation induced by various nitrovasodilators. Sodium nitroprusside (SNP), which releases NO, induced a relaxation of canine trachealis that was accompanied by a concentration-related increase in cyclic GMP content (295). The time courses of relaxation and cyclic GMP accumulation were parallel. Zaprinast, a cyclic GMP-specific phosphodiesterase V inhibitor, potentiated both SNP-induced relaxation and cyclic GMP accumulation. 8-bromo-cyclic GMP, a cell-permeable analog of cyclic GMP, mimicked the relaxant effects of SNP. Methylene blue, a guanylate cyclase inhibitor, and haemoglobin, that binds NO-containing compounds, suppressed both cyclic GMP accumulation and relaxation induced by S-nitroso-N-acetyl-penicillamine (SNAP) and glyceryl trinitrate (295). In canine tracheal smooth muscle, 3-morpholinosydnonimine (SIN-1) caused a concentration-dependent decrease in force produced by acetylcholine, which was correlated with the increase in intracellular cyclic GMP (134). Methylene blue proportionately attenuated both relaxation and cyclic GMP accumulation induced by SIN-1. These observations demonstrated that NO caused airway smooth muscle relaxation, mediated by cyclic GMP-dependent mechanisms. However, not all studies support the role of cyclic GMP in mediating nitrovasodilator induced relaxation of airway smooth muscle. Methylene blue and haemoglobin inhibited SNP-induced cyclic GMP

accumulation but, potentiated SNP-induced relaxation (295). These observations have prompted the suggestion that a cyclic GMP-independent mechanism also contributes to SNP-induced airway smooth muscle relaxation.

1.1.3.2 Cyclic GMP and Airway Smooth Muscle Relaxation

Cyclic GMP is synthesized from GTP in a reaction that is catalyzed by guanylate cyclase. Guanylate cyclase is localised to both the particulate and soluble cell fractions. Atrial natriuretic factor (ANF), brain natriuretic peptide and C-type natriuretic peptide (242), guanylin, and the heat stable enterotoxins from E. coli (58,251) bind to and stimulate the particulate guanylate cyclase, whereas NO binds to and activates soluble guanylate cyclase. Soluble guanylate cyclase has been purified from rat (93) and bovine (97) lung. The purified enzyme from bovine lung was stimulated 15 to 140 fold by SNP and catalyzed the formation of cyclic GMP at a high rate (8-15 μmol min^{-1}) (97).

Cyclic GMP relaxes airway smooth muscle in a variety of species (112,143,221,269,274). The potential target for cyclic GMP, cyclic GMP kinase which may mediate cyclic GMP-dependent relaxation, is present in all smooth muscle cells (44,146,168) including trachea (91). The relationship between kinase activation and airway smooth muscle relaxation has been examined in guinea pigs (83). The activation of cyclic GMP kinase correlated excellently with smooth muscle relaxation in carbachol contracted tracheal strips whereas activation of cyclic AMP kinase had little effect on airway smooth muscle tone. These results support a role for cyclic GMP kinase, but not

cyclic AMP kinase, in lowering smooth muscle tone and has established a link between activation of cyclic GMP kinase and relaxation of airway smooth muscle.

Three possible mechanisms have been suggested in cyclic GMP-cyclic GMP-dependent protein kinase-induced smooth muscle relaxation. Firstly, activation of cyclic GMP kinase by cyclic GMP may decrease the calcium sensitivity of the contractile proteins. This hypothesis is based on the observation that cyclic GMP kinase shifted the calcium-dependence of tension development and myosin light chain phosphorylation to the right in skinned smooth muscle strips which did not contain intact vesicular calcium stores (219,220). Since cyclic GMP kinase was observed to phosphorylate myosin light chain kinase (198), it was suggested that cyclic GMP kinase suppressed MLCK activation by the calcium/calmodulin complex, which in turn would reduce myosin light chain phosphorylation and thereby decrease force generation. However, elevation of cyclic GMP levels also decreased the maximal extent of myosin light chain phosphorylation which could not be explained by a shift in the calcium sensitivity of the contractile proteins. Other mechanisms such as stimulation of phosphatase were also proposed (143). These mechanisms need more experimental support.

A second potential explanation for cyclic GMP kinase-induced smooth muscle relaxation is that activation of cyclic GMP kinase may decrease cytosolic calcium concentrations. It has been shown in vascular and airway smooth muscle that cyclic GMP-elevating agents and cell permeant cyclic GMP analogues lower cytosolic free calcium levels (76,138,151,187,273). An active fragment of cyclic GMP kinase prevented the increase in cytosolic free calcium levels in bronchial smooth muscle evoked

by carbachol (76). The lowering of cytosolic free calcium levels by cyclic AMP was also suggested to result from activation of cyclic GMP kinase (132,167).

Several regulatory pathways by which cyclic GMP kinase lowers cytosolic free calcium have been identified. In vascular smooth muscle cells, cyclic GMP kinase activated plasma membrane Ca^{2+}-ATPase (91) which promoted calcium extrusion. As well, cyclic GMP kinase phosphorylated phospholamban in smooth muscle, up-regulating Ca^{2+}-ATPase in sarcoplasmic reticulum (SR) and stimulating calcium uptake into intracellular stores (53,226,247,279). It was reported recently that SNP and 8-bromo-cyclic GMP induced canine tracheal smooth muscle relaxation were greatly reduced by cyclopiazonic acid, a selective inhibitor of the SR Ca^{2+}-ATPase, indicating that pumping of Ca^{2+} into SR played an important role in cyclic GMP induced relaxation (179). Cyclic GMP kinase was also demonstrated to inhibit agonist-triggered breakdown of phosphatidylinositol 4,5 bisphosphate (PIP_2). NO, SNP and 8-Br-cyclic GMP inhibited agonist-induced accumulation of inositol phosphates in rat (228) and rabbit (157) aorta. Activation of cyclic GMP kinase suppressed the thrombin-induced calcium transient by inhibition of IP_3 synthesis in cyclic GMP kinase transfected Chinese hamster ovary cells (28). These cyclic GMP kinase regulatory pathways lead to a decrease in the cytosolic calcium concentration and contribute to cyclic GMP induced smooth muscle relaxation.

A third mechanism of action of cyclic GMP kinase is the induction of membrane hyperpolarization. NO and SNP hyperpolarize the membrane in certain vascular beds (135). Hyperpolarization of the vascular smooth muscle membrane decreases the open probability of the voltage-dependent calcium channels, reduces the sustained calcium

influx and thereby decreases tension development. Ca^{2+}-activated K^+ channels contribute significantly to the membrane potential in vascular and bronchial smooth muscle. In arterial smooth muscle, Ca^{2+}-activated K^+ channels were found to be phosphorylated and stimulated by cyclic GMP kinase (238,272), which may lead to a membrane hyperpolarization, and thereby, an inhibition of voltage-dependent Ca^{2+} channels. It is likely that a similar mechanism exists in airway smooth muscle, though direct evidence remains to be provided.

1.1.3.3 Endogenous Nitric Oxide Regulates Airway Smooth Muscle Tone

Endogenous NO plays a role in regulation of airway smooth muscle tone. The evidence for such a role is largely derived from studies using NOS inhibitors. NO is suggested to be a transmitter of inhibitory nonadrenergic noncholinergic (NANC) induced airway relaxation. Non-neural sources of NO may also act as a negative regulator of airway responsiveness to bronchoconstrictors.

The presence of NANC inhibitory nerves, which cause relaxation of smooth muscle, has been demonstrated in the airway in many species (discussed in section 1.2.2.3). The neurotransmitter of inhibitory NANC system in the airway is not known, and may vary with the species. In guinea pig trachea, vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI) have been suggested to account, in part, for the NANC relaxant responses (70,71,178). However, a non-peptide neurotransmitter has also been suggested by the same authors.

NO has been found to be a mediator for NANC neurotransmission in the gastrointestinal tract (246). In the airway, it has also been shown that NO plays an important role in NANC-induced relaxation in several species including humans. Li and Rand demonstrated that part of the NANC relaxant response of trachea was mediated by NO in guinea pigs (166). NOS inhibitors reduced the VIP-independent component of NANC-induced relaxation. A similar inhibitory effect of NOS inhibitor on NANC-induced relaxation was also found in pig (141) and horse (292) airway smooth muscle. In human airways, it is believed that NO plays a more important role in the mediation of NANC-induced relaxation than does VIP since N^G-nitro-L-arginine methyl ester (L-NAME) partly inhibited the NANC-induced relaxation whereas α-CT did not (10,283). Contrary to the above species, NANC-induced relaxation in feline bronchi was not inhibited by NOS inhibitors. This is of interest in that the first observations of a NANC relaxant system in vivo were made in the cat (62,125). The localization of NOS in airway neurons (25,38) provides further supporting evidence that NO is a neurotransmitter in NANC-induced airway relaxation.

The role of endogenous NO from epithelium in modulation airway responsiveness to bronchoconstrictors has recently been demonstrated in guinea pigs in vivo and in vitro. The administration of NOS inhibitors by aerosol to spontaneously breathing anaesthetized guinea pigs resulted in a significant enhancement of airway responsiveness to intravenous histamine infusion (197). NOS inhibition also increased the basal tone of tracheal tubes and the responsiveness to carbachol and MCh in vitro. Removal of the epithelium resulted in an upward shift in the histamine concentration-response curve and a NOS

inhibitor did not further increase tracheal responsiveness (197). Similar results have also been observed by other investigators (92) confirming that endogenous NO is involved in the regulation of airway responsiveness to bronchoconstrictors, at least in guinea pigs, and suggesting that airway epithelium may be the site where NO is produced. Further, it has been shown that airway tissue cyclic GMP concentrations increase after stimulation with histamine (163) and carbachol (254). It can be hypothesized that cholinergic stimulation and histamine activate NOS to produce NO which in turn increases intracellular cyclic GMP levels and down regulates airway responsiveness.

1.1.3.4 Nitric Oxide and Pulmonary Vascular Tone

NO is a potent dilator of vascular smooth muscle and plays an important role in regulating pulmonary vascular tone (13,124). Inhaled NO caused sustained pulmonary vasodilation in late gestation lambs (148). In blood-perfused isolated rat lungs, inhaled NO and SNP dilate small resistance arteries and veins, while SNP also dilates larger capacitance arteries and veins (241). Involvement of endogenous NO in the regulation of lung vascular function in vivo has been proposed, based on the observation that inhibition of NOS increases pulmonary vascular resistance (216). NOS inhibitors increase pulmonary vascular resistance and enhance hypoxic pulmonary vasoconstriction (262). Chronic NO inhibition in lamb in utero by continuous infusion of N$^{\omega}$-nitro L-arginine (LNNA) produces persistent pulmonary hypertension in newborn lambs (79). Since

inhaled NO selectively relaxes pulmonary vessels, it has been used in the treatment of pulmonary hypertension in recent years (see section 1.1.4).

1.1.3.5 Other Functions of Nitric Oxide in the Lung

A recent study has shown that NO is involved in the modulation of airway epithelial ciliary beat frequency (129). NOS inhibitors caused a decrease in ciliary beat frequency of cultured bovine bronchial epithelial cells stimulated by isoproterenol, bradykinin or substance P, indicating that NO may up-regulate ciliary motility in response to stimulation.

NO is known as an regulator of immunological reactions. The immune suppression mediated by the alveolar macrophage, a source of the inducible form of NOS in the lung, is eliminated by NOS inhibition, suggesting immunosuppressive activity of NO in alveolar macrophages (144). In rat lung macrophages, NO induced by interferon-gamma and lipopolysaccharide has been shown to reduce early growth response-1 gene expression, which is required for macrophage differentiation (114). This mechanism may be involved in the regulation of macrophage differentiation in inflammatory lung disease. Further, NO has also been proved to inhibit T cell-macrophage interactions (255), to regulate interleukin-4-induced IgE production by normal human peripheral blood mononuclear cells (211), and to modulate peripheral polymorphonuclear leucocyte responses (234). These immunological regulatory functions in the airway have not been studied.

Nitrovasodilators and 8-bromo-cyclic GMP have also been shown to inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells (94). However, the role of NO and cyclic GMP in modulating airway smooth muscle cell growth has not been addressed.

1.1.4 Current Therapeutic Applications of Nitric Oxide in Pulmonary Disease.

Inhaled NO has been used as a therapeutic substance in recent years. Inhaled NO in low concentrations (<80 ppm) is rapidly taken up by the capillary blood of the lung. Some inhaled NO can also diffuse through the resistance pulmonary arterioles at the precapillary level. In the blood, NO is rapidly inactivated by binding to haemoglobin and thus will not have any generalised vascular effects (231). Therefore, inhaled NO has a selective vasodilatory effect in pulmonary blood vessels and may be used in the treatment of pulmonary hypertension (6).

Pulmonary hypertension is commonly observed in neonatal and adult respiratory distress syndrome, after cardiopulmonary by-pass surgery and as an idiopathic condition. Existing therapy for pulmonary hypertension is not selective for the pulmonary circulation and therefore, can decrease systemic arterial pressure. In contrast, inhalation of NO gas in low concentrations (50-80ppm) causes selective pulmonary vasodilation. Beneficial effects of administration of NO in inhaled air to patients suffering from pulmonary hypertension have been demonstrated. Early studies of patients with primary pulmonary hypertension with extremely high pulmonary vascular resistance showed that

inhaled NO at a concentration of 40 ppm caused a selective pulmonary vasorelaxation (214). Similar observations were made in experimental acute lung injury and adult respiratory distress syndrome (ARDS) patients. In patients with ARDS, continuously inhaled NO (5 to 20 ppm) for 3 to 53 days reduced pulmonary arterial pressure and improved oxygen exchange (243). It has been shown that the ventilation-perfusion mismatch caused by acute lung injury can be alleviated by inhaled NO which improves gas exchange by redistributing blood flow from shunt units to lung units with a nearly ideal ventilation-perfusion ratio, without affecting total pulmonary or systemic vascular resistance (223). Improved ventilation-perfusion matching and gas exchange were most pronounced when NO was inhaled at the same time endogenous NO, which opposes hypoxic pulmonary vasoconstriction, was inhibited by systemic L-NMMA. Pulmonary hypertension induced by group B streptococcal sepsis in piglets was reversed by inhaled NO without changing cardiac output and systemic arterial pressure (27). Another disease which can be improved by inhaled NO is the persistent pulmonary hypertension of the newborn (PPHN). Roberts et al reported that low levels of inhaled NO increased pre- and post-ductal oxygen saturation and oxygen tensions in infants with PPHN (237). An improved oxygenation by inhaled NO in patients with PPHN was also reported by Kinsella et al (149). NO inhalation also reduces the pulmonary hypertension associated with cardiopulmonary by-pass (86).

NO may not only cause vascular smooth muscle relaxation but can also relax airway smooth muscle. The therapeutic effect of NO in asthma is discussed in section 1.1.6.

The toxicity of inhaled NO depends on the concentration of NO and the duration of inhalation. Rabbits breathed 43 ppm NO and 3.6 ppm NO_2 for up to 5 days without evidence of lung injury as measured by light or electron microscopy (119). Little evidence exists for NO toxicity at low concentrations in animals (86,119). It has been shown that there is no significant elevation in methaemoglobin levels in patients with adult respiratory distress syndrome treated by continuous inhaled NO for up to 53 days (243). However, concerns remain regarding potential toxicities, such as lung injury due to NO_2, peroxynitrite, and hydroxyl radical formation (22) (also see section 1.1.5).

In summary, at present, NO inhalation therapy remains promising but experimental. Some important questions, such as for how long and at what concentrations it is safe to expose the normal or acutely injured lung to NO and what the effect of inhaled NO on pulmonary structure and function is, remain to be answered.

1.1.5 Toxicity of Nitric Oxide in the Airway.

Nitric oxide is a common air pollutant, being a product of the combustion of a variety of compounds, including fossil fuels (188,289) and cigarette smoke (200). In the gas phase and in the presence of oxygen, NO is converted to NO_2, and then may react with NO_2 to form N_2O_3, a potent nitrosating species. Under certain conditions, NO may react with the superoxide anion ($O_2^{\cdot}$) to form the peroxynitrite anion ($ONOO^{\cdot}$), which is an extremely potent oxidising agent (22,23,224). These products and the products of reactions with amines, thiols, heme-porphyrin moieties, unsaturated lipids, and other

compounds in the airway lining layer under conditions of varying pH, reactant concentration, and temperature may induce toxicity in the lung.

The cytotoxicity of NO may be mediated by its combination with iron-containing enzymes of the mitochondrial respiratory chain and by affecting the synthesis of DNA in the target cells. It has been shown that induction of NOS in rat neonatal astrocytes in primary culture produced 25% to 56% inhibition of cytochrome c oxidase activity. The inhibition of the enzyme activity was prevented by incubation in the presence of the NOS inhibitor N^G-monomethyl-L-arginine (31). NO, can damage DNA at physiological pH by deaminating deoxynucleosides, deoxynucleotides and intact DNA. This deamination of DNA by NO may contribute to some genetic disease and smoke related cancer (286).

NO, when present in the air at high concentrations or for a long time, is toxic to the lung. Exposure to the air containing 0.5 to 1.5 ppm NO for 9 weeks induced alveolar septal injuries in rats (181). NO in higher concentrations may, at least theoretically, have pro-inflammatory effects such as an adverse influence on capillary permeability (6). The reaction of NO with hemoglobin can also lead to the formation of methemoglobin. Inhaling NO at higher levels caused marked methemoglobinemia and pulmonary edema, and has caused death in humans (47). Methemoglobinaemia, if excessive, can be treated with methylene blue without blocking the vasodilatory effect of NO on pulmonary vasculature (128,291).

Recent observations indicate that activated alveolar macrophages secrete large quantities of peroxynitrite, a product of NO and superoxide anion, into the epithelial lining liquid (126). Peroxynitrite was found to play a role in the initiation and

propagation of injury to alveolar epithelial cells and thus contribute to NO induced lung injuries. Peroxynitrite injures the pulmonary surfactant system to a much larger extent than reactive oxygen species generated by supraphysiological concentrations of xanthine and xanthine oxidase in the presence of iron (106). The simultaneous generation of NO and superoxide by SIN-1 resulted in a production of peroxynitrite and a dose-dependent decrease in the activity of surfactant protein A from the bronchoalveolar lavage of patients with alveolar proteinosis (105). Peroxynitrite also decreases alveolar type II cell Na^+ transport by damaging apical amiloride-sensitive Na^+ channels (118). Peroxynitrite is a major cytotoxic agent produced by inflammatory cells of the immune system and has been implicated in immune complex-stimulated pulmonary edema (189).

Another oxidative product of NO which may contribute to NO induced cytotoxicity in the lung is nitrogen dioxide (NO_2), which is also a common air pollutant. It has been reported that NO_2 can cause airway epithelial cell damage in different species (43,113,145,227) including human subjects (40). When rats were exposed to low levels of NO and nitrogen dioxide for 9 weeks, alveolar septal injuries were induced (181). Exposure to NO_2 also induced the synthesis of cytokines from human bronchial epithelial cells which may induce inflammation of airway epithelium (59).

1.1.6 Nitrogen Oxides and Asthma

Endogenous NO is known to be involved in the regulation of airway responsiveness in guinea pigs. A relationship to asthma in human subjects is suggested

by the finding that airway epithelial NOS is increased in bronchial biopsies from asthmatics relative to those from normal subjects (109). Further, the expired NO concentrations were higher in asthmatics than in normal subjects (95). The pathological role of NO in asthma is unclear. Since activation of alveolar macrophages, which is also a pathophysiological process in asthma, causes them to secrete large quantities of peroxynitrite, a product of NO and superoxide anion which may induce alveolar epithelial cell injury, into the epithelial lining liquid (127), NO produced by inducible NOS may, at least theoretically, be involved in the epithelial damage in asthma.

Nitrovasodilators, which release NO, have been used in the treatment of asthma (99,202,277). Recently, the effect of inhaled NO on induced airway constriction and asthma has been studied. Hogman et al. reported that inhalation of 80 ppm NO gas modulated the MCh-induced bronchoconstriction toward dilation in adult human subjects. Further, in patients with bronchial asthma, volume-corrected specific airway conductance increased from 0.4 ± 0.1 to 0.6 ± 0.2 ($kPa.s^{-1}$) after NO inhalation. This effect of NO is additive with $ß_2$-agonist inhalation (116). In asthmatic human subjects, 40 ppm inhaled NO gas increases airway conductance as well (85). The fact that NO is a weaker dilator of airway smooth muscle compared to vascular smooth muscle and that endogenous NO is already increased in asthmatics limit the therapeutic effect of inhaled NO in asthma. Indeed it might even benefit asthmatic patients if endogenous NO were inhibited.

In summary, nitric oxide is a recently discovered important messenger in the airway which has a variety of biological functions in the normal and diseased lung including regulation of airway smooth muscle tone. Inhaled NO has been employed as

a temporary treatment for some of the respiratory diseases such as pulmonary hypertension. Not all its actions are beneficial, however. NO is toxic under some physiological conditions. Further studies are required to elucidate the role of NO in the regulation of airway responsiveness, the source, metabolic fate and roles of endogenous NO in normal and diseased states such as asthma and cystic fibrosis, the effect of NO on lung cell proliferation and the potential therapeutic effects as well as the toxicity of inhaled NO in respiratory diseases including asthma.

1.2 Signal Transduction Pathways of Airway Smooth Muscle Contraction

1.2.1 Mechanisms of Airway Smooth Muscle Contraction

It is commonly accepted that contraction of smooth muscle mainly depends upon the cytoplasmic concentration of calcium ($[Ca^{2+}]_i$). The sources of Ca^{2+} are the extracellular space and the sarcoplasmic reticulum (SR). Two major types of calcium channels in the smooth muscle cell membrane account for the entry of Ca^{2+} in response to appropriate stimuli: voltage-dependent Ca^{2+} channels which are activated by membrane depolarization and receptor-regulated Ca^{2+} channels, which are opened by ligand binding of the receptor. The binding of ligand (airway constrictors) to receptors activates phospholipase C *via* G-proteins, which catalyze the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2). IP_3 in turn increases $[Ca^{2+}]_i$ by releasing Ca^{2+} from SR by binding to a specific IP_3 receptor.

The Ca^{2+}-calmodulin complex formed at increased $[Ca^{2+}]_i$ binds and activates myosin light chain kinase (MLCK), which phosphorylates the myosin light chain and results in actin activation of myosin Mg^{2+}-ATPase. Myosin undergoes cyclical interactions with actin with sliding of the thick and thin filaments driven by energy derived from ATP hydrolysis in each cycle, generating force and shortening. In addition to the primary activating effect of Ca^{2+} on MLCK, increased $[Ca^{2+}]_i$ also activates Ca^{2+}-

calmodulin kinase (CaM kinase II) which phosphorylates MLCK and decreases MLCK catalytic rate at a given concentration of Ca^{2+} and calmodulin. This pathway is thought to provide negative feedback that limits activation of crossbridges by large Ca^{2+} transients.

Break down of PIP_2 catalyzed by phospholipase C also produces DAG which in turn activates protein kinase C (PKC) in the presence of low levels of calcium. PKC has potentially important functions in the regulation of airway smooth muscle tone. Activation of PKC by phorbol esters induces slow airway smooth muscle contraction, which is associated with the phosphorylation of the same proteins as those observed during the late and prolonged phase of contraction induced by carbachol (210). Both contraction and protein phosphorylation induced by PKC activation are blocked by removal of extracellular Ca^{2+} (209,210). These observations suggest a role for PKC in maintaining smooth muscle contraction. In addition, airway smooth muscle agonists induce redistribution of PKC from the cytosol to the membrane by a Ca^{2+}-dependent mechanism (158). The translocation of PKC in response to bronchoconstrictors is inhibited by the bronchodilator isoprenaline (159) and SNP (158), indicating the involvement of PKC in airway smooth muscle constriction and relaxation. On the other hand, PKC has been shown to inhibit IP_3 production and Ca^{2+} transients by inhibition of phospholipase C activities (35). Thus PKC may also play a role in negative feedback to prevent over stimulation of the tissue and to limit the magnitude of the contractile response.

1.2.2 Autonomic Control of Airway Tone.

The autonomic nervous system is an important physiologic regulator of airway smooth muscle tone. It includes parasympathetic innervation, sympathetic innervation and nonadrenergic noncholinergic innervation.

1.2.2.1 Parasympathetic Innervation

Distribution of Nerve Fibres and Cholinergic Receptors

Parasympathetic nerve endings are found from the trachea to the bronchioles; their number decreases in the smaller airways. Acetylcholine, which binds to muscarinic receptors, is both the neural transmitter at the synapse between the preganglionic fibres and postganglionic neurons and the neural mediator between the postganglionic terminals and the airway smooth muscle. The stimulation of parasympathetic nerves results in the constriction of the airway. Moreover, parasympathetic nerve fibres appear to continuously release acetylcholine that contributes to the baseline tone of the airway (287).

Acetylcholine induced airway smooth muscle contraction is mediated by activation of muscarinic receptors, leading to phosphoinositide hydrolysis and release of calcium ions from intracellular stores. At least five subtypes of muscarinic receptor have now been cloned, although only three subtypes can be distinguished pharmacologically. M_1 receptors are involved in neurotransmission in airway parasympathetic ganglion cells and

have also been identified in airway submucosal glands and on the alveolar walls of human lung. M_2 receptors are located on postganglionic nerves and function as feedback inhibitory receptors that are likely to be involved in modulation of reflex bronchoconstriction. These receptors may be dysfunctional in asthmatic airways. M_3 receptors are present on airway smooth muscle and submucosal glands and mediate the classical muscarinic effects in airways (14).

1.2.2.2 Sympathetic Innervation

Distribution of Nerve Fibres and Adrenergic Receptors

Sympathetic nerve fibres that contain catecholamine have been found in the smooth muscle of human airways, but they are far fewer in number than cholinergic nerves. In the sympathetic system, acetylcholine is the neural transmitter at the synapse between the preganglionic fibres and postganglionic neurons as it is in the parasympathetic system. The postganglionic fibres of the sympathetic system, however, release primarily norepinephrine. ß-adrenoceptors are localized to many different cell types within lung (18,41,244) and are found in smooth muscle of all airways from trachea to terminal bronchioles (15,42). The density of ß-adrenoceptors increases in smaller airways in animals and humans (17,42). Stimulation of sympathetic nerves results in the relaxation of airway smooth muscle mediated by the activation of $ß_2$-adrenoceptors (293). There are relatively few α-adrenoceptors in the lung (16) and their roles in the regulation of airway functions are disputed.

The $ß_2$-adrenoceptor has generally been thought to induce airway smooth muscle relaxation through a cyclic adenosine 3`,5`-monophosphate (cyclic AMP)-dependent mechanism. The activation of the ß-adrenoceptor leads to the activation of adenylate cyclase, resulting in the formation of intracellular cyclic AMP. Recently, other mechanisms have been identified.

Increase of intracellular cyclic AMP attenuates the calcium transient in tracheal smooth muscle induced by bronchoconstrictors such as histamine and carbachol although isoprenaline and forskolin on their own appear to elevate intracellular calcium levels (271). The inhibitory effect of cyclic AMP on calcium mobilization in response to bronchoconstrictors in airway smooth muscle cells may be induced through multiple mechanisms. Firstly, elevation of intracellular cyclic AMP has been shown to inhibit PIP_2 hydrolysis in response to histamine in bovine and canine trachealis (107,108,175). However, PIP_2 hydrolysis in response to cholinergic stimulation is not inhibited by ß-adrenoceptor agonists (108), suggesting that other mechanisms are likely involved in modulating cholinergically induced Ca^{2+} transients. Secondly, isoprenaline opens Ca^{2+} activated K^+ channels (K_{Ca}) in airway smooth muscle cells (155), leading to hyperpolarization of the cell (4,87,117), which in turn inhibits the voltage sensitive Ca^{2+} channels of cell membrane (153). Thirdly, ß-adrenoceptor agonists produce airway smooth muscle relaxation by stimulation of Na^+/K^+ ATPase (103). This action could lower intracellular Ca^{2+} by a consequent increase in Na^+/Ca^{2+} exchange.

By activation of protein kinase A (PKA), cyclic AMP also induces phosphorylation of MLCK, and thus decreases the affinity of MLCK for Ca^{2+}/calmodulin

(52,57,198,199), leading to a reduction of MLC phosphorylation and force generation.

Cyclic AMP induced airway smooth muscle relaxation may also be mediated by protein kinase G (83). The functionally relevant substrate(s) for protein kinase G in airway smooth muscle has been discussed in section 1.1.3.2.

In addition to a cyclic AMP-dependent mechanism, ß-adrenoceptor agonist induced airway smooth muscle relaxation has also been suggested to be mediated by a cyclic AMP-independent mechanism. This is based on the observation that ß-adrenoceptor agonists activate airway smooth muscle K_{Ca} channels in the absence of ATP, which is necessary for the formation of cyclic AMP and protein phosphorylation (154). Moreover, in canine trachealis, forskolin induces relaxation to a lesser extent than isoprenaline, despite an equivalent cyclic AMP accumulation and PKA activation (294).

1.2.2.3 Nonadrenergic Noncholinergic Innervation

Distribution of Nerves

The presence of NANC inhibitory nerves, which cause relaxation of airway smooth muscle, has been demonstrated in several species (29,48,51,62,125) including humans (229). Transmural stimulation of isolated airway smooth muscle in the presence of muscarinic, α-adrenoceptor and ß-adrenoceptor antagonists produces a bronchodilation which is abolished by tetrodotoxin (51,229,230). In humans and cats, capsaicin inhalation or mechanical irritation of the larynx, under conditions of cholinergic and adrenergic

blockade, have been shown to cause a transient bronchodilator response (121,270). This NANC innervation may have a regulatory role in the control of bronchial smooth muscle tone (169).

The neurotransmitter of the inhibitory NANC system in the airway is not known and may vary with the species. In guinea pig trachea, vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI) account, in part, for the NANC relaxant responses evidenced by the observation that antisera to VIP and PHI and the nonspecific peptidase α-chymotrypsin (α-CT) partly inhibited electrically induced NANC relaxant responses (70,71). These findings also indicated the existence of a non-peptide neurotransmitter which accounted for the α-CT-resistant NANC relaxations of the guinea-pig trachea. More recent studies indicate that NO plays an important role in NANC-induced relaxation of airway from several species including humans (see section 1.1.3.2).

NANC excitatory innervation in airways has been found by electrical stimulation of the guinea pig bronchi, which produces a bronchoconstriction in the presence of atropine or adrenoceptor antagonists. These responses are mimicked by substance P and inhibited by substance P antagonists (172), suggesting that substance P may be the transmitter released from NANC excitatory nerves. Another candidate neurotransmitter for NANC excitatory innervation is calcitonin gene-related peptide (213).

1.2.2.4 Autonomic Dysfunction in Airways Diseases

The cholinergic nervous system might have a influence in asthma. In asthma,

there is hyperresponsiveness to cholinergic agonists, and chronic airway obstruction in asthmatic children can be reduced with atropine (32,54). Indirect indices of vagal tone (heart rate variability, sweating, and pupillary response to cholinergic agonists) are increased in allergic human subjects including those suffering from asthma (139,140). However, direct assessment of vagal tone in humans is not possible with current techniques. Cholinergic system abnormality is also involved in other diseases including cystic fibrosis (56).

An impaired ß-adrenergic response has been described in subjects with asthma (9), as well as in actively sensitized guinea pigs (20,183) and in the Basenji greyhound, a dog model of hyperresponsiveness (66). Alveolar macrophages from subjects with asthma accumulate less cyclic AMP in response to β-agonists compared to cells from control subjects without asthma, and the degree of hyporesponsiveness is related to the severity of asthma (7). A decreased adenylyl cyclase activity has been confirmed in membrane fractions of macrophages from asthmatic subjects in response to β-agonists (7).

It has been suggested that impaired NANC bronchodilatation may contribute to the pathogenesis of nocturnal asthma, since capsaicin-induced NANC bronchodilatation is significantly greater in the evening than early morning in both normal and asthmatic subjects (173). NO-dependent NANC relaxation in human bronchi was also diminished in patients with cystic fibrosis (24).

1.2.3 Other Modulators of Airway Smooth Muscle Tone

In addition to the autonomic neurotransmitters, a host of other substances influence the tone of airway smooth muscle. These mediators are synthesized in airway mast cells, eosinophils, neutrophils as well as epithelial cells, and play an important role in the regulation of airway tone in normal and diseased subjects.

Eicosanoids

Prostaglandins (PG) are the derivatives of arachidonic acid whose formation is catalyzed by cyclooxygenase. In the airway, prostaglandins are produced by epithelial and smooth muscle cells as well as inflammatory cells such as mast cells and macrophages (287). Some prostaglandins ($PGF_{2\alpha}$, PGD_2, PGG_2 and PGH_2) contract while others (PGE_1, PGE_2 and PGI_2) relax the smooth muscle of the airway (287). The role of prostaglandins in the airway is not completely understood. Cyclooxygenase inhibitors fails to relax human bronchi in vitro (34), indicating that constitutively formed prostaglandins do not contribute to the basal tone of the airway. However, PGE_2 has been suggested as a component of epithelium derived relaxant factor in the airway (222). In asthma and antigen-sensitized animals, prostaglandins levels are increased. In sensitized guinea pigs, prostaglandins are released within 10 min after challenged with ovalbumin (278). As well, prostaglandins in bronchoalveolar lavage fluid from patients with chronic stable asthma increase an average of 150-fold in nine min after local instillation of the antigen, indicating that the release of prostaglandins into the airways is an early event after the instillation of antigen in asthmatics (192). In children with acute asthma,

prostaglandin metabolite levels are significantly higher than those of the same children after resolution of asthma and those of normal children (257). Prostaglandins in bronchoalveolar lavage fluids are also higher in subjects with mild asthma than in normal subjects (170). In addition, cyclooxygenase inhibition reduces allergen induced airway hyperresponsiveness in human subjects (150) as well as exercise-induced airway constriction in asthmatics (80) and decreases the late response (73), suggesting the involvement of prostaglandins in asthma.

Leukotrienes

Leukotrienes are another group of arachidonic acid derivatives catalyzed by lipoxygenase. These compounds are formed in response to a number of physiologic and pathologic events from neutrophils, eosinophils, alveolar macrophages, monocytes, and mast cells, as well as tracheal epithelial cells. Leukotrienes are potent constrictors of airway smooth muscle. LTC_4 has been found to be 600 to 9500 times more potent than histamine (284). Airway hyperresponsiveness to leukotrienes has also been found in asthmatic subjects (101).

Biogenic Amines

Histamine is stored and released from either sensitized or unsensitized mast cells. With the exception of the rat, histamine contracts airway smooth muscle from varies species, including guinea pigs (280), cats (12), sheep (11,45) and humans (236,281) through the histamine (H_1) receptor.

Serotonin is released from platelets following aggregation during inflammation, infection and thromboembolic events. Serotonin induces the contraction of airway smooth muscle in humans and animals through the 5-HT_2 receptor. This is an important mast cell mediator in the rat.

Others

Bradykinin and related kinins are mediators of the inflammatory response. In humans bradykinin causes bronchoconstriction in asthmatics but not in normal subjects (88,256). The release of bradykinin has been found in asthma patients (1). Inhibition of bradykinin receptors attenuates the antigen-induced late bronchial responses in allergic sheep, suggesting that bradykinin contributes to the development of the late response in the allergic sheep model (2).

Platelet activating factor can also induce bronchoconstriction in animals and humans. It increases airway responsiveness as well (55). The role of platelet activating factor in airway disease is unclear. Early clinical trials of platelet activating factor antagonists have not been very promising.

1.2.4 Epithelium-dependent Regulation of Airway Smooth Muscle Tone

It has been demonstrated that removal of the airway epithelium increases airway smooth muscle responsiveness to cholinergic agonists, histamine and serotonin in animals and humans (19,77,82,225,265), but has no effect on airway responsiveness to potassium

(19,259). In addition, following the removal of the epithelium the relaxant response to the catecholamine, isoprenaline is reduced (82,98,264,265). These observations suggest a role of epithelium in the regulation of airway smooth muscle tone in response to bronchoconstrictors and dilators. The possible underlying mechanisms may include the production of relaxing factors, the inactivation of agonists, or a physical barrier effect.

Eicosanoids

Airway epithelium has been found to be a source of cyclooxygenase products. A basal level of PGE_2 and $PGF_{2\alpha}$ has been measured in epithelium-intact but not epithelium-denuded tracheal tissues (111). PGE_2 is also generated in airway epithelial cells in response to bronchoconstrictors such as bradykinin (160), leukotrienes (161) and substance P (60). However, the inhibitory effect of epithelium on airway smooth muscle responsiveness to cholinergic agonists and histamine is not blocked by the cyclooxygenase inhibitor indomethacin (19,82). Therefore, non-prostanoid inhibitory factors are also involved in epithelium-dependent regulatory functions. The nature of these factors is unclear.

Enzymatic Degradation of Agonists

Neutral endopeptidase (NEP), which catalyses the degradation of neuropeptides, has been found in airway epithelial cells (133,193). Airway responsiveness to neuropeptides is increased by epithelial removal. This effect of epithelium is significantly

attenuated by the NEP inhibitors, phosphoramidon and thiorphan (61,65,78,84,133,195,252,253). These observations are consistent with the idea that epithelium inhibits the airway smooth muscle response to neuropeptides by their degradation *via* NEP. Airway epithelium has also been demonstrated as a site for degradation for adenosine (3) and isoprenaline (74).

Barrier Function

The ability of the epithelium to act as a diffusion barrier has been demonstrated using perfused airways segments. Airway contractions in response to bronchoconstrictors are greater when the agonists are applied to the serosal surface than to the luminal surface. Removal of the epithelium increases the contractions in response to bronchoconstrictors applied intraluminally to an equal extent as applied to the serosal surface (261), suggesting a physical barrier function of airway epithelium.

1.3 Airway Hyperresponsiveness

1.3.1 Regulation of Airway Responsiveness

Definitions

Airway responsiveness is defined as the degree to which airways constrict in response to nonsensitizing physical or chemical stimuli. Airway hyperresponsiveness, thus, is an increase above the normal responsiveness of the airways to these stimuli. When measured with histamine or methacholine, there is an increase both in the ease of airway narrowing (left shift of the dose-response curves) (50,288) and in the magnitude of the airway constriction (elevation and eventual loss of the maximal response plateau) (288) in hyperresponsive human subjects. There are many stimuli which can be used to measure airway responsiveness including direct-acting stimuli, i.e. cholinergic agonists, histamine, and indirect-acting stimuli, i.e. beta-adrenergic blockers, bradykinin, adenosine monophosphate (AMP), as well as physical stimuli such as exercise, cold air, hyperventilation, and nonisotonic aerosols.

Geometry of the Airway

Airway calibre has been suggested as a determinant of airway responsiveness to inhaled chemical mediators (26). The resistance to airflow in a tube is inversely proportional to the radius of the tube to either the fourth power (laminar flow) or the fifth power (turbulent flow). Therefore, a small reduction in radius will have a magnified

effect on the resistance in a small tube when compared with a larger tube. Airway responsiveness can be regulated by a variety of factors and the change of these factors may lead to airway hyperresponsiveness, a consistent feature of asthma.

Airway Smooth Muscle

The balance between the force of airway smooth muscle contraction versus the load imposed by the airways and lung structure is an important determinant of airway calibre. Constriction of airway smooth muscle may lead to airway narrowing or, in smaller airways, complete closure. Increased airway smooth muscle mass may increase the isometric force produced by the same stimulation and thus increase airway responsiveness. In addition, increased airway smooth muscle mass may also decrease airway calibre, which increases airway responsiveness by airway geometry as discussed above (see section 1.3.1 second paragraph). Smooth muscle hyperplasia and hypertrophy have been found in human asthmatic airways (68,245), indicating that increased airway smooth muscle mass may contribute to airway hyperresponsiveness in asthma.

Animal studies suggest that several bronchoconstrictors may serve as stimuli for smooth muscle proliferation and growth (208). In addition, cytokines and growth factors such as interleukin one and platelet-derived growth factor, which are produced during inflammatory responses in asthmatic airways, can also stimulate smooth muscle cell proliferation (171). This may account, at least in part, for the increase in smooth muscle mass in the airways of asthmatics. It has also been shown that airway smooth muscle, even when corrected for mass, produces greater isometric force (248) and degree of in

vitro responsiveness (8) in asthmatics than in normal subjects. Smooth muscle from sensitized dogs has an increased maximal velocity of shortening (263). These observations indicate that intrinsic factors in airway smooth muscle other than smooth muscle mass may also contribute to airway hyperresponsiveness.

Airway and Parenchymal Interdependence

The tissues surrounding airways can influence airway responsiveness through the parenchymal attachments to the airway walls, which may act as an impedance to airway smooth muscle shortening. For a given stimulus, the magnitude of the bronchoconstriction is affected by lung volume, reflecting the mechanical interaction between the airways and surrounding tissues (63,174,180,194,233,240,258). The cartilage elasticity may also be an important determinant of the ability of airway smooth muscle to shorten in major airways and produce airway narrowing in vivo (186). When the external tethering of the airways is diminished, such as in the case of peribronchial edema (180), a given degree of force generation may lead to greater length changes and thus increase airway responsiveness.

Mucosal or submucosal edema or increased secretions of airways also induce airway thickening and influence airway calibre. A morphological study showed an increased cross-sectional area of the airway wall, including both increased cellularity and fluid, in asthmatics (130). The results of mathematical modelling have suggested that increased airway wall thickness could have a substantial effect on airway calibre when the airway smooth muscle contracts (285) while having only a minimal effect on baseline

airway resistance.

Epithelial Damage

Airway epithelium is known to protect airways from hyperresponsiveness through various mechanisms (see section 1.2.4). Epithelial damage has been found in airways of asthmatic patients including asymptomatic subjects (21). This may lead to a change of epithelium-derived relaxing factors and contribute to airway hyperresponsiveness in asthmatics.

Imbalance Between Endogenous Bronchoconstrictor and Dilator Mechanisms

In airway hyperresponsiveness, the balance of factors that influence airway calibre might be changed in favor of an excessive constrictor response to stimulation. An impaired ß-adrenergic agonist-cyclic AMP-relaxation pathway has been previously described in association with airway hyperresponsiveness and asthma (7,20,36,110,183). A reduced responsiveness of the airway smooth muscle to ß-adrenergic agents (9) has been found in subjects with asthma, in actively sensitized guinea pigs (20,183) and in the Basenji greyhound, a dog model of hyperresponsiveness (66). Alveolar macrophages from patients with asthma accumulated less cyclic AMP in response to ß-agonists compared to cells from control subjects without asthma, and the degree of the hyporesponsiveness was related to the severity of asthma (7). A decreased adenylyl cyclase activity in response to ß-agonists has been confirmed in membrane fractions of macrophages from asthmatic subjects (7).

An imbalance between the NANC inhibitory system and constrictor influences has also been suggested (239). NANC inhibitory function stimulated by capsaicin (an NANC stimulant) after parasympathetic and ß-adrenergic blockade is less effective in the early morning in both normal and asthmatic human subjects. This may contribute to nocturnal bronchoconstriction in asthma (173). Immunocytochemical studies have show a deficiency of VIP and an excess of substance P in asthmatic lungs (203). Further studies are required to define the pathophysiological roles of NANC inhibitory system in airway hyperresponsiveness and asthma.

NO is a recent addition to the list of endogenous bronchodilators. Endogenously produced NO is involved in the regulation of airway responsiveness to bronchoconstrictors (92,197). Its function in the hyperresponsiveness of the airways in such diseases as asthma has not been studied. Whether an impaired endogenous NO regulatory mechanism contributes to airway hyperresponsiveness deserves investigation.

1.3.2 Difference in Airway Responsiveness in Outbred and Inbred Strains.

Airway responsiveness is highly variable among (177,212) and within (10,49,120,260) species so far examined including humans (50,176). Highly inbred rats exhibit significant strain-related differences in airway responsiveness. In one such study, the Fisher strain was shown to be consistently hyperresponsive to inhaled methacholine compared with other strains such as Lewis and ACI rats. (69,177). Strain-related

differences in airway responsiveness are also described among inbred strains of mice and are postulated to be autosomal recessive (164,165). However, the mechanisms of strain-related differences in airway responsiveness are poorly understood. Both hereditary and environmental influences appear to be important. Theoretically, any difference in determinants of airway responsiveness including smooth muscle, epithelium, airway-parenchymal interdependence and neural control of the airways may result in strain-related differences in airway responsiveness. An increased smooth muscle mass has been found in airways of Fisher compared with Lewis rats (69,177), and a significant correlation between responsiveness and the amount of smooth muscle in the airway has been noted (69,177). However, only about 30% of the variability in responsiveness of both Fisher and Lewis strains could be accounted for by airway smooth muscle (69). Other mechanisms such as differences in signal transduction pathways, or the contractile apparatus in smooth muscle cells also need to be considered.

1.4 Summary

In summary, NO-cyclic GMP pathway is a recently discovered endogenous inhibitory system in the airway and could act to maintain airway smooth muscle and pulmonary vascular smooth muscle in a state of low tone. In addition, epithelial derived NO could have relaxant effects on the underline airway smooth muscle. Since the imbalance between bronchoconstricting and dilating systems is a possible mechanism for airway hyperresponsiveness, we hypothesized that an impaired NO-cyclic GMP pathway is involved in airway hyperresponsiveness. The current studies were designed to investigate the role of NO-cyclic GMP in the regulation of airway responsiveness and its contribution to the strain-related difference in airway responsiveness in rats. The NOS inhibitor, L-NNA, was used to test the role of endogenous NO. The effect of L-NNA on airway responsiveness was compared between Fisher and Lewis rats. To examine the pathway downstream of NO, the effects of exogenous NO (SNP) and cyclic GMP (8-bromo-cyclic GMP) on responsiveness of airway smooth muscle were also studied. In addition, we investigated the capacity of airway smooth muscle cells to produce NO, through NOS and cytochrome P450 enzymes.

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Chapter 2

Endogenous Nitric Oxide Contributes to Strain-related Differences in Airway Responsiveness in Rats.

A strain-related difference in airway responsiveness has been found in rats. However, the mechanism underlying this observation is unclear. Endogenous NO has been reported to play a role in the regulation of airway responsiveness in guinea pigs. Its role in other species such as rat has not been studied. In this chapter, we tested the hypothesis that endogenous NO was also involved in the regulation of airway responsiveness in rats and that an impaired NO-cyclic GMP pathway contributed to strain related airway hyperresponsiveness in rats.

2.1 ABSTRACT

The effects of N^{ω}-nitro-L-arginine (L-NNA), a nitric oxide synthase (NOS) inhibitor, on airway responsiveness were studied in the spontaneously hyperresponsive Fisher and the control normoresponsive Lewis rat strains to investigate the role of the endogenous nitric oxide (NO) pathway in strain-related differences in airway responsiveness. Responsiveness to inhaled methacholine (MCh) was significantly increased in L-NNA-treated Lewis rats but not in Fisher rats. L-NNA increased carbachol-induced tracheal contractions *in vitro* to a larger extent in Lewis rats compared with Fisher rats. The effect of L-NNA was abolished by removal of the epithelium. Carbachol induced a NO-dependent increase in cyclic GMP levels in tracheal tissues, but to a lesser extent in Fisher (2.1 fold increase) than in Lewis (3.7 fold increase) rats. In conclusion, endogenous NO is involved in the regulation of airway responsiveness to cholinergic agonists in rats. A relatively ineffective NO-cyclic GMP regulatory mechanism in Fisher rats contributes, in part, to strain related differences in airway responsiveness between Fisher and Lewis rats.

2.2 INTRODUCTION

Strain-related differences in airway responsiveness have been found in rats (13,27). The Fisher strain of rat is consistently hyperresponsive to inhaled agonists compared to several other strains. The underlying mechanisms of the difference between Fisher and other strains are not completely understood. Our previous studies demonstrated a decreased relaxant response to sodium nitroprusside, an NO donor, in the hyperresponsive Fisher rat strain compared with the less responsive Lewis rat (38). This suggests that resistance to the effects of NO may be associated with airway hyperresponsiveness.

NO can be synthesized in the lung and has a variety of potential functions including the regulation of airway tone. The role of endogenous NO in the modulation of airway responsiveness to bronchoconstrictors has recently been studied in guinea pigs. The administration of NO synthase (NOS) inhibitors to guinea pigs resulted in a significant enhancement of airway responsiveness to intravenously infused histamine (31). A similar result has also been observed by other investigators (15), confirming that endogenous NO is involved in the regulation of airway responsiveness to bronchoconstrictors, at least in guinea pigs.

NO is known to relax airway smooth muscle by activating soluble guanylate cyclase and increasing intracellular cyclic GMP (21,39). It has been shown that cyclic GMP concentrations in airway tissue increase after stimulation with histamine (24) and carbachol (34). Therefore, we hypothesised that bronchoconstrictors, which increase intracellular calcium, may activate constitutive calcium-dependent NOS activity and produce NO, which

in turn increases intracellular cyclic GMP in smooth muscle cells and down regulates airway responsiveness.

The current studies were designed to investigate the role of endogenous NO in the regulation of airway responsiveness in rats and to test whether an impaired endogenous NO regulatory mechanism contributes to strain-related airway hyperresponsiveness. The effects of L-NNA, a NOS inhibitor, on airway responsiveness and cyclic GMP accumulation in tracheal tissues in response to cholinergic stimulation were tested and compared between hyperresponsive Fisher and normoresponsive Lewis rats.

2.3 MATERIALS AND METHODS

Animals. The highly inbred Fisher and Lewis rat strains (male, 7-9 week old) were purchased from a commercial source (Harlan Sprague Dawley Inc., Indianapolis, IN) and housed in a conventional animal care facility at McGill University prior to experimentation. The protocols were approved by an Animal Ethics Committee.

Measurements of airway responsiveness *in vivo*. The effects of inhibition of NOS were tested both acutely and chronically. For study of the acute effects of NOS inhibition rats were treated by L-NNA (bolus i.v. injection of 2 mg/kg followed by infusion of 2 mg/kg/h for 30 min. In chronic study, Fisher and Lewis rats were treated with vehicle, L-NNA (5mg/day, Sigma Chemical Co. St Louis Mo), or L-NNA (5mg/day) + L-arginine (35mg/day, Nutritional Biochemical Co. Cleveland Ohio) by gavage for 4 weeks. Airway responsiveness was determined from changes in pulmonary resistance (R_L). R_L was measured at baseline and following progressively doubling concentrations of aerosolized MCh until a doubling in pulmonary resistance was obtained. Responsiveness was defined as the concentration of MCh required to double R_L ($EC_{200}R_L$). Aerosols were generated using a disposable nebulizer (Model 1400, Hudson Inc., Temecula, CA) and a compressed air source delivering an airflow of 8 l/min.

Pulmonary resistance were measured as previously described (7). Briefly, intubated rats were placed in the supine position. The tip of the tracheal tube was inserted into a Plexiglas box (volume, 265 ml). A Fleisch no. 0 pneumotachograph coupled to a piezoresistive differential pressure transducer (Micro-Switch 163PC01D36; Honeywell,

Scarborough, Ont., Canada) was attached to the other end of the box to measure airflow. Transpulmonary pressure (Ptp) was measured using a water-filled catheter placed in the lower third of the oesophagus connected to one port of a differential pressure transducer (Transpac II disposable transducer, Sorenson, Salt Lake City, Utah) and the other port was connected to the Plexiglas box. The pressure and flow signals were amplified, passed through eight-pole Bessel filters (Model 902LPF; Frequency Devices, Haverhill, MA), with their cutoff frequencies set at 100 Hz, and recorded by a 12-bit analog to-digital converter at a rate of 200 Hz. The data were stored on a computer. Pulmonary resistance was calculated by multiple linear regression by fitting the equation: $Ptp=E_L V + R_L V + K$, to 10-s segments of data, where V is flow, R_L is resistance, V is volume, E_L is elastance, and K is a constant value. A commercial software package (RHT Infodat Inc, Montreal, QC, Canada) was used.

Mechanical responses of tracheal rings. Following the measurement of lung resistance, rats were killed by a lethal overdose of pentobarbital and their tracheas were immediately excised and cut into rings of approximately 3 mm. Only the rings from the lower end of the trachea were used to measure the mechanical responses. Tracheal rings were mounted on hooks, connected to force transducers (Grass FT03, Quincy, MA) and incubated in a physiological saline solution (PSS), containing (mM): NaCl 118; KCl 4.5; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25.5; and glucose 5.6, bubbled with 95 % oxygen/5% carbon dioxide in 25 ml organ baths at 37°C. The passive tension was set at 1 gram and the tissue was equilibrated for 60 min in all the experiments. Isometric force of the tracheal rings in response to cumulative concentrations of carbachol (Sigma Chemical Co. St Louis MO) was recorded. Only one dose-response curve for carbachol was performed in each rat. In

another group of naive rats, the epithelial layer of the trachea was removed by passing a cotton gauze strip through the tracheal tube 2 times. Either tracheal rings with the epithelium intact or denuded rings were pre-incubated with or without L-NNA ($5x10^{-4}$ M) in PSS for 30 min before the recording. The width and dry weight of each of the tracheal rings were measured at the end of the experiment.

Cyclic nucleotide measurements. Rats were killed by a lethal overdose of pentobarbital and their tracheas were immediately excised. All extraneous tissues were carefully stripped from the tracheas in PSS bubbled with 95% oxygen/5% carbon dioxide. Epithelial intact tracheas were cut longitudinally into two equal parts and incubated for 30 min with or without L-NNA in PSS containing 0.5 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (Aldrich Chem. Co. Milwaukee. WI), to inhibit the degradation of cyclic nucleotide. Then each part of the tracheal tube was incubated with either carbachol ($5x10^{-5}$ M) or vehicle for 10 min. The reactions were stopped by freezing the tissues rapidly in liquid nitrogen. Frozen tissues (stored at -70°C) were homogenized in 1 ml cold trichloroacetic acid (TCA 10%, Sigma Chemical Co. St Louis MO) on ice followed by centrifugation at 2000 xg for 10 min at 4° C. TCA was removed from the supernatant by ether extraction. Cyclic GMP levels (following acetylation of the cyclic GMP (10)) in the tissue extracts were determined by radioimmunoassay (19) and expressed as femtomoles of cyclic GMP per milligram of tissue.

Statistical Analysis. Data are expressed as mean ± s.e. Significant differences between group means were tested for by Kruskal-Wallis one-way analysis of variance and post hoc comparisons were done using Fisher's LSD test for data on responsiveness *in vivo*

($EC_{200}R_L$, Figure 1) and by Student t test for all other data. $p<0.05$ was set as the level of significance.

2.4 ESULTS

Effect of acute L-NNA treatment on airway responsiveness *in vivo*. As showed in table 1, acute L-NNA treatment had no significant effect on baseline R_L (p=N.S) as well as airway responsiveness to MCh (P=N.S.) in either Fish or Lewis strains.

Table 1. Effect of acute L-NNA on airway responsiveness to inhaled methacholine in Fisher and Lewis rats *in vivo*.

		R_L (Baseline) $cmH_2O.l^{-1}.s$	EC_{200} $mg\ ml^{-1}$
Fisher	Control (n=6)	0.255 ± 0.026	1.07
	L-NNA (n=6)	0.291 ± 0.017	1.29
Lewis	Control (n=6)	0.261 ± 0.036	2.73
	L-NNA (n=6)	0.309 ± 0.037	1.79

EC_{200} is expressed as the geometric mean.

Effect of acute L-NNA treatment on airway responsiveness *in vivo*. As shown in Table 1, acute L-NNA treatment had no significant effect on the baseline R_L or the airway responsiveness to MCh in either the Fisher or Lewis strains. There was a trend towards an increase in responsiveness in Lewis after L-NNA that was not seen in Fisher rats.

Effect of chronic L-NNA treatment on airway responsiveness *in vivo*. Baseline R_L was not significantly different between Lewis and Fisher rats. Fisher rats exhibited hyperresponsiveness to inhaled MCh compared with Lewis rats ($p<0.0001$). In Lewis rats, L-NNA significantly increased airway responsiveness to MCh as indicated by a decrease in MCh concentration required to double the lung resistance; $EC_{200}R_L$ changed from 10.93 mg/ml to 4.13 mg/ml after treatment ($p<0.01$, Fig 1). This effect of L-NNA was reversed by L-arginine (p=NS compared with control). In contrast, L-NNA has less effect on airway

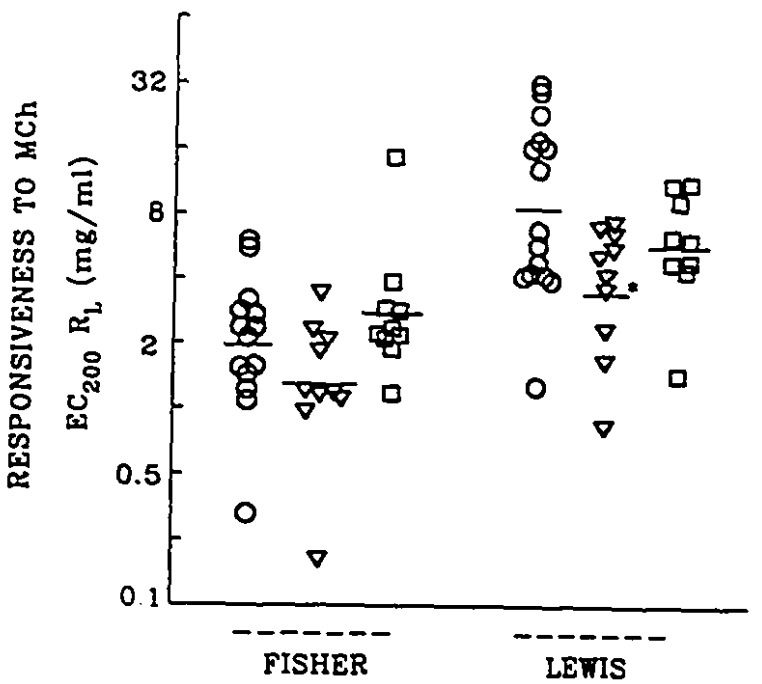

Figure 1. Effect of chronic L-NNA on airway responsiveness t0 inhaled methacholine in Fisher and Lewis rats *in vivo*. $EC_{200}R_L$: MCh concentration needed to double the pulmonary resistance. Each symbol represents an individual animal. Circles: control animals treated by vehicle (Fisher: n=14; Lewis: n=15) . Triangles: L-NNA treated animals (n=10). Squares: L-NNA+L-arginine treated animals (Fisher: n=10; Lewis: n=9). *: $p<0.01$ compared with control.

responsiveness to inhaled MCh in Fisher rats; $EC_{200}R_L$ changed from 2.45 mg/ml to 1.57 mg/ml after treatment, however, there is no significant difference among control, L-NNA and L-NNA + L-arginine treated Fisher groups (p=N.S.). $EC_{200}R_L$ for Lewis rats was 5 times higher than that for Fisher rats ($p<0.0001$) in control group. The difference was less in L-NNA treated animals; $EC_{200}R_L$ for L-NNA treated Lewis rats was only 2.6 times higher than that for L-NNA treated Fisher rats ($p<0.01$).

Effect of acute treatment with L-NNA and epithelial removal on tracheal responsiveness *in vitro*. In epithelium intact tracheal rings, Fisher rats were hyperresponsive

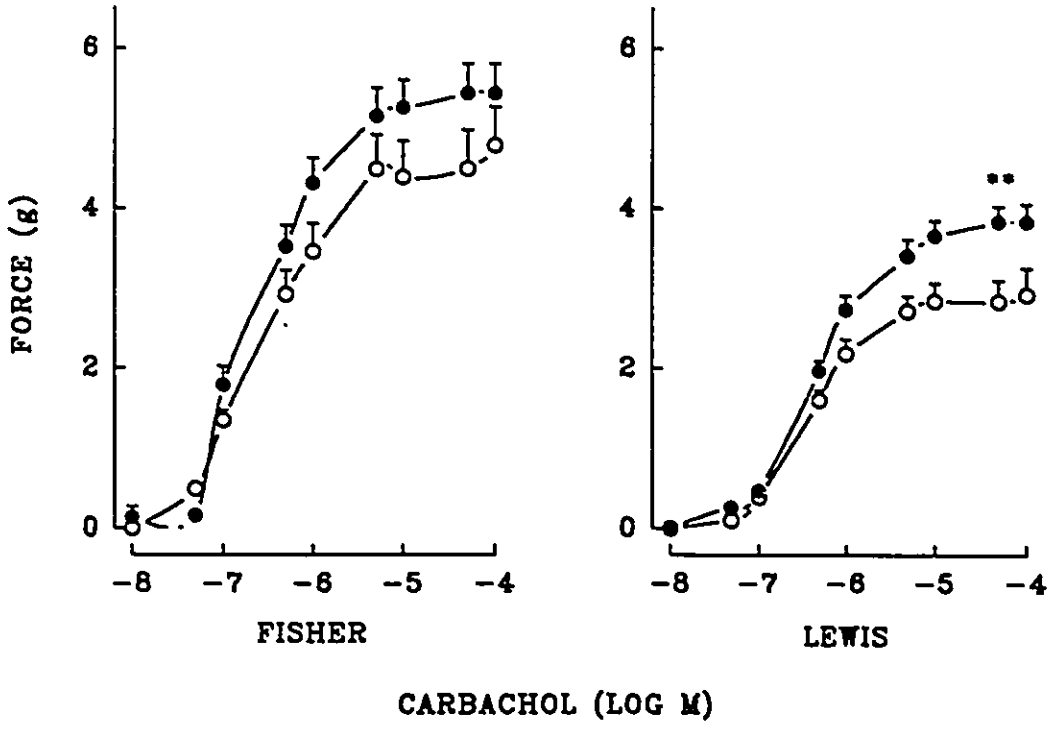

Figure 2. Effect of L-NNA pre-incubation on carbachol induced contractions of epithelium intact tracheal rings. Isometric tension (gram) of epithelium intact tracheal rings from rats was measured in response to progressively increasing concentrations of carbachol (log M). Open circles: Control conditions without L-NNA treatment (n=6); Closed circles: pre-incubated with 5×10^{-4} M L-NNA for 30 min before the addition of carbachol (Fisher n=8, Lewis n=7). Data are expressed as mean ± s.e. **: $p<0.01$ compared with control.

to carbachol compared with Lewis rats (Figure 2). Incubation of tracheal rings with L-NNA did not change baseline tone in either strain, but increased carbachol induced contractions in Lewis rats. Isometric force induced by 5×10^{-5} M carbachol was increased by pre-incubation with L-NNA (5×10^{-4} M) from 2.83 ± 0.26 to 3.84 ± 0.2 gram ($p<0.01$). However, the same concentration of L-NNA had less effect on tracheal ring contractions in response to carbachol in Fisher rats and in contrast to the effect of chronic L-NNA, the acute treatment did not elicit a statistically significant change. (Figure 2).

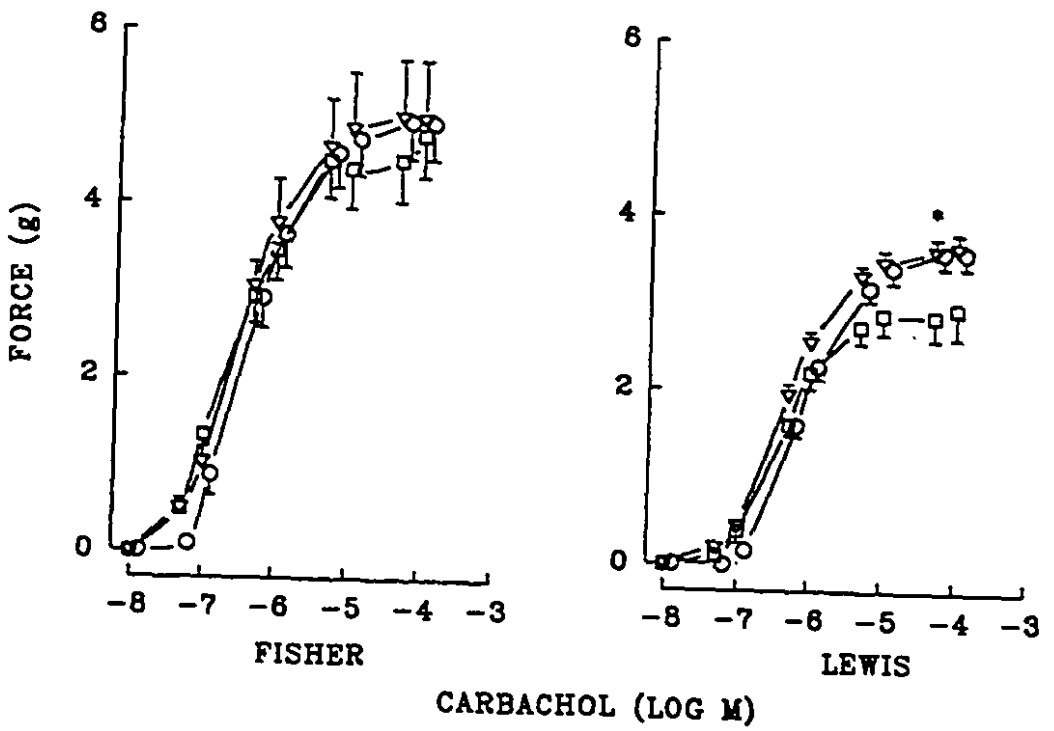

Figure 3. Effect of L-NNA pre-incubation on carbachol induced contractions of epithelium denuded tracheal rings. Squares: Control conditions with intact epithelium (n=6). Triangles: Epithelium-denuded tracheal rings (n=8). Circles: Epithelium-denuded tracheal rings pre-treated with 5×10^{-4} M L-NNA for 30 min (n=8). Data are expressed as mean ± s.e.; *: $p<0.05$ compared with control.

Stripping of epithelial cells increased contraction of the tracheal rings produced by carbachol as well (Figure 3). This change mimicked the effect of NOS inhibition by L-NNA (Figure 2). The isometric force of epithelium denuded tracheal rings induced by 5×10^{-5} M carbachol (3.60 ± 0.15 gram) was significantly increased compared with that of epithelium-intact tracheal rings (2.83 ± 0.26 gram, $p<0.02$) in Lewis rats. Removal of epithelium had less effect on carbachol induced contractions in Fisher rats (P=N.S.). L-NNA (5×10^{-4} M) did not further increase contractions produced by carbachol in epithelium denuded tracheal rings from both rat strains. The width and dry weight of each of the tracheal rings were measured at the end of the experiment and there was no significant difference between Fisher and Lewis rats.

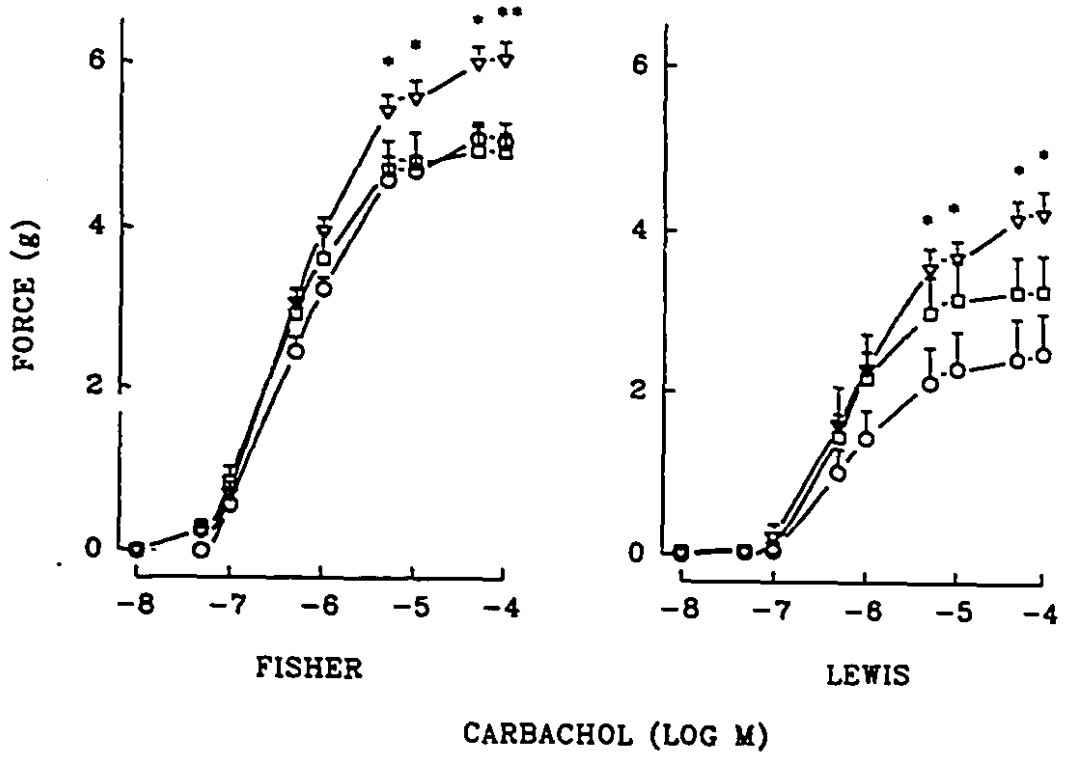

Figure 4. Effect of chronic L-NNA on tracheal responsiveness to carbachol *in vitro*. Isometric tension (gram) of isolated tracheal rings from rats treated by vehicle (circles, n=4), L-NNA (triangles, Fisher n=7, Lewis n=4) or L-NNA+L-arginine (squares, Fisher n=11, Lewis n=7) was measured in response to progressively increasing concentrations of carbachol (log M). Data are expressed as mean ± s.e. *: $p<0.05$; **: $p<0.01$ compared with control.

Effect of chronic L-NNA treatment on tracheal responsiveness *in vitro*. Isometric force in response to carbachol was greater in isolated tracheal rings from Fisher than that from Lewis rats (Figure 4). L-NNA treatment (5mg/day) increased carbachol-induced tracheal contractions in both strains which effect was reversed by L-arginine. In Lewis rats, isometric force induced by 5×10^{-5} M carbachol was increased by L-NNA treatment from 2.43 ± 0.5 to 4.17 ± 0.23 gram (71 %, $p<0.05$), whereas, L-NNA treatment increased isometric force from 5.08 ± 0.15 to 6.03 ± 0.22 gram (14%, $p<0.05$) in Fisher rats.

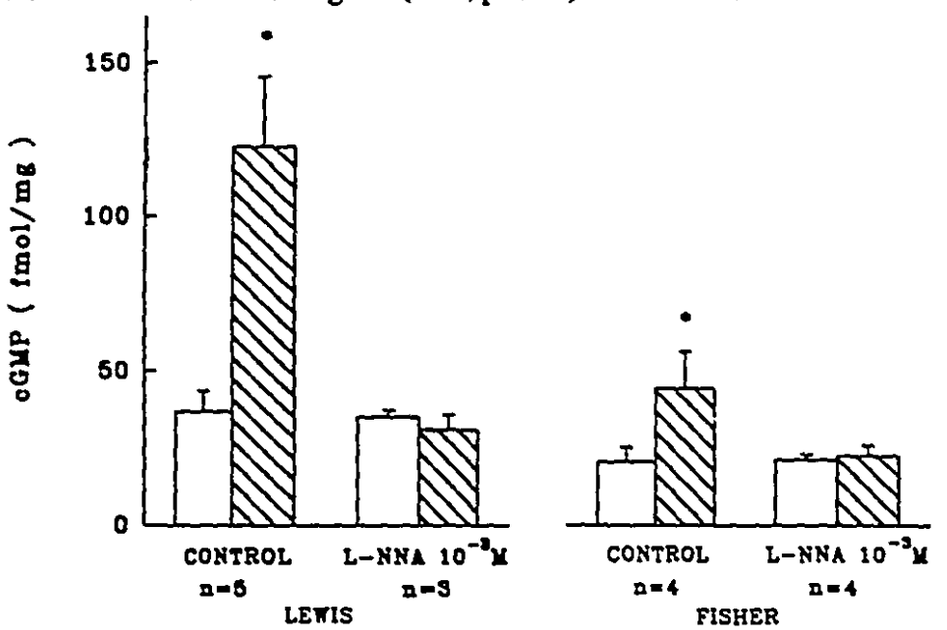

Figure 5. L-NNA inhibited carbachol induced cyclic GMP accumulation in rat trachealis. Open squares: Baseline conditions without carbachol. Dashed squares: incubated with 5×10^{-5} M carbachol for 10 min. Data are expressed as mean ± s.e.; *: $p<0.05$ compared with baseline.

Effect of carbachol on cyclic GMP levels in tracheal tissues. Figure 5 showed that carbachol (5×10^{-5} M) induced an accumulation of cyclic GMP in tracheal tissues of both Fisher and Lewis rats. Carbachol increased cyclic GMP levels to a lesser extent in Fisher (44.3±11.9 fmol/mg tissue, 2.1 fold increase, $p<0.05$) than in Lewis rats (112.6±23 fmol/mg

tissue, 3.7 fold increase, $p<0.05$). Inhibition of NOS by L-NNA (10^{-3} M) abolished carbachol induced cyclic GMP accumulation in tracheal tissues in both rat strains.

2.5 DISCUSSION

In the present studies, inhibition of NOS by L-NNA induced airway hyperresponsiveness to cholinergic stimulation *in vivo* and *in vitro* in Lewis rats. L-NNA was less effective in Fisher rats in increasing airway responsiveness than in Lewis rats. Removal of the epithelium from trachealis increased tracheal ring contractions in response to carbachol *in vitro* in Lewis rats, but did so to an insignificant extent in Fisher. L-NNA did not further increase airway responsiveness in epithelium-denuded trachealis from either strain. Cholinergic stimulation increased cyclic GMP levels in tracheal tissues from both strains of rat but did so to a lesser extent in Fisher than in Lewis rats. Carbachol induced cyclic GMP accumulation in tracheal tissues was abolished by L-NNA, indicating that the increase in cyclic GMP was through a NO-dependent mechanism.

Airway hyperresponsiveness to inhaled bronchoconstrictors is highly variable from animal to animal within a given species (3,12,20,36) and also among different species (27,32). The factors which result in airway hyperresponsiveness are not completely understood. Since endogenous airway relaxation mechanisms, such as the cyclic AMP-relaxation pathway, are involved in the regulation of airway tone (37), one of the possible mechanisms may be the imbalance between bronchoconstrictors and dilators. An impaired ß-adrenergic agonist-cyclic AMP-relaxation pathway has been previously described in association with airway hyperresponsiveness and asthma (2,6,11,18,28). NO is a recent addition to the list of endogenous bronchodilators.

In the current study, we tested the role of endogenous NO in airway responsiveness in rats by inhibition of endogenous NO production. We fed rats with L-NNA, a NOS inhibitor, for 4 weeks, using a protocol similar to that devised by Bank et al.(4). This treatment effectively inhibited endogenous NO production as evidenced by a significant reduction in urinary excretion of the stable metabolic products of NO, ie, NO_2^- and NO_3^- (4). We assumed that this treatment would *inhibit* NO production from the airway tissues as well. Indeed the results of our experiments demonstrated a substantial effect on airway responsiveness to a cholinergic agonist. The effect of L-NNA was reversible by L-arginine, the substrate of NOS, indicating the specific inhibition of NOS by L-NNA. Cholinergic stimulation increased cyclic GMP levels in rat tracheal tissues through a NO dependent mechanism, demonstrating the involvement of an endogenous NO-cyclic GMP pathway. These results are consistent with the report that NOS inhibitors increase airway responsiveness to histamine in guinea pigs (31).

Acute L-NNA treatment *in vitro* had similar but less effect on carbachol induced tracheal ring contractions. Using a treatment that had been proven to reduce pancreatic blood flow in rats (8,23), we were unable to show a significant effect of acute L-NNA treatment *in vivo* on airway responsiveness to inhaled MCh. It appears that this treatment *in vivo* is not effective in inhibition of NOS in the airway.

Endogenous NO is synthesised from L-arginine catalysed by NOS. Two distinct types of NOS have been purified: a constitutive Ca^{2+}-calmodulin-dependent enzyme and an inducible Ca^{2+}-independent enzyme. Inducible NOS is unlikely to account for the current observations since either airway responsiveness or cyclic GMP accumulation was determined

within 15 minutes after cholinergic stimulation, whereas it takes at least several hours to induce enzyme production. Constitutive NOS, which can be activated by increases in intracellular Ca^{2+}, seems more likely to account for the production of NO involved in the modulation of airway responsiveness.

There are several possible cell sources of NO in the airway including vascular smooth muscle cells, endothelial cells, neurons and airway epithelial cells (22). Airway epithelium has been known for some time to have an important influence on airway responsiveness to bronchoconstrictors (9,29,33). More recently, immunohistochemical studies have demonstrated the presence of both constitutive and inducible NOS in airway epithelial cells (1,16), adding NO to the candidate molecules involved in the observed modulatory effects of epithelium. Since some G protein coupled receptors such as muscarinic receptors (25,26) and histamine receptors (17) are also present in airway epithelium and the activation of these receptors can increase intracellular Ca^{2+} level in airway epithelial cells (17), it is possible that the endogenous NO which is involved in the regulation of airway responsiveness is produced in airway epithelial cells. The current observation that removal of epithelial cells increased tracheal contractions in a manner similar to NOS inhibition, and abolished the NO-dependent down-regulation of tracheal contractions supports this possibility. Presumably, cholinergic agonists activated constitutive Ca^{2+}-dependent NOS in airway epithelial cells and produced NO, which diffused into adjacent smooth muscle cells. Smooth muscle contraction was in turn inhibited by activation of guanylate cyclase and the consequent increase in cyclic GMP levels. Since NOS inhibition did not further increase carbachol-induced contractions in epithelium denuded tracheal rings, epithelium appears to be the only significant source of NO involved

in the regulation of airway contractions in response to carbachol. This does not exclude the involvement of NO from other sources, such as neurons, in the regulation of airway responsiveness to other bronchoconstrictors.

Some studies suggest that epithelium-derived relaxant factor EpDRF) is PGE_2 (33), while others indicate the involvement of non-prostanoid inhibitory factors in EpDRF; the inhibitory effect of epithelium is not inhibited by cyclooxygenase inhibitor indomethacin in a number of species (5,14,30,35). Our observations in rats and studies by Nijkamp et al. in guinea pigs (31) suggest that NO is a predominant component of tracheal epithelium-derived relaxant factors in these species. It is possible that the relative importance of these factors may vary among species.

Interestingly, NOS inhibition by L-NNA did not alter airway responsiveness in Fisher rats as much as in Lewis in which responsiveness was significantly increased both *in vivo* and *in vitro*. Chronic L-NNA had a small but significant effect on Fisher isolated tracheas. Consistent with the apparent lack of sensitivity of Fisher to inhibition of NOS, carbachol increased cyclic GMP levels to a lesser extent in Fisher than in Lewis tracheal tissues. These results indicate an impaired endogenous NO-cyclic GMP-dependent regulatory pathway in Fisher rats. Our previous studies demonstrated an impaired relaxation and reduced cyclic GMP accumulation in Fisher trachealis exposed to sodium nitroprusside compared with Lewis, suggesting decreased guanylate cyclase activity in trachealis from Fisher rats (38). This may account, at least in part, for the impaired endogenous NO-cyclic GMP-dependent regulatory pathway in Fisher rats. The difference in responsiveness between Fisher and Lewis rats to inhaled MCh was reduced but not entirely abolished after L-NNA treatment. Clearly

the impaired NO-dependent pathway in Fisher rats is an important but not the only contributor to the strain related difference in airway responsiveness between Fisher and Lewis rats. Morphometric studies have demonstrated a greater quantity of airway smooth muscle in Fisher than Lewis rats (13,27) which finding may account for the balance of the difference in responsiveness between these strains.

In summary, our present results demonstrate the involvement of endogenous NO-cyclic GMP-relaxation pathway in the regulation of airway responsiveness in Lewis rats. Our data also indicate that this NO-dependent regulatory pathway is impaired in Fisher rats which accounts, in part, for the strain related airway hyperresponsiveness. The current study does not establish whether the impaired NO-cyclic GMP-regulatory pathway in Fisher rats is caused by a decreased response of smooth muscle to NO or by a decreased NO generation in the airway. In view of the previously observed resistance of Fisher rats to sodium nitroprusside, an NO donor (38), a possible mechanism may include a decreased guanylate cyclase activity. However, reduced NO production by Fisher tracheal tissues is not excluded.

2.6 ACKNOWLEDGEMENT

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Chapter 3

Airways of a Hyperresponsive Rat Strain Show Decreased Relaxant Responses to Sodium Nitroprusside.

As detailed in chapter 2, endogenous NO has been found to play a role in regulation of airway responsiveness in normoresponsive Lewis rats. The NO-dependent regulatory pathway is less effective in hyperresponsive Fisher rats and may contribute to strain-related differences in airway responsiveness. It is not clear whether reduced production of NO or resistance to its effects are responsible for the findings. NO is known to activate guanylate cyclase and increase the production of cyclic GMP, which in turn inhibits smooth muscle contraction in response to bronchoconstrictors. Chapter 3 investigated this NO-cyclic GMP-relaxation pathway in Fisher rats and compared with Lewis rats, using an exogenous donor.

3.1 ABSTRACT

The aim of the current studies was to investigate the possibility that a decreased relaxant response to nitric oxide (NO) might contribute to strain-related differences in airway responsiveness in the rat. Isolated tracheal rings from hyperresponsive Fisher rats were confirmed to be more responsive to carbachol (EC_{50}=2.45x10^{-7}M) than those from Lewis (EC_{50}=3.60x10^{-7}M, $p<0.03$) and ACI (EC_{50}=3.85x10^{-7}M, $p<0.01$) rats. Sodium nitroprusside (SNP), a NO donor, caused relaxation of the carbachol (10^{-6}M) contracted tracheal rings but the IC_{50} SNP in Fisher rats (5.60x10^{-6}M) was significantly higher than that in Lewis (1.34x10^{-6}M, $p<0.001$) and ACI rats (1.13x10^{-6}M, $p<0.0005$). The inhibitory effect of SNP on airway responsiveness to inhaled methacholine (MCh) *in vivo* was also less pronounced for Fisher than Lewis rats. SNP induced an accumulation of cyclic GMP in cultured tracheal smooth muscle cells (TSM). Fisher TSM produced less cyclic GMP on exposure to SNP compared with ACI ($p<0.01$) and Lewis ($p<0.0001$). A decreased guanylyl cyclase activity may account for the impaired relaxant effect of SNP in Fisher rats.

3.2 INTRODUCTION

Airway responsiveness to methacholine and other bronchoconstrictors is variable within (14,33) and among species (22,29). The predominant determinant of the differences in responsiveness is not known. Suggested explanations include an increased amount of smooth muscle in the airways (11), disruption of the usual mechanical interaction between the airways and the surrounding parenchyma (15,23), alterations in neural control of the airways, and imbalance between endogenous bronchoconstrictors and dilators. Nitric oxide (NO) is a recent addition to the list of potential endogenous modulators of bronchomotor tone.

NO appears to play an important role in several biological functions of the lung including the regulation of airway smooth muscle tone (6). NO is known to relax tracheal smooth muscle by activating soluble guanylyl cyclase and increasing intracellular guanosine 3':5'-cyclic monophosphate (cyclic GMP) (16). NO is also partly responsible for the phenomenon of non-adrenergic, non-cholinergic relaxation of excised airways from both guinea pigs and humans (3,19). Recently it has been shown that inhibition of NO synthesis increases the airway responsiveness of the guinea pig to histamine and the cholinergic agonists carbachol and MCh both *in vitro* and *in vivo* (28), indicating an involvement of NO in the regulation of airway responsiveness to these bronchoconstrictors.

The current studies were designed to test the hypothesis that an impaired relaxant response to NO is involved in airway hyperresponsiveness. We took advantage of the reported strain related differences in airway responsiveness in the rat (22,29). Fisher rats have

been observed to exhibit a greater degree of airway responsiveness to methacholine compared with Lewis (12) and ACI rats (22). The relaxant effects of SNP, which is known to release NO (5,21), were tested on the airways of the relatively hyperresponsive Fisher rat and were compared with its effects on the less responsive Lewis and ACI strains.

3.3 MATERIALS AND METHODS

Animals. The highly inbred Fisher, Lewis and ACI rat strains (male, 7-9 week old) were purchased from a commercial source (Harlan Sprague Dawley Inc., Indianapolis, IN) and housed in a conventional animal care facility at McGill University prior to experimentation. The protocols were approved by an Animal Ethics Committee.

Mechanical responses of tracheal rings. Rats were killed by a lethal overdose of pentobarbital and their tracheas were immediately excised and cut into 3 mm rings approximately. Care was taken not to damage the epithelium. Only the rings from the lower end of the trachea were used to measure the mechanical responses. Tracheal rings were mounted on hooks, connected to force transducers (Grass FT03, Quincy, MA) and incubated in a physiological saline solution (PSS) bubbled with 95 % oxygen/5% carbon dioxide in 25 ml organ baths at 37°C. The passive tension was set at 1 gram and the tissue was equilibrated for 60 min. The isometric force of the tracheal rings in response to different concentrations of carbachol (Sigma Chemical Co. St Louis MO) was recorded. The magnitude of relaxation induced by SNP (Sigma Chemical Co., St Louis MO) was measured on rings that were pre-constricted with 10^{-6} M carbachol and calculated as the percent decrease in the isometric force developed with carbachol. The effects of LY-83583 (Calbiochem. San Diego, CA.) on SNP induced relaxation of tracheal rings were also recorded. The width and weight of each of the tracheal rings were measured at the end of the experiment.

Measurements of airway responsiveness *in vivo*. F344 (n=7) and Lewis strains (n=7) were given an intravenous infusion of SNP (3.4 μg/kg/min, in 5% glucose). Propranolol, a β-adrenergic receptor blocker, was injected intravenously (1mg/kg) before SNP infusion to counter any effects of catecholamines on airway responses. Pulmonary resistance (R_L) was measured at baseline and following aerosols of methacholine (MCh). MCh was administered in progressively doubling concentrations until a doubling in pulmonary resistance was obtained. Responsiveness was defined as the concentration of MCh required to double R_L ($EC_{200}R_L$). Each animal had a measurement of responsiveness to MCh measured after an infusion of SNP and also after an infusion of 5% glucose. Three animals from each strain received SNP first and 2 hrs later a glucose infusion whereas 4 rats from each strain had responsiveness measured in the reverse order. Aerosols were generated using a disposable nebulizer (Model 1400, Hudson Inc., Temecula, CA) and a compressed air source delivering an airflow of 8 l/min.

Pulmonary resistance were measured as follows. Intubated rats were placed in the supine position. The tip of the tracheal tube was inserted into a Plexiglas box (volume, 265 ml) to which a Fleisch no. 0 pneumotachograph coupled to a piezoresistive differential pressure transducer (Micro-Switch 163PC01D36; Honeywell, Scarborough, Ont., Canada) was attached to measure airflow. Transpulmonary pressure (Ptp) was measured using a differential pressure transducer (Transpac II disposable transducer, Sorenson, Salt Lake City, Utah) connected to a water-filled catheter whose tip was in the lower third of the oesophagus. The other port of the transducer was connected to the Plexiglas box. The pressure and flow signals were amplified, passed through eight-pole Bessel filters (Model 902LPF; Frequency

Devices, Haverhill, MA), with their cutoff frequencies set at 100 Hz, and recorded by a 12-bit analog to-digital converter at a rate of 200 Hz. The data were stored on a computer. Pulmonary resistance was calculated by multiple linear regression by fitting the equation: $Ptp=E_L V + R_L V + K$, to 10-s segments of data, where Ptp is transpulmonary pressure, V is flow, R_L is resistance, V is volume, E_L is elastance, and K is a constant value. A commercial software package (RHT Infodat Inc, Montreal, QC, Canada) was used.

Tracheal smooth muscle cell cultures. Rat tracheal smooth muscle cells were cultured as previously described (8,13). Briefly, tracheas were dissected rapidly and rinsed with ice cold Hanks' balanced salt solution (HBSS). All extraneous tissues were carefully stripped from the tracheas. Tracheas were cut longitudinally through the cartilages opposite to the strip of smooth muscle and incubated in HBSS containing 0.05% elastase (type IV, Sigma Chemical Co., St Louis MO) and 0.2% collagenase (Type IV, Sigma) for 30 min at 37°C with gentle shaking. The solution was centrifuged at room temperature at 1200 rpm for 6 min. The pellet was resuspended in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, GIBCO Grand Island, NY) and Ham's F12 nutrient mixture (ICN Biomedicals, Costa Mesa, CA), containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (GIBCO), and cultured in 25 cm^2 cell culture flasks at 37°C in humidified air containing 5% CO_2. When confluent, cells were detached from the flasks by incubation with 0.125% trypsin in HBSS containing 0.02% EDTA and subcultured in 24-well or 6-well plates. Only confluent cells from the first passage were used for experiments. Immunohistochemical staining for smooth muscle specific α-actin was done in preliminary experiments to confirm that cells

were in fact smooth muscle.

Cyclic nucleotide measurements. Cultured tracheal smooth muscle cells in 24-well plates were incubated with 1 ml Hepes-buffered culture medium containing 2% FBS for 30 min at 37°C as an initial period of equilibration which was followed by a 15 min incubation with 0.5 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX, Aldrich Chem. Co. Milwaukee. WI). IBMX was added so as to inhibit the degradation of cyclic nucleotide. Then cells were incubated with either test agents or vehicle for 10 min. The reactions were stopped by replacing the medium with 1 ml of ice cold 0.5 N hydrochloric acid. The cells were then sonicated and adenosine 3':5'-cyclic monophosphate (cyclic AMP) and cyclic GMP (following acetylation of the cyclic GMP) (7) were measured by radioimmunoassay. Experiments were repeated at least 3 times in quadruplicate.

Nitrite assay. Cultured tracheal smooth muscle cells in 6-well plates were incubated with 2 ml Hepes-buffered culture medium containing 2% FBS for 30 min at 37°C. SNP was added and cells were incubated for a further 10 min at 37°C. Nitrite in cell culture medium was determined by a spectrophotometric method based on the Griess reaction (35). Briefly, 0.4 ml samples of supernatant were mixed with 0.8 ml Griess reagent containing 0.5% sulphanilamide (Sigma Chemical Co.), 0.05% N-1-naphthyl-ethylenediamine-dihydrochloride (Sigma Chemical Co.), and 0.4 N HCl and incubated for 10 min at room temperature. The absorbance of the reaction product was read at 543 nm. The nitrite production was quantitated from values interpolated from a standard curve using sodium nitrite. The

sensitivity of the assay was approximately 10^{-6} M nitrite. Experiments were repeated 3 times and all measurements were made in triplicate.

Statistical Analysis. Data are expressed as mean ± s.e. Significant differences among group means were tested for by one-way analysis of variance (ANOVA) and post hoc comparisons were done using Fisher's LSD test. Comparisons of two means were made using Student's t test. The concentrations of carbachol required to effect 50% of the maximal contraction (EC_{50}) or of SNP required to cause 50% relaxation (IC_{50}) were expressed as the geometric means and were logarithmically transformed prior to statistical analysis. $p<0.05$ was considered significant.

3.4 RESULTS

Contractile responses of isolated tracheal preparations to carbachol. The isometric force generated by tracheal rings on exposure to carbachol is shown for Fisher, Lewis and ACI rat strains in Fig. 1. Carbachol (10^{-7} M to 10^{-4} M) induced an increase in isometric force in tracheal rings of all three strains in a concentration-dependent manner. Fisher tracheal preparations exhibited a greater responsiveness to carbachol compared with either Lewis or ACI rats. The maximal isometric force generated by Fisher trachealis in response to carbachol was 4.58±0.42 gram, which was significantly higher than that of the trachealis from either Lewis (3.03±0.25 gram, $p<0.005$) or ACI (2.82 ± 0.16 gram, $p<0.001$) rats. EC_{50} of Fisher trachealis (2.45×10^{-7} M; geometric mean) was lower than both that of Lewis (3.60×10^{-7} M, $p<0.03$) and ACI (3.85×10^{-7} M, $P<0.01$).

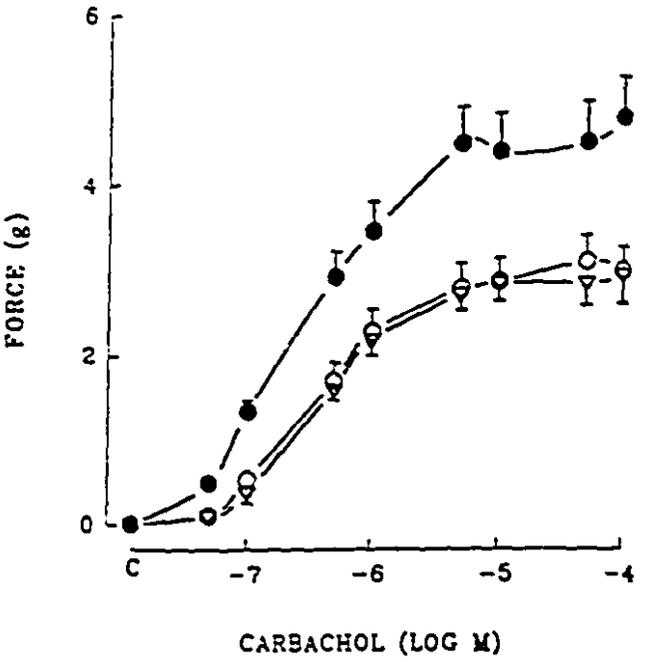

Figure 1. Isometric tension response (grams) of tracheal rings plotted against progressive increasing concentration of carbachol (log molars). Open circles: Lewis rats (n=6); closed circles: Fisher rats (n=6); open triangles: ACI rats (n=8). C: control condition without treatment. Data are expressed as mean ± s.e.

The width of tracheal rings was 3.2 ± 0.1 mm in Fisher, 3.0 ± 0.1 mm in Lewis (P=NS compared to Fisher rats) and 2.8 ± 0.2 mm in ACI rats (P=NS compared to Fisher rats). The weight of the tracheal rings was 7.21 ± 0.35 mg in Fisher, 7.95 ± 0.63 in Lewis (P=NS compared to Fisher rats) and 6.09 ± 0.26 in ACI rats ($p<0.05$, compared to Fisher rats).

Relaxant effects of sodium nitroprusside on carbachol precontracted tracheal preparations. Tracheal rings were precontracted with 10^{-6}M carbachol; this concentration effected contractions of the tracheal rings equivalent to 75±2%, 71±4% and 74±2% of the maximal isometric force in Fisher, Lewis (p=N.S.) and ACI (p=N.S.) rats, respectively. SNP (10^{-7} M to 10^{-3} M) induced a concentration-dependent relaxation of carbachol-contracted rat tracheal rings from the three strains (Fig. 2). However, Fisher rats were less

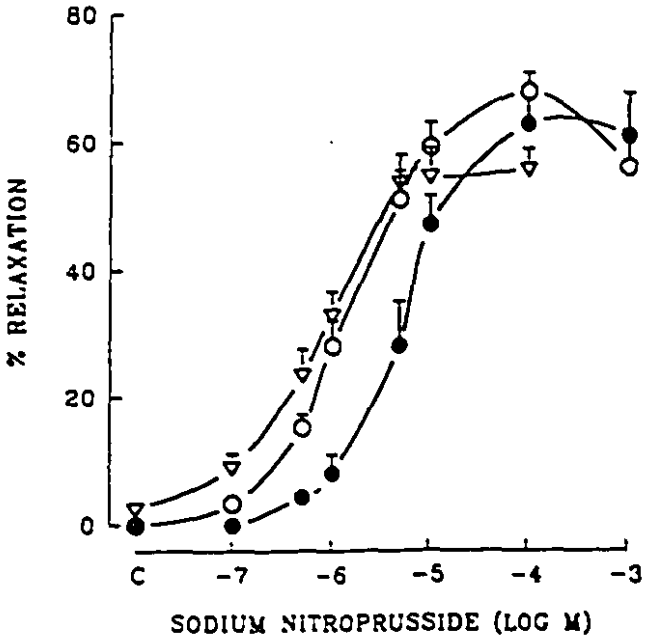

Figure 2. Relaxation of tracheal rings in response to sodium nitroprusside. The tracheal rings was pre-contracted by 10^{-6} Molar of carbachol. Relaxation is expressed as the % decrease in isometric tension developed with carbachol. Open circles: Lewis rats (n=9); closed circles: Fisher rats (n=7); open triangles: ACI rats (n=8). C: control condition without treatment. Data are expressed as mean ± s.e.

sensitive to SNP than Lewis and ACI rats, as evidenced by a reduced magnitude of relaxation for a given concentration of SNP. The IC_{50} for SNP in Fisher rats (5.6×10^{-6} M) was significantly higher than that in Lewis (1.3×10^{-6} M, $p<0.001$) and ACI (1.1×10^{-6} M, $p<0.0005$) rats.

Effect of sodium nitroprusside on pulmonary responsiveness to methacholine *in vivo*. Fisher rats were hyperresponsive to inhaled MCh compared with Lewis rats (Fig. 3). The concentration of methacholine needed to double pulmonary resistance ($EC_{200}R_L$) for Lewis rats (4.89 mg/ml) was 2.6 times higher than that of Fisher rats (1.86 mg/ml, $p<0.02$; Fig. 3). SNP reduced the pulmonary response to MCh in Lewis rats; $EC_{200}R_L$ increased from 4.9 to 11.4 mg/ml ($p<0.02$). In

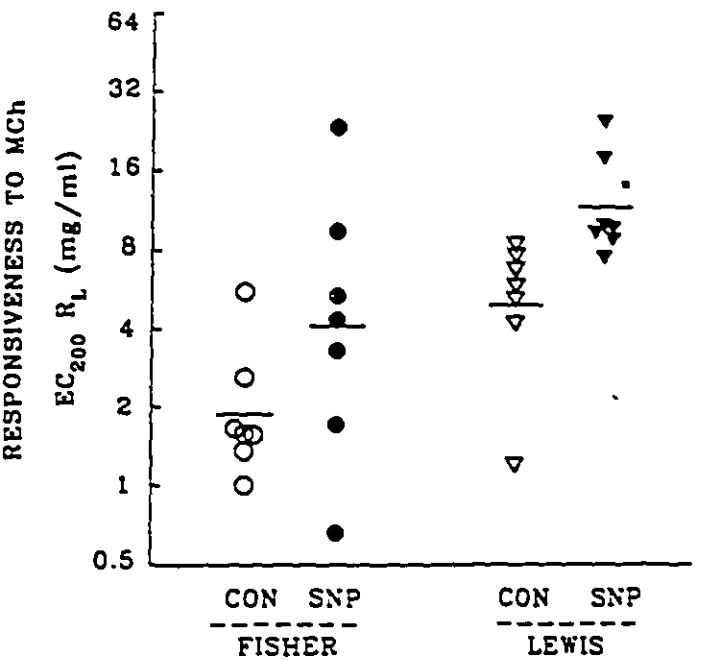

Figure 3. Effect of sodium nitroprusside on pulmonary resistance in response to methacholine *in vivo*. $EC_{200}R_L$: MCh concentration needed to double the pulmonary resistance. Each symbol represents data from an individual animal.

Fisher rats, although pulmonary responsiveness to MCh showed a similar trend, the changes in responsiveness were not significantly different before and after SNP infusion ($EC_{200}R_L$: 1.9 vs 4.1 mg/ml, p=N.S.).

Cyclic nucleotide production by tracheal smooth muscle in response to sodium nitroprusside. Fig. 4 shows the levels of intracellular cyclic nucleotides in cultured tracheal smooth muscle cells exposed to SNP. With increasing concentrations of SNP, in the presence of IBMX, there was a progressive increase in the levels of cyclic GMP in tracheal smooth muscle cells. Less cyclic GMP was produced for any given concentration of SNP

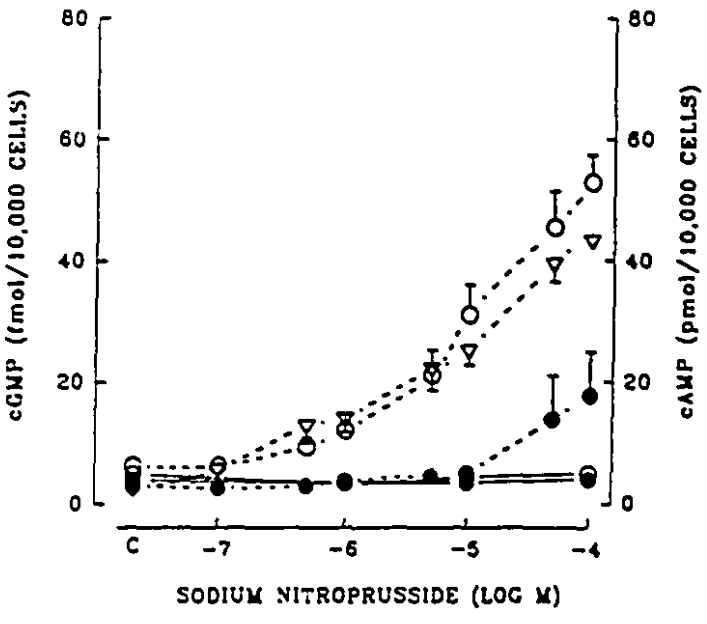

Figure 4. Cyclic GMP (dashed curves, unit: femtomoles) and cyclic AMP (solid curves, unit: picomoles) accumulation in cultured tracheal smooth muscle cells in response to sodium nitroprusside. Open circles: Lewis rats (n=6); closed circles: Fisher rats (n=6); open triangles: ACI rats (n=8). C: control condition without treatment. Data are expressed as mean ± s.e.

in the cultured tracheal smooth muscle cells from Fisher rats than from either Lewis ($p<0.0001$) or ACI rats ($p<0.01$). SNP did not effect any increase in intracellular cyclic AMP production by tracheal smooth muscle cells from any of the three strains (Fig. 4).

Sodium nitroprusside, cyclic GMP and relaxant responses. To further investigate whether SNP-induced relaxation was through a cyclic GMP-dependent mechanism, the effects of LY83583, a selective suppressor of cyclic GMP formation (20,32), on SNP-induced

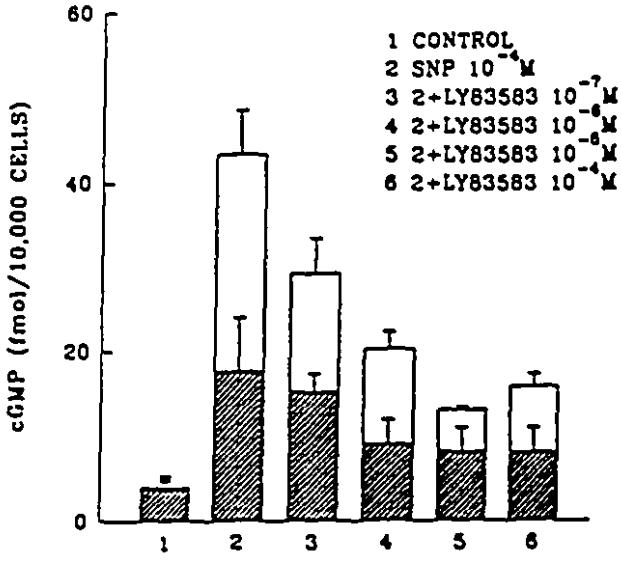

Figure 5. Effect of LY-83583 on SNP-induced cyclic GMP accumulation in cultured tracheal smooth muscle cells. Open bar: Lewis rats; hatched bar: Fisher rats. Data shown are mean ± s.e.

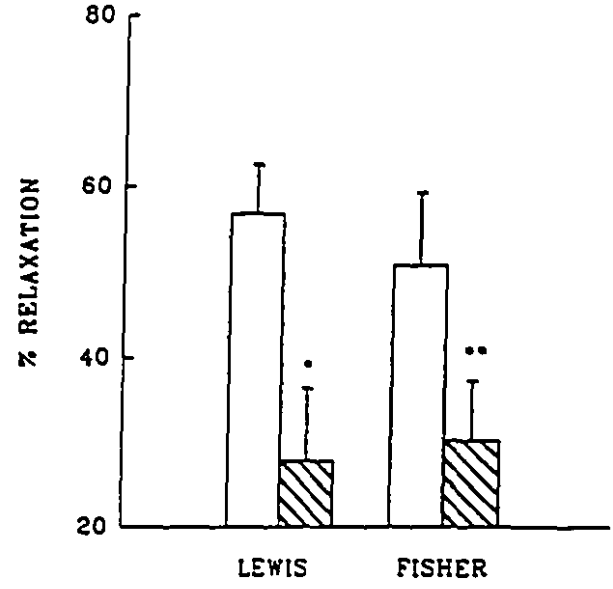

Figure 6. Effect of LY-83583 (10^{-5} M) on sodium nitroprusside (10^{-4} M)-induced relaxation of tracheal rings from Lewis and Fisher rats. The tracheal rings were pre-contracted by carbachol (10^{-6} M). Relaxation is expressed as % decrease in isometric tension developed with carbachol. Open bar: sodium nitroprusside only; cross-hatched bar: sodium nitroprusside plus LY-83583. Data are mean ± s.e. n=7, * $p<0.05$; ** $p<0.01$

intracellular cyclic GMP accumulation in cultured smooth muscle cells and relaxation of tracheal rings were tested. LY83583 (10^{-7}M to 10^{-4}M) inhibited cyclic GMP accumulation induced by SNP (10^{-4}M) in cultured cells (Fig. 5). LY83583 (10^{-5}M) was also found to inhibit SNP-induced tracheal ring relaxation in both Fisher (Fig. 6, $p<0.01$) and Lewis rats ($p<0.05$).

In addition, 8-bromo-guanosine 3':5'-cyclic monophosphate (8-bromo-cyclic GMP), a cell-permeable cyclic GMP analog, induced relaxation in carbachol (10^{-6} M)-constricted tracheal rings (Fig. 7). The tracheal rings from Fisher rats showed a lesser degree of

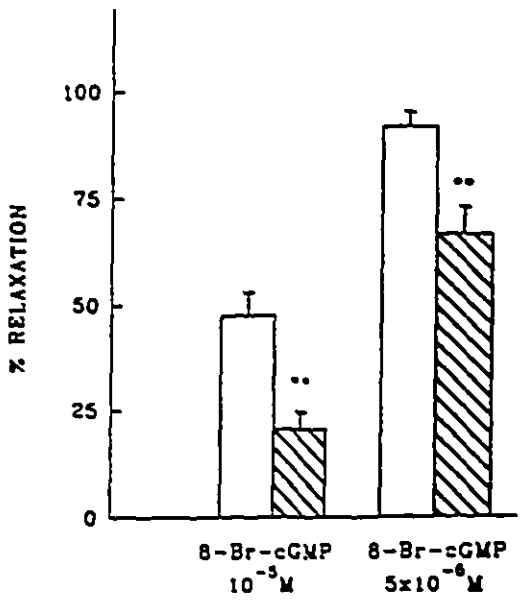

Figure 7. 8-Br-cyclic GMP induced relaxation of tracheal rings from Lewis (open bar) and Fisher rats (hatched bar). The tracheal rings were pre-contracted by carbachol (10^{-6} M). Relaxation in response to 8-bromo-cyclic GMP was recorded and expressed as % decrease in isometric tension developed with carbachol. n=5, ** $p<0.01$; ** $p<0.01$

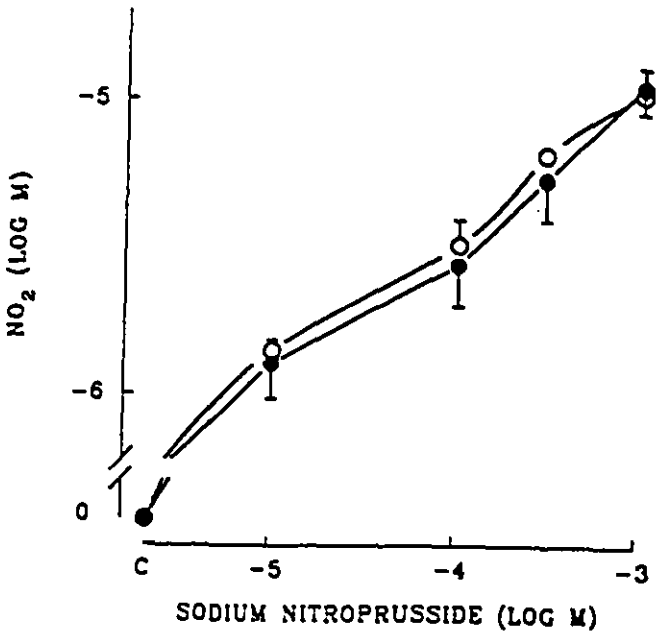

Figure 8. Nitrite production in cultured rat tracheal smooth muscle cells on exposure to SNP. Open circles: Lewis rats; closed circles: Fisher rats. C: control condition without treatment. Data are expressed as mean ± s.e. of 3 experiments in triplicate.

relaxation in response to the same concentrations of 8-bromo-cyclic GMP ($p<0.01$) than those from Lewis rats.

Nitrite assay. SNP produced nitrite when incubated with cultured airway smooth muscle cells in a concentration-dependent manner. There was no significant difference in the level of nitrite in the culture medium from Fisher and Lewis airway smooth muscle cells (Fig. 8). In SNP solution, nitrite levels increased with increasing concentrations of SNP in the absence of cells (data not shown) and were comparable to measurements made on the medium of the smooth muscle cultures from each of the strains.

3.5 DISCUSSION

In this study, the effects of sodium nitroprusside on the relaxation of carbachol pre-contracted tracheal rings and intracellular cyclic GMP accumulation in cultured tracheal smooth muscle cells were compared among Fisher, Lewis and ACI rats. Tracheal rings from Fisher rats were hyperresponsive to carbachol relative to Lewis and ACI rats, confirming an association between *in vivo* and *in vitro* differences in airway responsiveness among these strains (12,22). SNP induced a concentration-dependent relaxation of carbachol-contracted tracheal rings. However, Fisher rats demonstrated a lower sensitivity to SNP in terms of relaxation of pre-contracted tracheal rings as well as cyclic GMP accumulation in cultured airway smooth muscle cells than either Lewis or ACI rats. Further, Fisher tracheal smooth muscle relaxed less well on exposure to the cell permeable cyclic GMP analogue, 8-bromo-cyclic GMP, indicating a lower sensitivity of the contractile mechanisms to the relaxant effects of this cyclic nucleotide.

Differences in responsiveness among rat strains have been shown in response to airway challenge *in vivo* (12,22). Different responses *in vivo* to inhaled agonists could be caused by a number of factors other than intrinsic differences in the contractile properties of airway smooth muscle. Among these factors are altered lung parenchyma-airway interdependence (27), the loads against which airway smooth muscle contracts (9,26) and the airway geometry (25). In evaluating the possibility that airway hyperresponsiveness might be associated with impaired relaxation of smooth muscle to SNP, it was important to demonstrate that differences in smooth muscle responsiveness were present *in vitro*. Our

findings showing an association between *in vivo* and *in vitro* responsiveness are in contrast to previous studies that failed to show any relationship between airway responsiveness to provocation testing *in vivo* and the responsiveness of excised airway tissues *in vitro* (34). It is possible that the use of tissues from diseased lungs may have introduced confounding factors in earlier studies that masked relationships which might have been present had normal tissues been studied. *In vitro* responses of human airways are also difficult to evaluate for changes in the absolute magnitudes of response of human airways because of the lack of convenient methods for ensuring that the orientation of tissue strips corresponds to the long axis of the muscle bundles. The use of tracheal strips circumvents this problem because of the consistent transverse arrangement of the muscle bundles.

To allow comparisons not only of the positions of the concentration-response curves to carbachol but also the maximal responses we attempted to study tracheal rings of comparable sizes from each strain. There was no difference in the size or weight between Fisher and Lewis tracheas. However, rings were slightly lighter from ACI than Fisher rats which could account for part of the observed differences in maximal responses, assuming that the quantity of muscle was in proportion to the weight of the rings. However, in the absence of any measurements of the quantity of airway smooth muscle in the tracheal preparations, we cannot exclude the possibility that such differences were present and may have caused the differences in maximal responses. We have previously demonstrated an increase in smooth muscle in the intraparenchymal airways of Fisher compared to Lewis rats (12), so it is quite conceivable that such differences may be present also in the trachea. Although the predominant difference in responsiveness *in vitro* resided in the maximal responses, there

were also differences in the positions of the concentration-response curves that suggest intrinsic differences in contractile responses to carbachol. The finding of strain-related differences in tracheal responses and the association with measurements of airway responsiveness *in vivo* was surprising given the indirect link between the two measures. However, it does suggest that tracheal responses may be representative of the responsiveness of more distal parts of the airway tree.

Sodium nitroprusside was used to mimic the effects of nitric oxide, an endogenous modulator of airway tone (6). The relaxant effect of SNP on smooth muscle is likely to be mediated by a NO-dependent mechanism because SNP releases NO after photolysis or after the addition of reducing agents (5,21). It has been demonstrated also that a smooth muscle membrane-associated activity catalyses the release of NO from SNP (18). However, since our experiments were performed in such a way that photolysis of SNP could occur, catalytic conversion of SNP was not necessary. Indeed, nitrite levels assayed in solutions of SNP were comparable to levels in medium from smooth muscle cultures. Consistent with the idea that SNP induced relaxation involved cyclic GMP, LY83583, an agent that decreases intracellular cyclic GMP (20,32), inhibited both SNP induced cyclic GMP accumulation in the smooth muscle cells in culture as well as the relaxation of tracheal rings.

The Fisher rat trachea was less sensitive to the relaxant effects of SNP than either Lewis or ACI tissues. Maximal responses to SNP were similar among the three strains. The changes in cyclic GMP in SNP exposed cultured myocytes are consistent with these observations, in that levels of cyclic GMP were less in the Fisher cells for any concentration of SNP. Maximal relaxation of the tracheal rings was effected by concentrations of SNP

around 5 X 10^{-6} M in Lewis rats and 10^{-5} M in Fisher; these concentrations resulted in equivalent levels of cyclic GMP (20 femtomoles/10^4 cells). However, at lower concentrations of SNP there was a measurable relaxant effect in tissues from Fisher rats even though cyclic GMP levels were not detectably elevated. This suggests that compartmentalization of cyclic GMP may occur and that total cell levels may not be a sufficiently sensitive way to detect increases in intracellular concentrations. Although we have not established the exact mechanism for the decreased production of cyclic GMP in response to SNP in Fisher rats, nitrite measurements exclude the possibility that SNP might have released less NO in Fisher rat tracheal smooth muscle cells. It is more likely that guanylyl cyclase activity is lower in the smooth muscles of Fisher rats, or that the sensitivity of the enzyme to NO itself is different between strains. Lower levels of cyclic GMP do not appear to be the sole explanation for differences in tracheal responses to SNP, since in Fisher rats the tracheal rings also relaxed relatively less to any given concentration of the cell permeant cyclic GMP analog, 8-bromo-cyclic GMP. This finding indicates that a decreased sensitivity of the contractile process to cyclic GMP may also contribute in part to the differences in tracheal responses to SNP in Fisher rats.

Impaired relaxant responses of airway smooth muscle have been previously described in association with hyperresponsiveness. A reduced responsiveness of the airway smooth muscle to β-adrenergic agents (2) has been found in subjects with asthma, in actively sensitized guinea pigs (4,24) and in the Basenji greyhound, a dog model of hyperresponsiveness (10). Alveolar macrophages from patients with asthma accumulate less cyclic AMP in response to β-agonists compared to cells from control subjects without

asthma, and the degree of hyporesponsiveness is related to the severity of asthma (1). A decreased adenylyl cyclase activity has been confirmed in membrane fractions of macrophages from asthma patients in response to β-agonists (1). The current studies suggest that decreased guanylyl cyclase activity may be associated with hyperresponsiveness of the rat tracheal smooth muscle, indicating that other relaxant pathways may be affected in hyperresponsive animals.

The link between decreased guanylyl cyclase activity and airway hyperresponsiveness is not immediately obvious, but it is consistent with a role for NO as a determinant of airway responses to contractile agonists (28). Airway smooth muscle does not appear to have been evaluated as a possible source of NO, but it is possible that other cells such as epithelium may synthesize it (17,30). Recent evidence indicates that a cyclic GMP-dependent protein kinase reduces agonist-stimulated Ca^{++} increase by inhibiting inositol 1,4,5-triphosphate (IP_3) production (31), suggesting that the altered regulation of IP_3 levels intracellularly may be the mechanism by which cyclic GMP inhibits airway smooth muscle contraction. We postulate that an imbalance between NO-cyclic GMP-induced relaxation and contractile agonist-stimulated IP_3 production may be a determinant of differences in airway responsiveness *in vivo*.

Other explanations for strain-related differences in airway responsiveness in the rat have been proposed. An increased amount of airway smooth muscle has been found by morphometry in Fisher rats compared to Lewis and has been postulated to account for the observed inter-strain differences in responsiveness (12). However, the hyperresponsiveness of Fisher rats *in vitro* involves both an increased maximal response and sensitivity (EC_{50}) to

carbachol. The increased maximal isometric responses *in vitro* observed in the present study are consistent with the possibility that the amount of tracheal smooth muscle may also be increased in Fisher rats (12). However, the increased sensitivity of the tracheal rings in Fisher rats to carbachol cannot be explained by changes in the amount of airway smooth muscle, but, it must have been caused by other mechanisms of which sensitivity to endogenous NO is one possibility.

In summary, our present results and previous studies (12,22) demonstrate that the Fisher rats have a degree of airway responsiveness that is greater than certain other rat strains. Our data also indicate that the functional integrity of the airway NO-cyclic GMP-relaxant pathway is impaired in Fisher rats, suggesting the possibility of an imbalance between contraction and relaxation as a potential mechanism leading to airway hyperresponsiveness.

3.6 ACKNOWLEDGEMENT

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Chapter 4.

P450 MEDIATED NITRIC OXIDE SYNTHESIS BY TRACHEAL SMOOTH MUSCLE CELLS

Studies reported in Chapter 2 and 3 are investigations of the role of NO in the regulation of airway smooth muscle tone. In the airway, NO is believed to be synthesized in cells such as epithelium and diffuse into adjacent smooth muscle cells where it regulates airway responsiveness. Since no constitutive NOS has been detected so far in airway smooth muscle cells, an alternative pathway for NO production, which has been reported in vitro previously, is investigated in airway smooth muscle cells and results are presented in this chapter.

4.1 ABSTRACT

Nitric oxide (NO) is known to be synthesized from L-arginine in a reaction catalyzed by NO synthase. Liver cytochrome P450 enzymes also catalyze the oxidative cleavage of C=N bonds of compounds containing a -$C(NH_2)$=NOH function, producing NO *in vitro*. The current study was designed to investigate whether the P450-catalyzed pathway for production of NO is also present in tracheal smooth muscle cells. The effects of formamidoxime, a compound containing -$C(NH_2)$=NOH, on airway relaxation, intracellular cyclic GMP accumulation and NO production in cultured tracheal smooth muscle cells were tested. Formamidoxime (10^{-2}M to 10^{-4}M) relaxed carbachol contracted tracheal rings and increased intracellular cyclic GMP in cultured tracheal smooth muscle cells in a concentration-dependent manner while L-arginine had no effect. NO was detectable in the medium containing cultured tracheal smooth muscle cells when incubated with formamidoxime. Ethoxyresorufin (10^{-7}M to 10^{-4}M), a P450 inhibitor, inhibited formamidoxime induced cyclic GMP accumulation in cultured tracheal smooth muscle cells as well as tracheal ring relaxation. The NO-synthase inhibitors, N^{ω}-nitro-L-arginine (10^{-3}M) and N^{G}-monomethyl-L-arginine (10^{-3}M), had no effect on formamidoxime induced cyclic GMP accumulation. In conclusion, these results suggest that NO can be synthesized from formamidoxime in tracheal smooth muscle cells by a reaction catalyzed by P450.

4.2 INTRODUCTION

It has been well established that endogenous NO is synthesized from L-arginine catalyzed by NO synthase (NOS). NOS catalyzes the formation of NO from L-arginine in two steps. Firstly, it catalyzes the N-oxygenation of L-arginine to form N^{ω}-hydroxy-arginine (NOHA), which contains a $-C(NH_2)=NOH$ function. Secondly, the oxidative cleavage of the C=N bonds of NOHA produces NO and L-citrulline (26). Liver cytochrome P450s have also been found to catalyze the oxidative cleavage of C=N bonds of compounds containing a $-C(NH_2)=NOH$ function, producing the corresponding derivatives containing a $-C(NH_2)=O$ function and NO *in vitro* (1).

NO appears to play an important role in regulating several biologic functions in the lung including modulation of airway smooth muscle tone (4,10,14,19). NO relaxes airway smooth muscle by activating soluble guanylate cyclase leading to the accumulation of intracellular cyclic GMP. In the lung, NO can be synthesized in a number of cell types including macrophages, neutrophils, mast cells, nonadrenergic noncholinergic inhibitory neurons, fibroblasts, vascular smooth muscle cells, pulmonary arterial and venous endothelial cells, and airway epithelial cells (11). However, no constitutive NOS activity has been found in airway smooth muscle cells. Since cytochrome P450 isoenzymes have been identified in rat lung (27), the current study was designed to test whether the P450-catalyzed pathway for NO production was also present in airway smooth muscle cells. We chose formamidoxime ($HC(NH_2)=NOH$), a compound containing $-C(NH_2)=NOH$, as the substrate for this pathway. Therefore, the effects of formamidoxime on airway relaxation and intracellular cyclic GMP

accumulation in cultured tracheal smooth muscle cells were investigated. The role of P450 enzymes in the production of cyclic GMP was explored using ethoxyresorufin and miconazole, P450 inhibitors (21,22).

4.3 METHODS

Animals. Lewis rats (male, 7-9 week old) were purchased from Harlan Sprague Dawley Inc.(Indianapolis, IN) and housed in a conventional animal care facility at McGill University prior to experimentation. The protocol was approved by an Animal Ethics Committee.

Mechanical responses of tracheal rings. Rats were killed by overdose with pentobarbital and their tracheas immediately excised and incubated in a physiological saline solution (PSS, containing (mM): NaCl 118; KCl 4.5; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25.5 and glucose 5.6) bubbled with 95% oxygen/5% carbon dioxide. Tracheas were dissected from surrounding tissues and cut into 3 mm rings approximately. Only those rings from the lower end of the trachea were used to measure the mechanical responses. Tracheal rings were mounted on hooks, connected to force transducers (Grass FT03, Quincy, MA), and incubated in PSS bubbled with 95 % oxygen/5% carbon dioxide in 25 ml organ baths at 37°C. The passive tension was set at 1 gram and the tissue was equilibrated for 60 min. The isometric force of the tracheal rings in response to carbachol (Sigma Chemical Co. St Louis MO) was recorded. The magnitude of relaxation induced by formamidoxime ($HC(NH_2)$=NOH, Aldrich Chemical Co. Milwaukee WIS) was measured on rings that were pre-constricted with 10^{-6} M carbachol and calculated as the percent decrease in the isometric force developed with carbachol. The effects of LY-83583 (Calbiochem. San Diego, CA.), ethoxyresorufin (ER, Molecular Probes Inc. Eugene, OR), N^{ω}-nitro-L-arginine (L-NNA,

Sigma Chemical Co. St Louis MO) and N^G-monomethyl-L-arginine (L-NMMA, Calbiochem Co. La Jolla CA) on formamidoxime induced relaxation of tracheal rings were also recorded.

Tracheal smooth muscle cell cultures. Rat tracheal smooth muscle cells were cultured as previously described (8,9). Briefly, tracheas were dissected rapidly and rinsed with ice cold Hanks' balanced salt solution (HBSS). All extraneous tissues were carefully stripped from the tracheas. The anterior aspect of the tracheas were cut longitudinally through the cartilages and incubated in HBSS containing 0.05% elastase (type IV, Sigma) and 0.2% collagenase (Type IV, Sigma) for 30 min at 37°C with gentle shaking. The solution was centrifuged at room temperature at 1200 rpm for 6 min. The pellet was resuspended in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, GIBCO Grand Island, NY) and Ham's F12 nutrient mixture (ICN Biomedicals, Costa Mesa, CA), containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (GIBCO, Grand Island, NY), and cultured in 25 cm^2 cell culture flasks at 37°C in humidified air containing 5% CO_2. When confluent, cells were detached from the flasks by incubation with 0.125% trypsin in HBSS containing 0.02% ethylenediaminetetraacetic acid and subcultured in 24-well or 6-well plates. Only confluent cells from the first passage were used for experiments. Immunohistochemical staining for smooth muscle specific α-actin was done to confirm that the cells obtained in this way were smooth muscle cells.

Cyclic nucleotide measurements. Cultured tracheal smooth muscle cells were incubated in 24-well plates with 1 ml Hepes-buffered culture medium containing 2% FBS for

30 min at 37°C as an initial period of equilibration, followed by a 15 min incubation with 0.5 mM of the phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX, Aldrich Chem. Co. Milwaukee. WI). Test agents or vehicle were added in the presence of 0.5 mM IBMX and incubated for 10 min at 37°C. The reactions were stopped by replacing the medium with 1 ml of ice cold 0.5 N hydrochloric acid. The cells were then sonicated and cyclic AMP and cyclic GMP (following acetylation of the cyclic GMP) (6) were measured by radioimmunoassay (RIA) (12). Experiments were repeated at least 3 times in quadruplicate.

Nitric oxide measurement. NO levels were measured using an NO chemiluminescence analyzer (Sievers Research, Boulder, CO). Cultured tracheal smooth muscle cells in 6-well plates were incubated with formamidoxime for 10 min at 37°C in 1ml Hanks' buffer. The incubation buffer was subsequently transferred to test tubes for measurement of NO as follows; samples (100 µl) were injected into a modified purging chamber containing 5 ml sodium iodide (NaI, 1% in glacial acetic acid), which was continuously being purged by a stream of argon (30-40 ml/min). Sodium iodide converted any NO that may have been transformed to nitrite by interaction with O_2 back to NO (2). The argon stream was drawn into the analyzer and mixed with internally generated O_3 (by electrostatic discharge). The light emission was detected at an integration time of 0.25 s by a cooled Hamamatsu red-sensitive photomultiplier tube after the light passed through a red filter interposed to eliminate chemiluminescence due to volatile sulfides (2). The detection limit of this technique is 1 picomole. The background signal produced by the control Hanks'

buffer was subtracted from the signal obtained from the formamidoxime treated samples. The standard were constructed using potassium nitrite at the same integration time.

Statistics. Data were expressed as mean ± SE. Differences between means of dose-response curves were tested by Dunnett t test for testing several treatment with a common control. Others were tested by Student t test. $p<0.05$ was set as the level of significance.

4.4 RESULTS

Relaxant effect of formamidoxime on rat tracheal rings. Isolated tracheal rings were precontracted with 10^{-6} M carbachol. The addition of formamidoxime to the organ bath induced a progressive relaxation of the pre-contracted rings (Figure 1). A relaxation of 32% was induced by 10^{-2}M formamidoxime

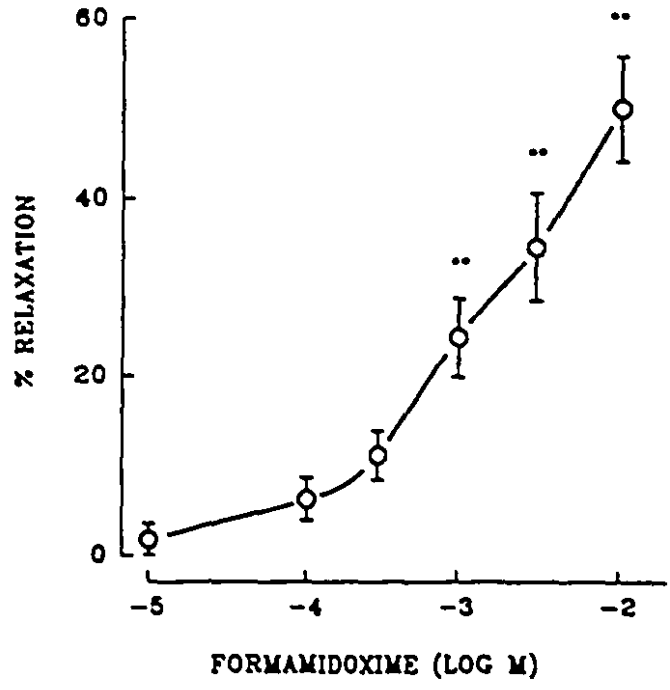

Figure 1. Formamidoxime induced relaxation of carbachol contracted rats tracheal rings. Rat tracheal rings were precontracted by 10^{-6}M carbachol (n = 6). Relaxation was calculated as decrease of isometric force and expressed as % of maximal isometric force induced by carbachol. Data are expressed as mean ± SE. * $p<0.05$; ** $p<0.01$.

Effect of formamidoxime on intracellular cyclic nucleotide production. Figure 2 shows the levels of intracellular cyclic nucleotides in cultured tracheal smooth muscle cells exposed to formamidoxime. With increasing concentrations of formamidoxime, there was a progressive accumulation of cyclic GMP in tracheal smooth muscle cells; 10^{-2} M formamidoxime increased intracellular cyclic GMP from 6.7±1.0 to 17.6±4.2 fmol/10,000 cells. In contrast, L-arginine (10^{-4}M to 10^{-2}M) had no effect on intracellular cyclic GMP levels

under the same experimental conditions. Intracellular cyclic AMP levels in tracheal smooth muscle cells were unaffected by formamidoxime.

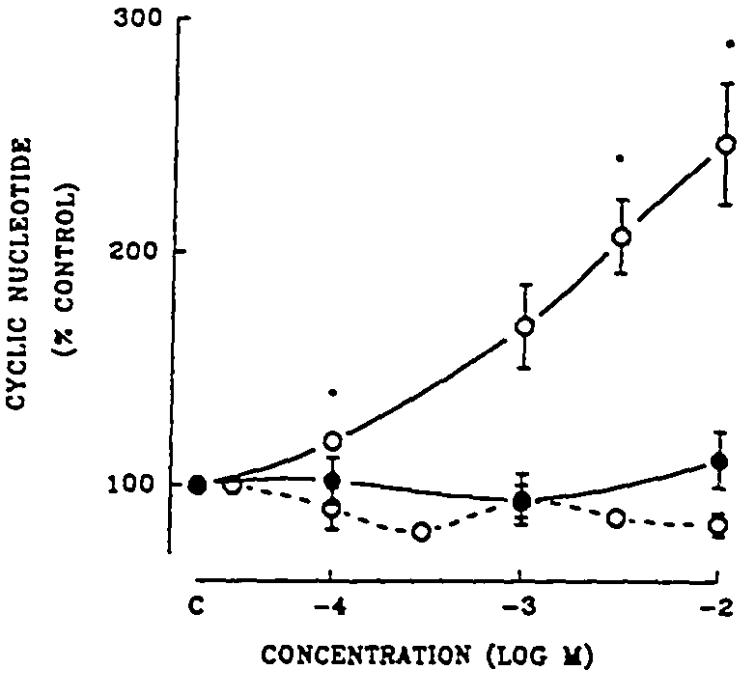

Figure 2. Formamidoxime induced cyclic GMP accumulation in cultured tracheal smooth muscle cells. Tracheal smooth muscle cells were incubated with formamidoxime or L-arginine for 10 min at 37° C. Cyclic AMP and cyclic GMP in the cells were measured. Open circle: formamidoxime; closed circle: L-arginine; solid line: cyclic GMP; dashed line: cyclic AMP. C: Control (vehicle) for formamidoxime of L-arginine. Data are expressed as mean ± SE from 3 experiments in quadruplicates. * $p < 0.05$

Formamidoxime, cyclic GMP and relaxant responses. To further investigate whether the formamidoxime-induced relaxation was mediated through a cyclic GMP-dependent mechanism, the effects of LY83583, a selective suppressor of cyclic GMP

formation (18,25), on formamidoxime-induced intracellular cyclic GMP accumulation in cultured tracheal smooth muscle cells and on relaxation of tracheal rings were tested. Increasing concentrations of LY83583 progressively inhibited formamidoxime-induced cyclic GMP accumulation in cultured tracheal smooth muscle cells (Figure 3); 10^{-2} M formamidoxime increased cyclic GMP levels from 3.58±0.91 to 6.26±1.5 fmol/10,000 cells which was inhibited by LY83583 (10^{-5} M) to 3.86±1.0 fmol/10,000 cells. 10^{-5} M LY83583 also inhibited formamidoxime-induced tracheal ring relaxation significantly as shown in Figure 4 ($p<0.01$).

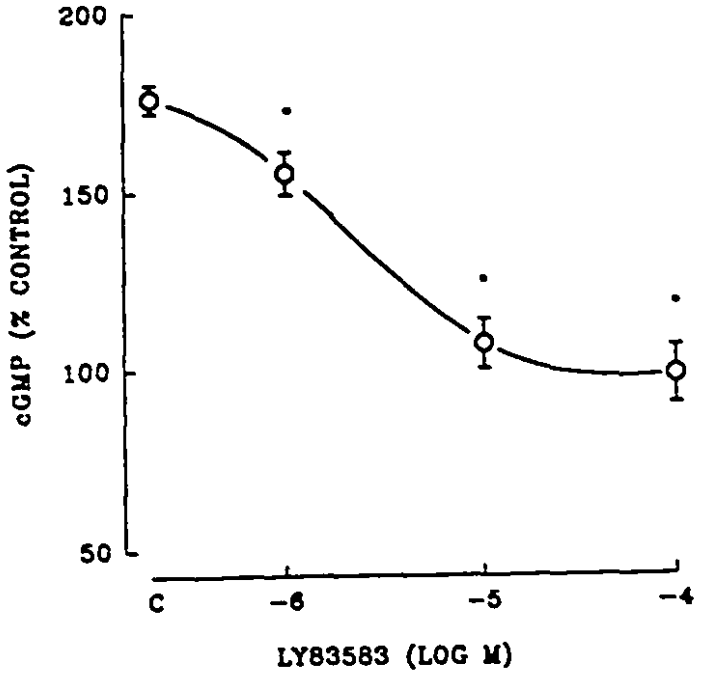

Figure 3. LY83583 inhibited formamidoxime induced cyclic GMP accumulation in tracheal smooth muscle cells. Cultured tracheal smooth muscle cells were preincubated with increasing concentrations of LY83583 for 15 min, followed by a 10 min incubation with formamidoxime (10^{-2}M). C: Control (vehicle) for LY83583 at the presence of formamidoxime (10^{-2} M). Data are expressed as mean ± SE from 3 experiments in quadruplicate. * $p<0.05$

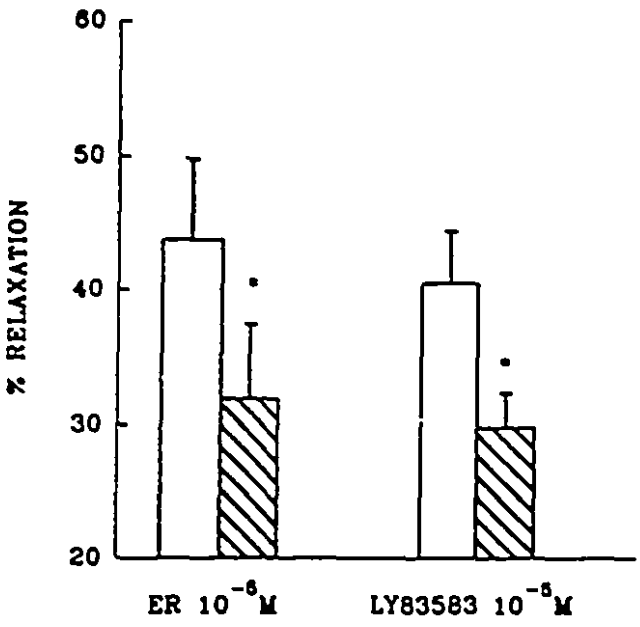

Figure 4. Effects of ethoxyresorufin (ER) and LY83583 on formamidoxime induced relaxation. Isolated tracheal rings were preincubated with vehicle (open bar) or ER or LY83583 (hatched bar) for 15 min. Relaxant effects of formamidoxime (10^{-2} M) on carbachol (10^{-6}M) contracted trachea were recorded. Data are expressed as mean ± SE. n=6. * $p<0.05$.

Effect of NO-synthase inhibitors on formamidoxime induced cyclic GMP accumulation in cultured tracheal smooth muscle cells. When cultured tracheal smooth muscle cells were preincubated with the NOS inhibitors, L-NNA (10^{-4} M) and L-NMMA (10^{-4} M), formamidoxime (10^{-2} M) induced cyclic GMP accumulation in the cells was not affected (Figure 5).

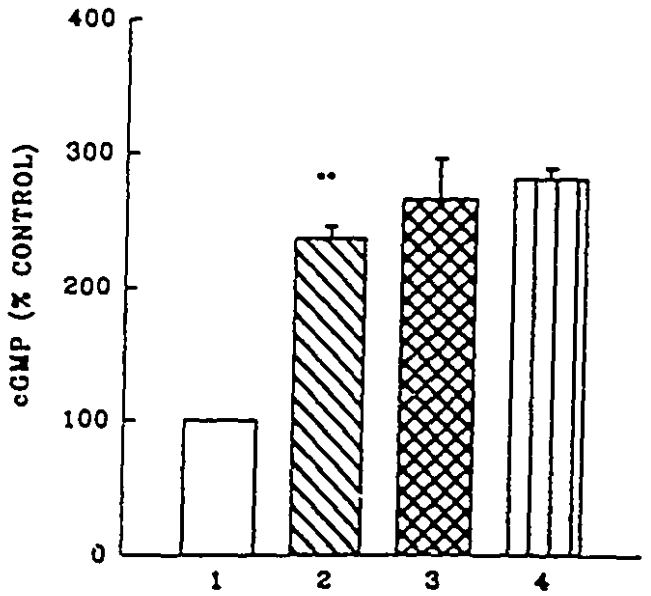

Figure 5. Effect of NOS inhibitors on formamidoxime induced cyclic GMP accumulation in tracheal smooth muscle cells. The experiments were performed in the same way as in the legend of Figure 3. 1. Control; 2. Formamidoxime 10^{-2}M; 3. L-NNA 10^{-4}M; 4. L-NMMA 10^{-4}M. Data are expressed as mean ± SE from 3 experiments in quadruplicate. ** $p<0.0001$ compared with Control

Effect of P450 inhibitors on formamidoxime induced cyclic GMP accumulation in cultured tracheal smooth muscle cells. Ethoxyresorufin (10^{-7} to 10^{-4} M), a cytochrome P450 inhibitor, inhibited formamidoxime (10^{-2}M) induced cyclic GMP accumulation in cultured tracheal smooth muscle cells in a concentration-dependent manner (Figure 6); Formamidoxime (10^{-2} M) increased cyclic GMP levels in cultured tracheal smooth muscle cells from 2.51±0.29 to

5.58±0.93 fmol/10,000 cells; this effect was inhibited by Ethoxyresorufin (10^{-5} M) to 3.44±0.47 fmol/10,000 cells. Ethoxyresorufin (10^{-5}M) also inhibited formamidoxime (10^{-2} M) induced relaxation in carbachol contracted tracheal rings (Figure 4). Miconazole

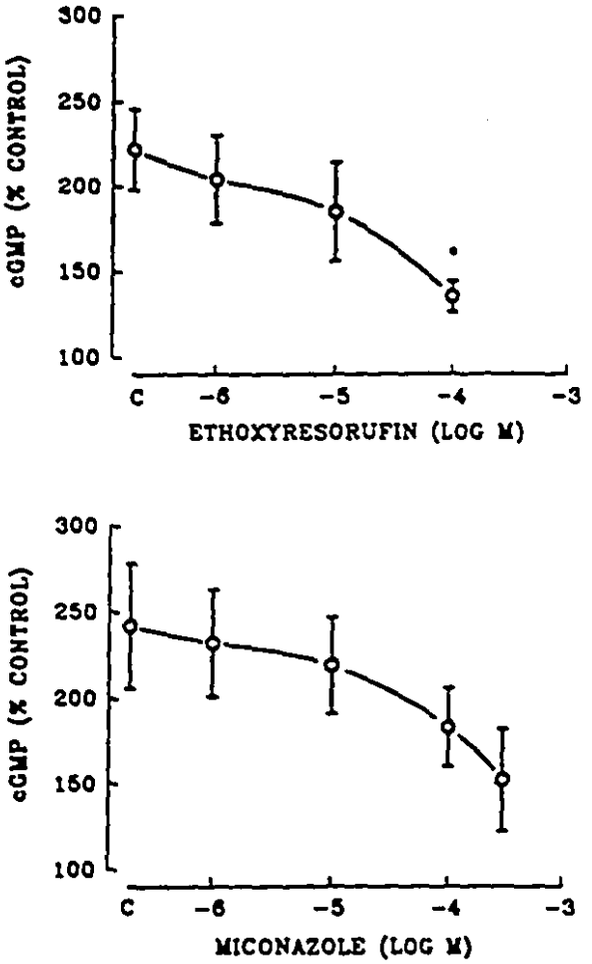

Figure 6. P450 inhibitors inhibited formamidoxime induced cyclic GMP accumulation in cultured tracheal smooth muscle cells. The experiments were done in the same manner as described in the legend of Figure 3. C: Control (vehicle) for ethoxyresorufin or miconazole at the presence of formamidoxime (10^{-2}). Data are expressed as mean ± SE from 3 experiments in quadruplicate. * $p<0.05$

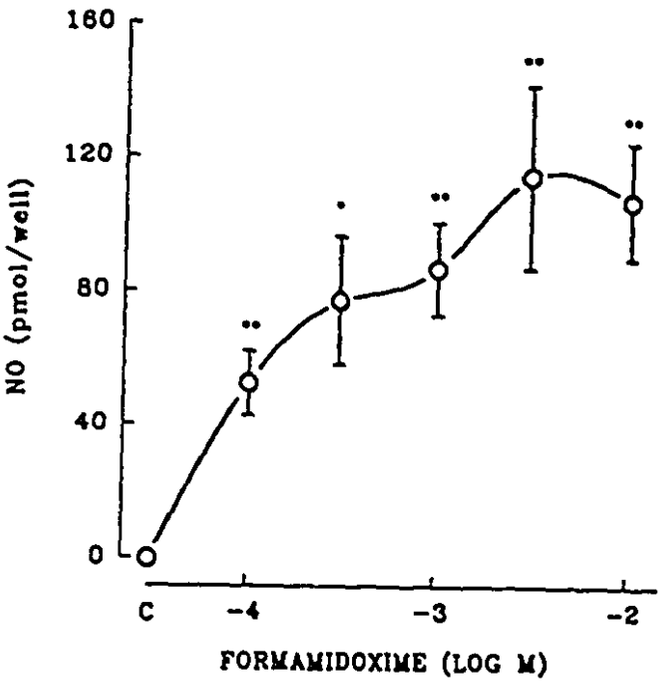

Figure 7. Formamidoxime induced NO production in cultured tracheal smooth muscle cells. Cultured tracheal smooth muscle cells were incubated with formamidoxime in Hanks' buffer for 10 min at 37° C. NO in the Hanks' buffer was measured by NO chemiluminescence analyzer. Data are expressed as mean ± SE from 3 experiments in quadruplicate. * $p<0.05$; ** $p<0.01$

(Janssen Biotech N.V., Cedarlane Laboratories Limited, Hornby, ONT), another P450 inhibitor, had a similar inhibitory effect on formamidoxime induced cyclic GMP accumulation in tracheal smooth muscle cells in culture (Figure 6).

Nitric oxide production from formamidoxime. A concentration-dependent production of NO was detected in the culture medium when tracheal smooth muscle cells were incubated with increasing concentrations of formamidoxime (10^{-4} to 10^{-2} M, Figure 7). The maximal NO produced at 3 mM formamidoxime was about 110 pmol/well. The same concentration of formamidoxime in Hanks' buffer without cells did not produce NO. At higher concentrations NO was detectable in medium in the absence of cells.

4.5 DISCUSSION

We have shown that formamidoxime was able to induce tracheal ring relaxation and accumulation of cyclic GMP in cultured tracheal smooth muscle cells, while L-arginine had no effect on intracellular cyclic GMP levels in similar cells. Formamidoxime also produced measurable levels of NO in the culture medium of tracheal smooth muscle cells. The effects of formamidoxime were inhibited by P450 inhibitors but not by NOS inhibitors, strongly suggesting that a P450 catalyzed pathway for the production of NO was present in tracheal smooth muscle cells.

NO is known to relax airway smooth muscle by activating guanylate cyclase and increasing intracellular cyclic GMP. Formamidoxime is a convenient commercially available compound containing a -C (NH_2)=NOH function, the oxidative cleavage of C=N bond of which is able to produce NO. In this study, we found that formamidoxime induced relaxation of carbachol contracted tracheal rings. To investigate whether this relaxation was potentially caused by the production of NO, we measured the intracellular cyclic GMP and cyclic AMP levels in cultured tracheal smooth muscle cells after exposure to formamidoxime. Formamidoxime stimulated cyclic GMP but not cyclic AMP accumulation in cultured tracheal smooth muscle cells, which is consistent with the production of NO. To further investigate the link between the formamidoxime induced-relaxation and cyclic GMP, we also evaluated the effect of LY83583, an agent that decreases intracellular cyclic GMP (18,25), on both formamidoxime-induced airway relaxation and cyclic GMP accumulation in cultured tracheal smooth muscle cells.

LY83583 inhibited both formamidoxime induced cyclic GMP accumulation in the tracheal smooth muscle cells in culture and the relaxation of isolated tracheal rings. These findings confirm that formamidoxime induced relaxation is induced through a cyclic GMP dependent mechanism, presumably stimulated by NO. Furthermore, we measured NO production by a chemiluminescence assay which confirmed the production of NO by cultured tracheal smooth muscle cells from formamidoxime. This NO production was a cell dependent process because 3 mM formamidoxime in the absence of cells did not produce NO. This concentration of formamidoxime was sufficient to cause the maximal NO production in cultured tracheal smooth muscle cells. These results provide direct evidence that NO is produced from formamidoxime and that it is likely responsible for the observed relaxation of tracheal smooth muscle.

NOS has been found in many cell types in the lung. However, so far no direct evidence of the constitutive form of NOS has been found in airway smooth muscle cells. It is believed that NO is produced in the adjacent cells such as epithelium (20) and/or neurons (3,15,17,18) and diffuses to airway smooth muscle cells where it may regulate smooth muscle tone. Interestingly, we found in the present study that formamidoxime stimulated NO production and cyclic GMP accumulation in cultured tracheal smooth muscle cells, implying that the site of production of NO from formamidoxime was within the airway smooth muscle cells. However, the lack of effect of NOS inhibitors on cyclic GMP levels after formamidoxime indicates that NO production from formamidoxime is catalyzed by another enzymatic pathway.

Both NOS and P450 (1) are able to catalyze the oxidative cleavage of C=N bonds of -C(NO_2)=NOH and produce NO. The endogenous compound containing -C(NH_2)=NOH, namely NOHA, is produced by N-oxidation of L-arginine catalyzed by NOS. Normally NOS is the only enzyme, for which L-arginine is the substrate, that is capable of producing NO *in vivo*. However, in the absence of NOS, formamidoxime is a compound which may be catalyzed by P450 to produce NO. To confirm that P450 but not NOS was involved in catalyzing the NO production from formamidoxime, we evaluated the effect of inhibitors of NOS and P450 on formamidoxime induced tracheal ring relaxation and cyclic GMP accumulation in cultured smooth muscle cells. The P450 inhibitor, ethoxyresorufin, inhibited formamidoxime induced cyclic GMP accumulation in airway smooth muscle cells as well as tracheal ring relaxation, while the NOS inhibitors L-NNA and L-NMMA had no effect on formamidoxime-induced cyclic GMP accumulation in cultured tracheal smooth muscle cells. This observation provides strong evidence that NO production from formamidoxime was catalyzed by P450. Although, some P450 inhibitors such as ethoxyresorufin may also inhibit NOS activity (5), a nonspecific inhibitory effect of the P450 inhibitor on NOS seems unlikely in the present study in view of the lack of other evidence of constitutive NOS activity and the lack of the effect of L-arginine on cyclic GMP levels in tracheal smooth muscle. We believe that these results provide evidence that NO production from formamidoxime is catalyzed by P450, and that the findings are consistent with the previous observations *in vitro* by Andronik-Lion and colleagues (1) that liver microsomal cytochrome P450 is able to

catalyze the cleavage of the C=N bonds of compounds containing a -C(NH_2)=NOH function and produce NO.

It is believed that the P450 3A subfamily is involved in catalyzing the production of NO from NOHA *in vitro* (24). We did not examine the subfamily of P450 in the current study. If formamidoxime produces NO from the same functional structure as NOHA, we predict that the same subfamily of P450, that is P450 3A, is involved in catalyzing the production of NO from formamidoxime. Some isoenzymes of cytochrome P450 such as P450 1A1, 2A3, 2B1, 4B1 (13,16,23,27) and 2E1 (7) have been identified in the lung. The presence of P450 3A subfamily in the lung does not appear to have been described as yet.

In summary, tracheal smooth muscle cells from Lewis rats may produce NO by a P450 pathway independent of NOS if formamidoxime, a compound containing a -C(NH_2)=NOH function is provided. The pharmacological significance and applicability of this finding deserves to be further investigated.

4.6 ACKNOWLEDGEMENTS

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Chapter 5

General Discussion and Conclusions

The aim of my studies was to investigate the contribution of the NO-cyclic GMP pathway in the regulation of airway responsiveness. The first part comprises chapters 2 and 3, in which the NO-cyclic GMP pathway is compared between hyperresponsive Fisher rats and the Lewis and ACI rats of lower responsiveness. Inhibition of NOS increased airway responsiveness significantly in Lewis but to a lesser extent in Fisher rats. Fisher rats also exhibited a resistance to SNP, an exogenous source of NO, and 8-bromo-cyclic GMP, a cell permeable analogue of cyclic GMP which mediates the effects of NO. The second part is contained in chapter 4, in which NO production was measured in cultured tracheal smooth muscle cells in the presence of formamidoxime, a compound containing a $-C(NH_2)=NOH$ function. The production of NO was inhibited by P450 inhibitors but not NOS inhibitors.

Strain-related differences in airway responsiveness have been reported in highly inbred rats. The underlying mechanisms are poorly understood. The possible explanations may include the difference in the amount of smooth muscle in the airways, altered epithelial function, the mechanical interactions between the airways and surrounding tissues and neural control. Since endogenous airway relaxant mechanisms, such as the cyclic AMP pathway, are involved in the regulation of airway tone (23), one of the possible mechanisms may be the imbalance between bronchoconstrictors and dilators. An impaired ß-adrenergic agonist-cyclic AMP pathway has been previously described in association with airway hyperresponsiveness and asthma (4,5,7,12,18). The current studies showed that inhibition of NO production by L-NNA increased airway

responsiveness in Lewis but to a lesser extent in Fisher rats, indicating the role of endogenous NO in the regulation of airway responsiveness and in strain-related airway hyperresponsiveness in rats. Consistent with this finding, carbachol increased cyclic GMP levels to a lesser extent in Fisher than in Lewis tracheal tissues. These observations suggested that the NO-dependent inhibitory pathway was deficient in the hyperresponsive Fisher strain.

The impaired NO-dependent inhibitory pathway in airways from Fisher rats may have resulted from a decreased response to NO. To further test whether a decreased relaxant response to NO was present in Fisher rats, the effect of SNP, which releases NO, was compared between Fisher and Lewis or ACI strains. The Fisher rat trachea was less sensitive to the relaxant effects of SNP than either Lewis or ACI tissues. The changes in cyclic GMP in SNP-exposed cultured myocytes are consistent with these observations, in that levels of cyclic GMP were less in the Fisher cells for any concentration of SNP. Although we have not established the exact mechanism for the decreased production of cyclic GMP in response to SNP in Fisher rats, nitrite measurements exclude the possibility that SNP might have released less NO in Fisher rat tracheal smooth muscle cells. It is more likely that guanylate cyclase activity is lower in the smooth muscles of Fisher rats, or that the sensitivity of the enzyme to NO itself is different between strains. Lower levels of cyclic GMP do not appear to be the sole explanation for differences in tracheal responses to SNP, since in Fisher rats the tracheal rings also relaxed relatively less to any given concentration of the cell permeant cyclic GMP analog, 8-bromo-cyclic GMP. This finding indicates that a decreased sensitivity of

the contractile process to cyclic GMP may also contribute in part to the differences in tracheal responses to SNP in Fisher rats. These observations that Fisher rats exhibited a decreased response to a NO donor and to the cyclic GMP analog provide an explanation for the impaired endogenous NO-cyclic GMP-inhibitory system in Fisher airways.

Endogenous NO is synthesised from L-arginine catalyzed by NOS. Two distinct types of NOS have been purified: a constitutive Ca^{2+}-calmodulin-dependent enzyme and an inducible Ca^{2+}-independent enzyme. Inducible NOS is unlikely to account for the current observations since either airway responsiveness or cyclic GMP accumulation was determined within 15 minutes after cholinergic stimulation, whereas it takes at least several hours to induce enzyme production. Constitutive NOS, which can be activated by increases in intracellular Ca^{2+}, seems more likely to account for the production of NO involved in the modulation of airway responsiveness.

There are several possible cell sources of NO in the airway including vascular smooth muscle cells, endothelial cells, neurons and airway epithelial cells (15). Airway epithelium has been known for some time to have an important influence on airway responsiveness to bronchoconstrictors (6,19,21). More recently, immunohistochemical studies have demonstrated the presence of both constitutive and inducible NOS in airway epithelial cells (3,9), adding NO to the candidate molecules involved in the observed modulatory effects of epithelium. Since some G-protein coupled receptors such as muscarinic receptors (16,17) and histamine receptors (11) are also present in airway epithelium and the activation of these receptors can increase intracellular Ca^{2+} level in

airway epithelial cells (11), it is possible that the endogenous NO which is involved in the regulation of airway responsiveness is produced in airway epithelial cells. The current observations that removal of epithelial cells increased tracheal contractions *in vitro* in a manner similar to NOS inhibition, and abolished the NO-dependent down-regulation of tracheal contractions supported this possibility. Presumably, cholinergic agonists activated constitutive Ca^{2+}-dependent NOS in airway epithelial cells and produced NO, which diffused into adjacent smooth muscle cells. Smooth muscle contraction was in turn inhibited by activation of guanylate cyclase and the consequent increase in cyclic GMP levels. Since NOS inhibition did not further increase carbachol-induced contractions in epithelium denuded tracheal rings, epithelium appears to be the only significant source of NO involved in the regulation of airway contractions in response to carbachol.

Endogenously produced NO is related to asthma by the finding that airway epithelial NOS is increased in bronchial biopsies from asthmatics relative to those from normal subjects (10). As well, the expired NO concentrations were higher in asthmatics than normal subjects (1,8,14,20). Theoretically, increased NO production may play both a pathological role in asthma, such as epithelium injury, or a beneficial one to induce airway relaxation. If a decreased relaxant response to NO is also present in asthmatic airways as in hyperresponsive Fisher rats, the increased NO in asthmatic airways may predominantly play a pathological role. if so inhaled NO may not turn out to be a useful treatment for asthma. Indeed, inhaled NO (80ppm) exerts only a weak bronchodilatory effect in bronchial asthma; volume-corrected specific airway conductance increased from 0.4 ± 0.1 to 0.6 ± 0.2 $(kPa.s)^{-1}$ by inhaled NO in patients with bronchial asthma (13). On

the contrary, NOS inhibition might benefit asthmatic subjects. However, further studies are required to elucidate the role of NO in asthmatic airways.

It has been well established that endogenous NO is synthesized from L-arginine catalyzed by NO synthase (NOS). NOS catalyzes the formation of NO from L-arginine in two steps. Firstly, it catalyzes the N-oxygenation of L-arginine to form N^{ω}-hydroxy-arginine (NOHA), which contains a $-C(NH_2)=NOH$ function. Secondly, the oxidative cleavage of the C=N bonds of NOHA produces NO and L-citrulline (22). Liver cytochrome P450s have also been found to catalyze the oxidative cleavage of C=N bonds of compounds containing a $-C(NH_2)=NOH$ function, producing the corresponding derivatives and NO *in vitro* (2). Thus, a compound containing a $-C(NH_2)=NOH$ function, such as formamidoxime, has the potential to produce NO catalyzed by both NOS and P450. NOS has been located in several cell types in the airway. However, so far no direct evidence of the constitutive form of NOS has been found in airway smooth muscle cells. We found in the present study that formamidoxime induced relaxation of isolated tracheal rings precontracted with carbachol and stimulated cyclic GMP accumulation NO production and in cultured tracheal smooth muscle cells, implying that NO can be generated from formamidoxime and the site of production of NO from formamidoxime was within the airway smooth muscle cells. The P450 inhibitor, ethoxyresorufin, inhibited formamidoxime induced cyclic GMP accumulation in airway smooth muscle cells as well as tracheal ring relaxation, while the NOS inhibitors L-NNA and L-NMMA had no effect on formamidoxime-induced cyclic GMP accumulation in cultured tracheal smooth muscle cells. This observation provides strong evidence that NO

production from formamidoxime was catalyzed by P450.

In conclusion, a NO-dependent inhibitory pathway is involved in the regulation of airway responsiveness in rats and contributes to strain related difference in airway responsiveness as indicated by a deficient NO-cyclic GMP-relaxation pathway in hyperresponsive Fisher rats. A P450-catalyzed NO production pathway is also observed in airway smooth muscle cells in rats.

The following are the contributions of this work to original knowledge:

1. Evidence that endogenous NO is involved in the regulation of airway responsiveness to cholinergic stimulation *in vivo* and *in vitro* in rats.

2. The endogenous NO-dependent inhibitory system is deficient in hyperresponsive Fisher rats as evidenced by both a lesser NOS-dependent cyclic GMP accumulation in response to carbachol and a lesser effect of NOS inhibitor on airway responsiveness in Fisher rats, and thus contributes to strain related differences in airway responsiveness in rats.

3. Isolated tracheal rings from Fisher rats are hyperresponsive to carbachol relative to Lewis and ACI rats in vitro, confirming an association between *in vivo* and *in vitro* differences in airway responsiveness among these strains.

4. The mechanism of a deficient NO-dependent inhibitory system in Fisher rats includes a decreased guanylate cyclase activity as indicated by a lesser cyclic GMP accumulation in response to SNP in cultured tracheal smooth muscle cells, as well as a decreased relaxation in response to a given concentration of cyclic GMP.

5. P450-dependent NO production was proved inside the cells in this study, while

previous studies only provided evidence of such a pathway in the test tube.

6. Airway smooth muscle can synthesize NO if an appropriate substrate is provided.

7. Formamidoxime, a compound containing the -$C(NH_2)NOH$ function, is a substrate for NO production.

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