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**STRAIN DEPENDENCE OF THE AIRWAY RESPONSE TO DRY
GAS HYPERPNEA CHALLENGE IN THE RAT**

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Abstract

The aim of the study was to investigate the mechanisms of bronchoconstriction induced by dry gas hyperpnea challenge in the rat. The interaction of airway hyperresponsiveness to contractile agonists and the atopic constitution of the animals with responsiveness to hyperpnea challenge was also evaluated. A hyperresponsive strain, Fisher 344, two control normoresponsive strains, Lewis and ACI, and an atopic but normoresponsive rat, Brown Norway (BN) to dry gas hyperpnea challenge were studied. The effects of the NK1 (CP-99994), the NK2 (SR-48968) receptor antagonists, and the selective LTD4 receptor antagonist pranlukast on responses to hyperpnea challenge were examined in BN rats. The animals were anesthetized with xylazine and pentobarbital sodium intraperitoneally and mechanically ventilated with a tidal volume of 8 ml/kg and a frequency of 150 breaths/min for hyperpnea challenge. Pulmonary responses to challenge were measured in spontaneously breathing animals. Dry gas hyperpnea challenge was performed with a dry mixture of 5% CO₂-95% O₂ for 5 min whereas wet gas was humidified prior to administration. Bronchovascular leakage was assessed by quantitating Evans blue in bronchoalveolar lavage (BAL) fluid. Pulmonary resistance (R_L) was significantly increased following dry gas hyperpnea challenge in BN and ACI but not in Fisher and Lewis rats. Hyperpnea with wet gas did not evoke changes in R_L . Evans blue dye extravasation in BAL fluid was significantly increased in all dry gas challenged animals and was not observed in three of the strains (BN, Lewis and F344) after wet gas challenge. CP-99994, SR-48968 and pranlukast significantly attenuated the increase in R_L in dry gas challenged BN rats. Evans blue in BAL fluid was significantly reduced in SR-48968 and pranlukast, but not in CP-

99994 pretreated BN rats following dry gas challenge. BN rats were more responsive to inhalational challenge with exogenous LTD4 than F344 and ACI strains. In conclusion, neurokinins and LTD4 mediate airway responses to dry gas hyperpnea in rats. The dissociation between R_L and airway microvascular leakage suggests that the latter does not contribute to airway narrowing induced by dry gas hyperpnea challenge.

Résumé

Le but de cette étude a été d'investiguer les mécanismes de la bronchoconstriction induite par l'hyperpnée de gaz sec chez le rat. L'interaction entre l'hyperréactivité bronchique aux agonistes contractiles et le statut atopique des animaux ayant une réponse bronchique positive au test de l'hyperpnée a également été évaluée. Quatre lignées de rats ont été étudiées: le Fisher 344, une lignée d'animaux avec hyperréactivité bronchique, le Brown Norway (BN), une lignée d'animaux atopiques avec réactivité bronchique normale et deux lignées avec une réactivité bronchique normale, les Lewis et les ACI. Nous avons étudié les effets de deux antagonistes des récepteurs NK1 et NK2, le CP-99994 et le SR-48968 respectivement, ainsi que les effets du pranlukast, un antagoniste sélectif du récepteur LTD4, sur la réponse à l'hyperpnée des rats BN. L'hyperpnée était produite en ventilant mécaniquement les animaux - anesthésiés par injection intraperitonéale de xylazine et de sodium pentobarbital - avec un volume courant de 8 ml/kg à une fréquence de 150 cycles/min pendant 5 minutes. La réponse pulmonaire était mesurée avant et après l'exposition à l'hyperpnée chez les animaux respirant spontanément. Le gaz respiré durant l'hyperpnée était un mélange gazeux contenant 5% CO₂ -95% O₂ administré sec, ou humidifié avant son administration. Les fuites vasculaires de la circulation bronchique ont été évaluées par quantification du Bleu d'Evans dans les lavages bronchoalvéolaires (LBA). Suivant la période d'hyperpnée au gaz sec, on a pu observer une augmentation de la résistance pulmonaire (R_L) chez les rats BN et ACI mais pas chez les rats Fisher et Lewis. L'hyperpnée au gaz humidifié n'a pas provoqué de changement de R_L . L'hyperpnée au gaz sec a provoqué une augmentation de l'extravasation de Bleu d'Evans dans le LBA de tous

les animaux exposés, alors que l'hyperpnée au gaz humide a produit une augmentation de la perméabilité vasculaire chez les rats ACI mais pas chez les animaux des trois autres lignées (BN, Lewis et F344). Le traitement des rats BN par le CP-99994, le SR-48968 ou le pranlukast a diminué de façon significative l'augmentation de la R_L induite par l'hyperpnée d'air sec. La concentration de Bleu d'Evans était diminuée de façon significative par le traitement au SR-48968 et au pranlukast mais pas par celui au CP-99994. Les rats BN ont eu une réponse bronchique plus marquée à l'instillation intratrachéale de LTD4 que les animaux des lignées F344 et ACI. En conclusion, ces résultats montrent que les neurokinines et la LTD4 sont impliquées dans la réponse bronchique induite par l'hyperpnée de gaz sec chez les rats. La dissociation entre la R_L et la perméabilité vasculaire suggère que cette dernière ne contribue pas à la bronchoconstriction induite par l'hyperpnée de gaz sec.

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Abbreviations

AHR	Airway hyperresponsiveness
FEV1	Forced expiratory volume in one second
PAF	Platelet activating factor
cys-LTs	Cysteinyl-leukotrienes
FLAP	5-lipoxygenase activation protein
Ig	Immunoglobulin
FcεRI	High affinity IgE receptor
FcεRII/CD23	Low affinity IgE receptor
BAL	Bronchoalveolar lavage
IL	Interleukin
TX	Thromboxane
GM-CSF	Granulocyte-macrophage colony-stimulating factor
TNF	Tumour necrosis factor
MBP	Major basic protein
ECP	Eosinophil cationic protein
NANC	Noncholinergic and nonadrenergic
NO	Nitric oxide
VIP	Vasoactive intestinal protein
NK	Neurokinin
NEP	Neutral endopeptidase
EIA	Exercise-induced asthma
HIB	Hyperpnea-induced bronchoconstriction

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CHAPTER 1

INTRODUCTION

1.1 Asthma

1.1.1 Definition of asthma

Asthma has been recognised as a disease for more than 1000 years. However, the term has been frequently used in the past in a general sense to refer to almost any sort of difficulty in breathing, especially if it was episodic in nature. Even if in modern times the definition of asthma has created controversy, the diagnosis of asthma is accepted in patients who complain of feeling abnormally breathless with episodes of wheezing and objective evidence of reversible airflow limitation. Asthma can be provoked by a variety of stimuli, both nonspecific and specific, which induce abnormal increases in airflow resistance and which usually do not result in effects of similar magnitude in normal subjects (163). This latter characteristic is referred to as hyperresponsiveness of the airways. More specific or immunological reactions to inhaled substances in concentrations having no effect in normal individuals can be demonstrated in some subjects.

In the past, definitions of asthma have included immunological hypersensitivity as a necessary feature. However, it is not a uniform feature for all of the patients who are labelled as asthmatic (92). When tests for bronchial hyperresponsiveness or hyperreactivity first received attention and were well standardized procedures, the association of bronchial hyperresponsiveness and asthma prompted the introduction of "nonspecific bronchial hyperreactivity" into the definition of asthma. The current definition of asthma proposed by the American Thoracic Society (ATS) not only includes airways hyperresponsiveness and intermittent airways obstruction as defining characteristics of asthma but also refers to the inflammatory nature of the disease (8). The expansion of the definition in this way may have

been motivated by the desire to emphasize the distinction between the rationale for the two sorts of therapeutic agents-pharmacological bronchodilators and agents such as corticosteroids and cromolyn sodium whose action may be regarded as anti-inflammatory.

1.1.2 The prevalence and incidence of asthma:

Asthma affects about 10 % of the population and its prevalence appears to rising (20). The increase in prevalence and severity and the rising mortality is occurring despite a substantial increase in prescribed asthma treatment. Asthma affects 9 to 12 million people in the United States according to the U.S. National Centre for Health Statistics. The overall disease prevalence is 3 percent, with slightly higher rates reported for younger (<6 years) and older (>65 years) males. Most studies that fulfil the criteria for a valid study suggest that asthma prevalence has been increasing in children and young adults since the end of the 1950s at least. This increase has been shown in different countries using different methods including studies of physician-diagnosed asthma based on the airway response to exercise. Data relative to hospital admissions for asthma, and to those of anti-asthma drugs, suggest that either the prevalence and severity of asthma, or both, have increased (34,114,147). An increase in the prevalence of severe asthma is therefore more likely.

1.2 Airway Hyperresponsiveness (AHR)

1.2.1 Definition of AHR

Hyperresponsiveness of the airway to physical and chemical stimuli is a consistent feature of asthma. Although airway hyperresponsiveness being synonymous with asthma can

be questioned, that airway hyperresponsiveness is very closely related to asthma is agreed unanimously. Attenuation of the increased resistance is usually demonstrable in response to bronchodilator drugs or to corticosteroids (63).

Non-specific airway responsiveness is defined as the degree to which the airways constrict to non-allergic stimuli. AHR is an increase in airway responsiveness above the normal to the varied range of physical and chemical stimuli. For example, when measured with histamine or methacholine, there are very distinct behaviours of the dose response curves between subjects who are normal and those with airway hyperresponsiveness. In the case of airway hyperresponsiveness, there is an increase in ease of airway constriction with any magnitude of stimulus or left shift of the dose-response curve and an increase and eventual loss of the maximal response (163).

Many non-specific stimuli can be used to provoke airway responsiveness such as cholinergic agonists (methacholine, acetylcholine), histamine, beta-adrenergic antagonists, as well as physical stimuli such as exercise, cold air hyperventilation and nonisotonic aerosols. Histamine and methacholine act on their specific receptors on the airway smooth muscle and cause airway narrowing directly. However, β -agonists, exercise, cold air hyperventilation and nonisotonic aerosols appear to cause airway narrowing through mediator release such as neuropeptides and inflammatory cell derived mediators including serotonin, histamine, leukotrienes and therefore, be considered as belonging to a category of indirect stimuli. Although direct and indirect acting stimuli involve different mechanisms there is correlation in responsiveness determined by the various stimuli and in particular for histamine, methacholine, cold-air hyperventilation (133), and probably for arachidonic acid

metabolites (126). Since bronchoconstriction under usual conditions in asthmatic subjects is likely to involve indirect mechanisms, it has been hypothesized that bronchoconstrictive responses to indirect stimuli may correlate better with other measures of the severity of naturally occurring asthma.

1.2.2 Possible mechanisms of AHR

1.2.2.1 Mechanical factors

Structural abnormalities caused by the asthmatic airway inflammation are usually considered to cause airway hyperresponsiveness. Reduction in baseline airway caliber has been suggested as a major determinant of hyperresponsiveness of the airways to inhaled chemicals. The resistance to airflow in a tube is inversely proportional to the radius of tube to either the fourth power for laminar flow or the fifth power for turbulent flow. A small reduction in radius will have a magnified effect on the resistance in a small tube when compared with a large tube. Since a 20 percent reduction in forced expiratory volume in 1 sec (FEV₁) in the presence of a pre-existing reduction in airway caliber will require relatively less airway narrowing, the airway may appear to be more sensitive. However, current data suggest that reduced baseline airway caliber is not the principle determinant of airway hyperresponsiveness in asthma (30,140,141,165), although it may be an important feature in other lung diseases such as chronic obstructive diseases.

1.2.2.2 Humoral mediators

The chemotaxis of inflammatory cells and their subsequent activation within the airways of asthmatics may cause the release of mediators that alter tissue responses or damage the airways, resulting in airway hyperresponsiveness. Many mediators have been considered as potential causes of airway hyperresponsiveness and airway inflammation. Examples of such mediators that can be released from activated inflammatory cells are histamine, lipid-derived mediators including the prostaglandins, thromboxanes, leukotrienes (89) and platelet-activating factor (PAF) (88) , as well as bronchoactive neuropeptides such as tachykinins that may be released from afferent nerve endings (96,119).

Among the lipid derived mediators, cysteinyl-leukotrienes (cys-LTs) appear to be the most important in patients with active asthma. Cys-LTs are lipid mediators formed from the action of 5-lipoxygenase on arachidonic acid. Arachidonic acid is a normal component of many cell membrane phospholipids. In the presence of appropriately activated phospholipase A₂, such as occurs after activation of mast cells and eosinophils, free arachidonic acid is cleaved from the cell membrane phospholipid and undergoes oxidation by the cyclooxygenase pathway to form prostaglandins and thromboxane. The arachidonic acid interacts with a specific 5-lipoxygenase (5-LO) activation protein (FLAP), which translocates 5-LO to the nuclear membrane facilitating the catalysis of the oxygenation of arachidonic acid to form cysteinyl-leukotrienes (40).

Cys-LTs have potent proinflammatory and spasmogenic properties. The biological actions of cys-LTs include the induction of airway obstruction, constriction of arterioles, hypotension, mucus secretion and plasma extravasation. Preexisting levels of airway

hyperresponsiveness appears to be increased slightly by leukotrien (LT) C₄ (123), and LT antagonists have been reported to improve airway function and to block substantially attacks of asthma provoked by exercise and cold air (47,71). LTs are also involved in inflammatory and anaphylactic reactions and more recently, they have also been shown to play an important role in hyperpnea-induced by bronchoconstriction (HIB) in human and in guinea pig.

1.2.2.3 Airway inflammation

Airway inflammation is perhaps the most frequently mentioned phenomenon suggested to account for airway hyperresponsiveness. Even in the mildest asthmatics with airway hyperresponsiveness, inflammatory cells such as eosinophils, metachromatic cells, and lymphocytes can be seen in bronchoalveolar lavage (BAL) fluid and bronchial biopsy specimen (121,137). Transient airway hyperresponsiveness and airway inflammation occur in humans following exposure to allergen (62,111), low-molecule-weight chemical sensitizers (83,104) noxious gases such as ozone (84) and viral respiratory tract infections (42). Animal models of allergen induced airway hyperresponsiveness (116) and ozone-induced hyperresponsiveness (122) have convincingly demonstrated the requirement for cells of the polymorphonuclear leukocyte series both for the appearance of late asthmatic responses and transient increases in airway responsiveness. Studies in both human asthmatics subjects and animal models show convincingly that several inflammatory cells interact in a complex manner and release multiple inflammatory mediators that act on various target cells of the airways to produce the characteristic pathophysiology of asthma.

1.3 Inflammatory cells and inflammatory mediators

1.3.1 Mast cells

Mast cells are distributed throughout the human respiratory tract and are found in large numbers in the walls of the alveoli and airways. They are superficially localized in the airway epithelium. Mast cells have long been recognized as the central effector cells of allergic reactions because of their stores of various preformed mediators, their capacity to synthesize additional mediators and their abundance of high-affinity immunoglobulin E (IgE) receptors. IgE-dependent release mechanisms are initiated by the interaction of a specific allergen with two or more divalent IgE molecules bound firmly to specific receptors on mast cells, resulting in a cascade of cellular events leading to exocytosis and secretion of preformed mediators such as histamine and heparin, and the generation of non-preformed mediators, such as prostaglandin(PG) D₂ and cys-LTs (89). The properties of these mast cell mediators are such that they could account for all of the manifestations of immediate allergic responses, including those elicited in the skin and airways.

More recently, it has been demonstrated that mast cells can be activated by a variety of stimuli pertinent to asthma that do not involve immune globulins. Substance P has been reported to cause histamine release from human pulmonary mast cells in vitro (62). Exogenous substance P and neurokinin A (NKA) have been shown to cause histamine and serotonin release from mast cell in Fisher 344 rats in vivo (76).

1.3.2 Macrophages

Macrophages are the most prevalent cells in the airways. They are present in the mucosa and submucosa throughout the respiratory tract from the alveolus to the larynx and are found both in the airway lumen and the airway walls. Macrophages are the major predominant cells of the BAL fluid (over 90%) and are derived from blood monocytes. There is a marked increase in the numbers of macrophages in BAL fluid after allergen challenge in asthmatic subjects and increased numbers of macrophages in biopsies from asthmatics.

Many stimuli can activate the airway macrophages including phagocytosis and the interaction of cell surface-bound IgE with relevant antigen. In contrast to high-affinity IgE receptors present on mast cells and basophils (FcεRI), low-affinity IgE receptors (FcεRII/CD23) are found on B-lymphocytes, macrophages (157), eosinophils, platelets, T-lymphocytes and Langerhans' cells. The functions of this receptor are the mediation of IgE-dependent cytotoxicity by macrophage, eosinophil, and platelet and IgE-dependent allergic/inflammatory responses. FcεRII/CD23 expression is enhanced on macrophages and platelets of asthmatics (162), and cromoglycate directly inhibits IgE-related activation of macrophages, platelets and eosinophils suggesting the involvement of other cell types, in addition to mast cells and basophils, in IgE-related allergic reactions.

After allergen exposure alveolar macrophages may release a variety of inflammatory mediators, including leukotrien (LT) B₄, PGF₂α, LTC₄, thromboxane (TX) B₂, and platelet-activating factor (PAF). Macrophages also release a large variety of cytokines, such as interleukin (IL)-1, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor (GM-

CSF), histamine-releasing factors, and tumor necrosis factor-alpha (TNF- α) (26,61). These cytokines and mediators have demonstrated their potential to play a very important role in inflammation and the communication between different inflammatory cells and could account for the pathogenesis of asthma.

1.3.3 Eosinophils

Eosinophilic infiltration is a characteristic feature of asthmatic airways and differentiates asthma from other inflammatory conditions of the airway. A close relationship between eosinophil counts in peripheral blood or bronchial lavage and airway hyperresponsiveness have been reported (21). There is a marked increase in eosinophils in all layers of the airway wall and in BAL fluid at the time of the late response following allergen challenge (35). Eosinophils may be activated in several ways including interaction with IL-5, their own cationic proteins, PAF and the interaction of cell surface-bound IgE with relevant antigen via Fc ϵ RII/CD23.

Eosinophil granule products such as major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin cause marked epithelial shedding (161). This may account for an increase in airway responsiveness by inhibition of the release of putative epithelial-derived relaxant factors. Newly synthesized lipid-derived mediators from the eosinophil are generated from arachidonic acid and include the prostaglandins, thromboxanes, leukotrienes and PAF. Most of these mediators are potent vasodilators and bronchoconstrictors and may thus cause microvascular leak, contraction of airway smooth muscle and secretion from bronchial glands. Eosinophils are not only a source of cytotoxic

and proinflammatory mediators but they can also generate cytokines and growth factors, including their own factors of differentiation, such as IL-5, IFN- γ as well as IL-10 and IL-4 (85).

1.3.4 Lymphocytes

Lymphocytes are present in the normal human lung in paratracheal and hilar lymph nodes, in the interstitial tissues of the bronchial tree in the form of nodules, aggregates and individual cells, in the alveolar walls in small groups or individually, and on the epithelial surface from the trachea to the alveoli. T-lymphocytes dominate, with at least 10-fold fewer B cells. Two surface proteins, CD4 and CD8, define the two naturally exclusive T-cell populations found throughout the body. CD4⁺ cells (helper/inducer) predominate over CD8⁺ cells (suppressor) in bronchial biopsies from mild asthmatics. CD4⁺ cells are further divided into Th1 and Th2 subtype cells based on their pattern of cytokines secretion. Th1-type cells produce predominantly IL-2 and IFN- γ , whereas Th2-type cells produce IL-5, IL-4, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Th2-type cells are proved to be involved in the development of allergic inflammation (159). IL-4 plays a critical role in the switching of B-cells to IgE production. IL-5 (together with IL-3, GM-CSF) is important in the maturation, maintenance, differentiation and recruitment of eosinophils in the airway tissue. IL-5 and substance P incubation together have been shown to induce the airway hyperresponsiveness in guinea pigs (156). A monoclonal antibody to IL-5 has been shown to inhibit airway hyperresponsiveness in sensitized monkeys and guinea pigs (3,108). The CD8⁺ cell functions include killing the cells expressing foreign antigens in conjunction

with MHC class I molecules (cells infected with viruses and or intracellular parasites), suppressing proliferative CD4+ cell responses and B cell immunoglobulin synthesis and release (64,138).

B-lymphocytes synthesize immunoglobulins (Ig), the soluble receptors for antigens. There is not much data about B-cells or immunoglobulin synthesis locally within the lung in human. The data from animals has demonstrated that specific immunoglobulin production in response to aerosolized antigens is compartmentalized to the lower respiratory tract, suggesting B cells home to the alveolar spaces where they are activated by antigens and mount a local immune response.

1.4 Airway microvascular leak

Microvascular leak is an important feature of airway inflammation. There is evidence for plasma exudation into the airway wall and lumen after exposure of the airway mucosa to inflammatory agents. The airway epithelial shedding and many bronchoconstrictive mediators of asthma, such as bioamines, peptides, and lipid products have the capacity to increase microvascular permeability and induce plasma exudation. Endothelial cells separate in exposure to inflammatory mediators and produce large gaps in the wall of postcapillary venule (67). The airway epithelium which is tightly sealed at the apical surface have been shown to open and close by direct action of proinflammatory agents such as allergen, histamine and leukotriene (130). The plasma exudate moves between the epithelial cells in the area of challenge and all around each cell in vitro (44). The extravasated plasma carries adhesive and leukocyte-activating proteins (such as fibronectin and fibrinogen), proteases,

immunoglobulins, cytokines and biologically active peptides (kinin system and complement system). These molecules present in the inflamed airway mucosa may have diverse effects including contributing to airway hyperresponsiveness. However, the stable airway hyperresponsiveness of asthmatics is not associated with hyperpermeability of the airway mucosa (120).

1.5 Abnormalities of airway neural control

The autonomic nervous system controls many aspects of airway function and plays an important role in the regulation of airway caliber in health and disease. The traditional view was that the autonomic nervous system exists as a balance with opposing actions of parasympathetic and sympathetic neurotransmitters on various target organs. This view has had to be modified in the face of evidence that there are other neural effects which are not either adrenergic or cholinergic. These effects have been attributed to other pathways which have been termed peptidergic to acknowledge the contribution of neuropeptides and is often referred to as nonadrenergic and noncholinergic (NANC) system.

1.5.1 Cholinergic airway control

Cholinergic nerves are the dominant neural bronchoconstrictor pathway in animal and human airways (11). In general, the dorsal motor nucleus of the vagus, the nucleus ambiguus, and nucleus dorsomedialis contribute fibres supplying the respiratory tract. Preganglionic fibres course via the left and right vagus nerves and innervate both left and right lung via cross-over in the posterior pulmonary plexus (parasympathetic ganglia) which

are located in the wall of bronchi and bronchioles. From these ganglia relatively short postganglionic fibres pass to target cells, such as smooth muscle, epithelium, and submucosal glands and vascular smooth muscle.

Efferent cholinergic stimulation releases acetylcholine from the granular vesicles in the nerve terminals. Acetylcholine is metabolised by the enzyme acetylcholinesterase. In normal circumstances, the function of the cholinergic efferents is believed to maintain the certain degree of resting bronchomotor tone in many animal species and in humans. This resting vagal tone is abolished by section of the vagus nerve in animals and by pretreatment with atropine in animals and normal human subjects.

Three subtypes of muscarinic receptor are present in the airways of several species, including human, although at least five subtypes of muscarinic receptors have been cloned. M1 receptors are localized in ganglia, on the postsynaptic cells, and their stimulation facilitates neurotransmission mediated by acetylcholine's actions on the nicotinic receptors. M2 receptors are 'autoreceptors' mediating negative feedback effects of acetylcholine on cholinergic nerves. M3 receptors are found on bronchial smooth muscle and glands and mediate contraction of the former and secretion from the latter.

Cholinergic nerve function can be modulated by many neurotransmitters, neuropeptides, and inflammatory mediators. For example, epinephrine and norepinephrine reduce cholinergic nerve contraction of canine tracheal smooth muscle at concentrations that have little effect on responses to exogenous acetylcholine, suggesting an inhibitory effect on acetylcholine release from postganglionic nerve terminals; this is mediated by prejunctional β_1 receptors. In human bronchi cholinergic neurotransmission is modulated by β_2 receptors

(139). Prostaglandin E2 inhibits, whereas serotonin and thromboxane potentiate, cholinergic neurotransmission in dog airways. Tachykinins facilitate cholinergic neurotransmission in guinea pig airways (160).

The hypothesis that cholinergic overactivity causes airway hyperresponsiveness has been vigorously pursued. Inflammation or irritation induced by inflammation of the airways stimulates afferent vagal fibres. The attenuation of induced bronchoconstriction by atropine indicates that reflex vagal pathways can contribute to bronchoconstriction. Anticholinergic drugs are effective bronchodilators in chronic obstructive airway disease and in asthma, confirming a cholinergic component to altered airway tone. However, vagal reflex-mediated bronchoconstriction can not be abolished by anticholinergic agents in guinea pigs (66,117,143), indicating that acetylcholine is not the sole neurotransmitter involved in airway narrowing caused by vagal stimulation at least in this species.

There are several possible mechanisms by which cholinergic responsiveness in the airways may be increased in asthma. There may be an increase in central vagal drive. There could be an increase in airway afferent receptor (irritant and C-fibre) discharge, either because afferent nerve endings below epithelial cells have been exposed by damage or loss of the epithelium and/or because inflammatory mediators (histamine, leukotrienes, prostaglandins, adenosine, bradykinin) are released which activate the cholinergic reflexes in the airways. C-fibres contain neuropeptide including substance P and NKA and are released via an axon reflex. Because substance P can increase acetylcholine release from postganglionic nerves (60), its liberation may cause cholinergically-mediated bronchial obstruction.

Asthmatic patients show an airway hyperresponsiveness to cholinergic agonists. Anticholinergic drugs are effective against those bronchoconstrictive stimuli that are known to activate afferent receptors and attenuate the bronchoconstriction induced by antigen, histamine, and exercise. However, this exaggerated airway response is not specific but is also manifested to other spasmogens, such as histamine, leukotrienes and prostaglandins, suggesting that an isolated enhancement of muscarinic receptor function is unlikely. Neutral endopeptidase, an ectoenzyme that inactivates neurokinins, including substance P and NKA is localized to the airway epithelium. Reduced neutral endopeptidase activity following removal of airway epithelium may heighten airway responses to substance P and thus may facilitate the cholinergic neurotransmission.

Despite the above, anticholinergic agents are significant but less effective bronchodilators than the sympathomimetic agents against antigen, histamine, and exercise induced bronchoconstriction (16,31,32,36,48). The therapeutic usefulness of anticholinergic drugs is limited because they give no protection against the direct effect of locally released mediators in the airways, unlike other relaxant agonists, which relax airway smooth muscle irrespective of the contractile agent used. Therefore, increased cholinergic tone in asthmatic airway does not appear to completely account for the airway hyperresponsiveness.

1.5.2 Sympathetic neural control

Compared to the parasympathetic nerve supply to airways in all species, the extent of sympathetic innervation is more variable and indeed direct innervation of airway smooth muscle by sympathetic fibers is not present in many species. It also is not present in human

respiratory epithelium, but the released norepinephrine can constrict the blood vessels and inhibit secretion by submucosal glands. Human airway smooth muscle is potently relaxed by β -agonists in vitro and the absence of direct functional adrenergic innervation suggests that circulating catecholamines might be important in regulating human airway tone. Exogenous epinephrine has been used as a bronchodilator in the treatment of asthma since the beginning of this century. The protective role of circulating epinephrine against bronchoconstrictive influences has also been revealed by several studies. For example, adrenergic β -receptor blockers potentiate the bronchoconstrictive response to infused histamine and to antigen in sensitized animals and similar results are produced by surgical adrenalectomy (113). Beta adrenoceptor blockade does not however influence airway responsiveness in human subjects or in many animal species.

The major subtype of adrenoceptors in the airways is the β receptor. Autoradiography shows β -adrenoceptors on the smooth muscle, epithelium and glands, and also in very large numbers, in alveoli (23). β_2 receptors are distributed on human airway smooth muscle, those in the alveoli are both β_1 and β_2 . The density of β receptors increases from trachea to bronchioles. Relaxation of airway smooth muscle to exogenous β -agonists including epinephrine is largely a β_2 receptor-mediated effect. In addition to relaxation of airway smooth muscle, β agonists have some other possible effects, such as reducing the release of mediators from mast cells (29), reducing cholinergic tone (139), increasing mucociliary clearance and possibly reducing plasma extravasation and mucosal edema (43).

A defect of β -adrenergic receptor function was proposed by Szentivanyi (152,153) to explain airway hyperresponsiveness by tipping the scale in the direction of enhanced

cholinergic tone. The hypothesis has been supported by observations of reduced responses to β -adrenergic agents in a number of airway and non-airway tissues in asthmatics. Because β adrenoceptor agonists reverse the bronchospasm in asthmatic airways, it was logical to suggest that there might be a defect in β -receptor function underlying asthma and bronchial hyperresponsiveness (25,59).

Adrenergic nerves may be present in parasympathetic ganglia, in which location they could influence cholinergic neurotransmission via prejunctional α - and β -receptors (10). Alpha-receptors which mediate bronchoconstriction have been demonstrated in the airways of several species, but may only be demonstrated under certain experimental conditions, such as exposure to inflammatory mediators. It is possible that inflammatory mediators, such as histamine, might impair the release of noradrenaline from adrenergic nerves although sympathetic neurotransmission is normal.

1.6 Noncholinergic and nonadrenergic mechanisms and neuropeptides

1.6.1 Inhibitory NANC (i-NANC)

Inhibitory NANC (i-NANC) nerves relax airway smooth muscle. They have been demonstrated *in vitro* by electrical field stimulation after adrenergic and cholinergic blockade in several species, including human (10). In human airway smooth muscle, the i-NANC system seems to be the only neural bronchodilator pathway. I-NANC nerves have also been demonstrated in human *in vivo* by capsaicin stimulation of the airway after cholinergic blockade (86).

1.6.2 Nitric oxide (NO) and vasoactive intestinal peptide (VIP) in i-NANC System

Nitric oxide (NO) and vasoactive intestinal peptide (VIP) have been shown as putative neurotransmitters in i-NANC nerves in the airway (90). NO is derived endogenously from the amino acid L-arginine by three forms of NO synthases (NOS). Endogenous NO regulates the airway functions in a dual manner with potentially both beneficial and harmful effects. The major beneficial effects of the NO are to mediate the neural inhibitory NANC response in the airway and to blunt cholinergic bronchoconstriction. However, there are several harmful effects of NO in the airways including bronchial vasodilation which may result in plasma extravasation. Selective inhibition of interferon- γ (IFN- γ) by NO may augment asthmatic inflammation because IFN- γ suppresses the proliferation of Th2 lymphocytes (164).

VIP has been demonstrated to be localized to nerves and ganglia in airways and pulmonary vessels. VIP-ergic fibers are found to be rich in the proximal airways, but few in bronchioles. The evidence favours VIP co-localization with acetylcholine in human airways. VIP-ergic fibers are found within airways smooth muscle, around bronchial vessels and surrounding submucosal glands (38,94,100). VIP acts as a potent relaxant of airway smooth muscle in human airways *in vitro* and causes bronchodilatation in animals *in vivo* (39,127,142). VIP potently stimulates mucus secretion in animals *in vitro*. It has also been shown that VIP acts as a potent pulmonary vascular dilator via a direct activation of VIP receptors on vascular smooth muscle.

1.6.3 Excitatory NANC (e-NANC)

Electrical stimulation of guinea pig bronchi, and, less consistently trachea *in vitro*, and the vagus nerve *in vivo* produces a component of bronchoconstriction that is not inhibited by atropine (7). This bronchoconstrictive response has been termed the excitatory NANC (e-NANC) and this response can be mimicked by substance P and inhibited by substance P antagonists. There is convincing evidence that tachykinins released retrogradely from a certain population of sensory nerves by nerve stimulation mediate e-NANC responses. A similar e-NANC response has occasionally been observed in human airway *in vitro*, but the effect seems less consistent than in other species.

1.6.4 Tachykinins in e-NANC System

Substance P and NKA have been demonstrated to be the neurotransmitters of the e-NANC system in animals. Various local axon reflexes may release tachykinins such as substance P, NKA and calcitonin gene-related peptide (CGRP). These agents have potent effects that mimic a number of the features of the asthma.

Tachykinins are a family of peptides with the common C-terminal sequence Phe-Gly-Leu-Met-NH₂. There are five tachykinins that have been identified in the nervous system of mammalian airways, namely, substance P, NKA, NKB, neuropeptide K (NPK) and neuropeptide-Y (NP-Y). They are all produced from a single preprotachykinin gene (77,101,154). By radioimmunoassay, the content of substance P in segmental bronchi was reported to be around 3 pmol·g⁻¹ tissue in human and guinea pig, but NKA is about 0.3 pmol·g⁻¹ (107). By immunocytochemistry, substance P-like immunoreactivity nerves in the

airways are found beneath and within the airway epithelium, around blood vessels, and submucosal glands, within the bronchial airway smooth muscle layer and around local tracheobronchial ganglion cells. These nerve fibres are around in and around bronchi, bronchioles and more distal airways, occasionally extending into the alveoli (95). Substance P and NKA have been demonstrated to be co-localized with CGRP in capsaicin-sensitive C-fiber afferents in the airways.

In the study of rodent airways, substance P and NKA have been elucidated as major neurotransmitters mediating the excitatory part of the NANC nervous system. These neuropeptides are likely to be released from peripheral and central branches of sensory nerves upon irritation. Certain chemical and physical stimuli, such as capsaicin, vapour-phase components of cigarette smoke, mediators of the allergic reaction, such as histamine have been shown to cause release of tachykinins in the airway and result in multiple local with individual patterns of bioactivity (145). For example, exposure to high concentrations of capsaicin leads to degeneration of C-fiber afferents and subsequent loss of neuropeptides (145). Pharmacological blockade of tachykinin-mediated responses can be achieved by substance P analogues with antagonistic activity. These treatments reduce markedly the cigarette-smoke-evoked protein extravasation in the airways in rats (57).

Substance P and NKA are degraded by two enzymes, neutral endopeptidase (NEP, enkephalinase) and angiotensin-converting enzyme (ACE, kininase II). ACE is predominantly localized to vascular endothelial cells and therefore breaks down intravascular peptides (146). NEP is present in the basal cells of the epithelium, alveolar type II cells, in submucosal glands, airway smooth muscle and postcapillary venules and nerves, and any

factors that inhibit the enzyme or its expression may be associated with enhanced tachykinin effects. The inhibition of NEP by using the potent and relatively selective inhibitors of NEP, thiorphan and phosphoramidon enhance various airway effects of exogenously administered and endogenously released substance P and tachykinins in animals and human, including airway smooth muscle contraction, plasma extravasation and airway mast cell activation (13,65,76). Moreover, NEP degrades substance P administered by aerosol only, but not that administered intravascularly in guinea pig suggesting that NEP may be the most important enzyme for the breakdown of tachykinins in tissues (146).

1.6.5 Tachykinin Receptors

Tachykinin effects on target cells are mediated by specific receptors. Three receptors for the tachykinins, the NK1, NK2 and NK3 receptors, have been characterized pharmacologically and cloned both in animal and human tissue (102). They are members of the super family of G protein-coupled receptors. All members of this family of receptors are glycoproteins with seven putative alpha-helical transmembrane segments, an extracellular amino-terminus and an intracellular carboxyl tail. They all interact with one or more G-proteins to promote high-affinity binding and signal transduction. The amino acid sequence of each receptor type is well conserved among species, with the greatest difference observed at the NH₂-and C-termini. The overall homology for the NK2 receptor among species (bovine, man, rat, murine) is greater than 85%. For the NK1 receptor (man, rat, mouse, guinea pig) and NK3 (man, rat) receptor the homology is approx 90%(102). Each tachykinin appears to selectively activate a distinct subtype of receptor: NK1 receptors are activated

preferentially by SP, NK2, by NKA, and NK3, by NKB. Autoradiographic studies have mapped the widespread distribution of SP-receptors in guinea-pig and human lung (24). SP-receptors are found in high density in airway smooth muscle from trachea down to small bronchioles and vascular endothelium, but less densely labelled in pulmonary vascular smooth muscle and epithelial cells. SP-receptors are also found in submucosal gland (9). NK1 receptors are observed on the apical surface of airway epithelium, from the trachea down to the respiratory bronchioles, but are not present on alveolar cells. However, both NK1 and NK2 receptors are not found to present on the airway smooth muscle of rats (49). From studies on animal airways, NK2 receptors, and to a lesser extent NK1 receptors have been shown to be involved in bronchoconstriction (107), whereas NK1 receptors have been found to be involved in mucus secretion, microvascular leakage, vasodilation and to mediate most of the neurokinins effects on inflammatory cells (53,98).

1.6.6 Biological effects of tachykinins

Substance P and NKA have various effects that could contribute to several of the features of asthmatic airways. Activation of e-NANC nerves can generate antidromic impulses and a local axon reflex, which leads to noncholinergic bronchoconstriction, increase in vascular permeability, extravasation and vasodilatation (97,98). There is increasing evidence to suggest that tachykinins interact with inflammatory and immune cells. Tachykinins have been shown to cause mast cell degranulation (79). Substance P has been shown to stimulate human peripheral blood lymphocyte proliferation and could amplify the response to the T-cell mitogens phytohaemagglutinin A and concanavalin A (33). It is also

reported that substance P enhances the expression of IL-2 mRNA and secretion of IL-2 (22,129). Airway macrophages are activated via NK2 receptors which stimulate production of cytokines such as IL-1, TNF- α and IL-6 as well as the release of LTB₄. Furthermore, substance P stimulate the chemotaxis of neutrophils and eosinophils.

1.7 Neurogenic inflammation

1.7.1 Definition

Pain, heat, redness, and swelling are the cardinal signs of inflammation. Sensory nerves may be involved in the generation of each of these signs. This "neurogenic inflammation" is due to the antidromic release of neuropeptides from nociceptive nerves or C fibers via an axon reflex, in which antidromic impulses pass down collateral branches of primary afferent sensory fibers. Such a phenomenon has been recognised for many years to be involved in local vasodilation of the skin. This concept has been extended recently to "axon reflex" activity in the stomach and it seems likely to be an important mechanism in other organs, including the lung and may contribute to the inflammatory response in asthma (109).

1.7.2 Neurogenic inflammation in the airways

Sensory neuropeptides mimic many pathophysiologic features of asthma. Release of peptides from sensory nerves in the airways may result in inflammation of the airways. Stimulation of the vagus nerve in rodents and other species causes microvascular leakage and stimulates mucus secretion from submucosal glands and epithelial goblet cells, which is

prevented by prior treatment with capsaicin or by tachykinin antagonists (99). In asthmatic subjects, the airways epithelium is often shed, thereby exposing sensory nerve endings. Therefore sensory nerves in asthmatic airways may be "hyperalgesic" as a result of exposure to inflammatory mediators such as prostaglandins and bradykinin (132). The feature of the axon reflex in the skin that may be relevant to the lung is that antidromic release of sensory neurotransmitters which may interact with inflammatory and immune cells, and subsequently lead to the release or synthesis of local proinflammatory mediators such as histamine, bradykinin, and arachidonic acid products.

1.8 Exercise-induced asthma (EIA)

Subjects in whom exercise induction of symptoms is a prominent feature of their asthma form a heterogeneous group in other respects; the wide variation in airflow resistance to which they are liable without the special factor of exercise may or may not be attributable to a common mechanism such as identifiable antigen-antibody reactions. Increased bronchial responsiveness to exercise does occur after allergen provocation in children with asthma. Furthermore, sensitized but not normal control rabbits showed significant airway obstruction after hyperpnea challenge with room temperature dry gas (73). Recently, the highly significant relationship between exercise-induced bronchospasm and specific IgEs for seasonal and perennial allergens has been reported (19).

EIA is important because of its prevalence, particularly in children, and because of the light it sheds on the pathophysiology of asthma. EIA resembles in some respects of the immediate response to an allergen inhalation challenge. Both are rapid in onset and pass off

relatively quickly, are easily inhibited by pretreatment with sympathomimetic agents and sodium cromoglycate, a mast cell stabilizer (17,87).

Hyperpnea-induced bronchoconstriction (HIB) and EIA resemble each other in many ways and follows a very similar time course. Airway narrowing in both instances usually peaks 2-10 min after a challenge and spontaneously recovers in 30-60 min. Although subtle differences may exist in the underlying mechanism triggered by these stimuli, there are numerous similarities in terms of the time-course of airway narrowing, the degree of evaporative heat and water loss, and the efficacy of a wide spectrum of drugs that attenuate their effects. Furthermore, an increase in the concentration of leukotrienes and histamine in BAL fluid, mucosal injury and mast cell degranulation occurs in asthmatic with HIB suggesting that the mediators of airway narrowing in EIA and HIB are the same.

The mechanisms responsible for EIA and/or HIB are not fully understood. However, there are several hypotheses have been proposed to explain the development of EIA and/or HIB in asthmatic subjects. It is widely accepted that the stimuli of EIA in asthmatics are directly related to the amount of water lost from the airways in conditioning inspired air (6). This hypothesis states that drying increases airway fluid osmolality, stimulates local release of mediators, and results in airway smooth muscle contraction (4). Another hypothesis is that rapid airway cooling and rewarming causes bronchovascular hyperemia and mucosal edema of the airway walls with the consequent narrowing of the bronchial lumen (37,110). A third suggests that exercise has two opposing effects, bronchodilatation due to catecholamine release and bronchoconstriction due to release of bronchoconstrictive mediators and that bronchoconstriction outweighs bronchodilator influences in EIA.

Some studies of EIA and HIB have been performed by several investigators (14,46,47,103). In these studies, the hyperventilation of exercise, or voluntary isocapnic hyperventilation *per se*, result in drying of the airways, which is dependent on the condition relative (humidity and temperature) of the inspired air. As a result of the changes in osmolarity, there is release of mediators from mast cells (or other storage cells) and nerve endings that act on the bronchial smooth muscle and cause bronchospasm or/and airway microvascular leak.

Finally, the airway responsiveness to EIA or HIB may in turn depend on the state of the airways as modified by such factors as recent allergenic exposure, viral infections, and air pollution. Previous studies also have pointed out that EIA is inherently variable and the magnitude of the response to exercise on any one occasion is largely unpredictable.

1.9 Animal models of EIA

1.9.1 Some animal species as models of EIA

For many reasons, invasive studies designed to elucidate mechanisms of EIA or test the efficacy of potential therapeutic agents are not possible in humans. Animal models of EIA have contributed considerable insights into potential contributions of the bronchial circulation, neuropeptides and various local mediators to the development of the pathological changes in structure and functions of airways in this condition. The development of animal models of EIA has been relatively recent compared to the many models of allergic asthma.

HIB had been studied in the guinea pig, rabbit, cat, dog, and monkey (12,27,52,72,124). In these models, the respiratory tract is subjected of studied animals are

exposed to hyperventilation with dry air, cold dry air or isocapnic gas. In guinea pigs, rabbits and dogs, HIB reportedly causes the maximal change of pulmonary resistance 2 to 10 minutes after challenge. In monkeys, pulmonary resistance shows the maximal changes about 25 minutes after exposure.

A variety of biochemical mediators have been implicated in the development of HIB in humans and animals (47,55,103). However, much of the evidence is derived from studies of pharmacological blockade with various antagonists and there is little direct evidence to support their role in the development of HIB. Pliss reported that isocapnic hyperpnea increased the concentration of leukotrienes in BAL fluid obtained from asthmatic patients (131). Furthermore, an increase in biliary leukotrienes after isocapnic hyperpnea challenge from guinea pigs has also been reported.

1.9.2 The rat as a potential model of HIB

There are several rat strains including Sprague-Dawley rat and Brown Norway (BN) rats that have been established in the studies of allergic responses of both skin and respiratory system (15,155). These highly inbred rat stains have an advantage over outbred strains because any aspects of the genetic constitution of the animal that may contribute to variability in responses will be largely eliminated. In addition to Sprague-Dawley rat and BN rats, airway responsiveness to bronchoconstrictive agents in some other rat strains has also been investigated. The Fisher rat reveals bronchial hyperresponsiveness in response to the bronchoconstrictive agonist methacholine compared to Lewis, ACI, BN, WF, BUF and WKY (158). Fisher rats have been extensively employed for the studies of exploration of the phenotypic characteristics responsible for airway hyperresponsiveness. BN rats have been

extensively used for the studies of allergen-induced bronchoconstriction because of their high baseline level of IgE and high IgE production after immunization (2,115). This strain of rat is a high responder to parainfluenza virus-induced bronchiolitis, pneumonia, and bronchiolar mastocytosis and has more mast cells and eosinophils in the airway walls compared to other rat strains (150). Allergen-immunized BN rats also reveal dual airway responses, early and late allergic bronchoresponses which mimic biphasic the asthmatic reactions of human subjects after specific antigen exposure (106).

The observations in guinea pigs such as HIB, hyperpnea-induced airway microvascular leak and inflammatory mediators release to other species such as the rat is somewhat uncertain. An important difference between the guinea pig and the rat is that neurokinin receptors are expressed on airway smooth muscle in the guinea pig but not in the rat (49,68). Despite the lack of neurokinin receptors on rat airway smooth muscle exogenous tachykinin does cause bronchoconstriction in some rat strains but the effect is mediated indirectly through effects on the mast cell (76).

1.10 Aims of this study

The purposes of the study described in this thesis were to establish whether the mechanisms of bronchoconstriction induced by dry gas hyperpnea challenge were particular to the guinea pig or whether similar mechanisms were also important to the rat. The lack of NK receptors on rat airway smooth muscle (49) is an important difference to the guinea pig and could have an important bearing on the responses to HIB. More specifically, the interaction of endogenous airway hyperresponsiveness to contractile agonists and the atopic

predisposition of the animals with responsiveness to hyperpnea challenge were evaluated. To do this, the responses of a strain hyperresponsive to inhaled contractile agonists, Fisher 344 (144), two control normoresponsive strains, Lewis and ACI (74), and an atopic but normoresponsive rat, Brown Norway (128,155) were examined. The four strains were examined for airway responses to dry gas hyperpnea challenge and the efficacy of neurokinin (NK) and LTD4 receptor antagonists on responses to challenge. To test the hypothesis that inter-strain differences in responsiveness to dry gas hyperpnea challenge may relate to airway responsiveness to the specific mediators released by this form of challenge, the responsiveness of three of the rat strains to LTD4 was tested. Although the inter-strain differences in responsiveness to methacholine have been described (41,80,105), it is possible that differences in responsiveness to methacholine might not reflect similar differences in responsiveness to LTD4. Responsiveness to LTD4 is potentially more relevant to responses to dry gas hyperpnea challenge given the role that LTD4 plays in hyperpnea induced bronchoconstriction in the guinea pig.

CHAPTER 2

METHODOLOGIES AND METHODS

2.1 Background to methodologies

The methodologies involved in the studies of HIB in this thesis were several and were designed to permit the measurement of pulmonary responsiveness, the evaluation of pulmonary synthesis and release of cysteinyl-leukotrienes and the quantification of airway microvascular leakage following isocapnic hyperpnea challenge.

2.1.1 Measurement of dynamic respiratory mechanics

Airway response to various challenges in vivo can be measured by analysis of the dynamic mechanical properties of the respiratory system. In moving air from outside the body into the peripheral pulmonary units for gas exchange, there are three opposing forces that need to be overcome, namely the elastic recoil of lung tissue and chest wall, the frictional resistances of airway and lung tissue and thorax and the inertance of the entire system and the gas it contains. The analysis of the mechanical properties of the respiratory system can be simplified because inertance has been found to be negligible during normal breathing and because the contributions of elastic forces and flow-resistive forces can be separated by studying the system under static and dynamic conditions, respectively. Airflow through a tube can be categorized into three types: laminar, turbulent, and convective acceleration. Laminar flow is streamline flow with a parabolic velocity profile with a maximum velocity at the center of the tube. Gas flow in the airway is usually laminar during quiet breathing (Dekker E. Transition between laminar and turbulent flow in human trachea, *J. Appl. physiol.* 16: 1060-1064). The relationships between the driving pressure across the

lung and the various impedances are embodied in the equation

$$\text{Pressure difference} = \text{resistance} \times \text{rate of flow} + \text{elastance} \times \\ \text{volume} + \text{inertance} \times \text{acceleration}$$

The airway resistance to airflow in the respiratory system is given by the ratio of the frictional pressure losses between the points of pressure measurement to the magnitude of airflow. However there is also a component of viscous pressure loss across the lung tissue that is independent of the airways. This pressure loss has been variously designated as tissue resistance, viscance, and tissue hysteresis. It is added to the airway resistance when the pressure drop across the lung is used for the determination of lung resistance. In various animal species this component of lung resistance has been determined and is greater than the actual airway resistance itself. Hence, the pulmonary resistance is the sum of lung tissue (viscous) resistance and airway resistance and is determined from the simultaneous measurements of the flow and the pressure difference between the airway opening and the pleural surface. It can be written by the equation

$$R_L = \text{Pao-Ppl}(\text{cmH}_2\text{O})/\text{flow}(\text{ml}/\text{sec}).$$

Pressure difference between the airway opening and the pleural surface (Pao-Ppl) is transpulmonary pressure (P_L). Pao remains near zero (the same as the atmospheric pressure). During inspiration, the alveolar pressure becomes negative and creates a driving pressure for airflow into the lung. If the airway narrows in response to various stimuli, either the rate of airflow in airway will decrease due to an increase in airway pulmonary resistance or the pressure drop across lung will increase in order to maintain airflow. In either case the ratio

of resistive pressure to flow will increase as a reflection of the reduction in airway calibre.

To make measurements of resistance, the flow and the pressure driving flow need to be measured. The measurement of R_L depends on obtaining a valid measurement of intra-pleural pressure (Ppl). Because the intra-pleural pressure measurement in vivo is an invasive process, esophageal pressures have been the preferred alternative to direct measurements of pleural pressure. Simultaneous recordings of pleural and esophageal pressure have shown that the correspondence between the two is quite close (28,112). The esophageal pressure can be measured in large animals using an air-containing thin cylindrical latex balloon sealed over a narrow-bore plastic catheter that transmits pressure from the balloon to an electromanometer. However the esophageal catheters that are used for small animals are fluid-filled because of the need to have an adequate frequency response of the measurement system. The Pao is zero when airway is open to the atmosphere.

The rate of airflow can be determined in various ways. The flow may determined directly by using a pneumotachograph or indirectly from displacements of the body surface by body plethysmography. In the experiment of this thesis, the airway flow rate was measured by placing the tip of the endotracheal tube of the spontaneously breathing rat into a small plexiglass chamber (250ml approximal) which was connected to a Fleisch type pneumotachograph.

The practical problem that is posed in trying to determine R_L is that the resistive and elastic pressures need to be separated in order to plot the relationship between the resistive pressures loss and flow. This problem is frequently solved by either the mid-volume technique of Neergard and Wirz, or the electrical subtraction technique of Mead and

Whittenberg. However in the experiments of this thesis, the technique of multiple linear regression analysis was used (41).

2.1.2 Measurement of cysteinyl-leukotrienes

When LTC₄ enters the blood stream it is rapidly metabolized to LTD₄ and LTE₄. These cysteinyl leukotrienes are taken up by the liver and are also excreted by the kidneys. After partial intracellular metabolism, they are excreted into bile and urine. In rodents LTE₄ metabolism continues in the liver cells and includes N-acetylation and ω -oxidation, followed by β -oxidation from the ω -end. About 40% of these metabolites are recovered in bile (78) and about 10% in urine within 5 hours. Urinary metabolites are the result of an enterohepatic circulation.

The technique of high-performance liquid chromatography (HPLC) was employed in the experiments of this thesis for the separation and quantitation of cysteinyl-leukotrienes in bile (106). Reversed phase HPLC (RP-HPLC) is commonly used for the separation and analysis of eicosanoids. The term "reversed-phase" is used to describe those packings in which the bound organic derivative is a straight chain alkyl group. The separation mechanism in RP-HPLC arises out of the fact the stationary phase now consists of a non-polar surface and the mobile phase is polar, normally a water/methanol or water/acetonitrile mixture. This non-polar surface /polar mobile phase combination gives the method its name in that it is the reverse situation to that of adsorption chromatography. The main advantages of HPLC are that it has wide sample applicability and for many analyses it is rapid when compared to other sequential techniques. The basic principle of chromatography is that when the

molecules of a compound are introduced to the top of a chromatographic column, they are subject to two opposing forces, that of the mobile phase tending to force them through the column, and that of the stationary phase tending to retain them. This dynamic equilibrium between the mobile and stationary phases causes the desired material to emerge at the end of the column as a band of molecules in the form of a Gaussian peak.

2.1.3 Airway microvascular leakage

Various cells such as endothelial cells and epithelial cells are joined to each other by tight junctions. Normally, the permeability of endothelial cells is very low and they only allow small molecules such water and some ions pass through along the direction of the driving force. There is only a small amount of plasma protein that passes through the endothelial barrier and consequently there is little plasma protein in bronchoalveolar lavage fluid in normal situations. Under conditions of lung inflammation where microvascular leak is present plasma proteins such as albumin, fibrinogen and α 2-macroglobulin are significantly increased in BAL fluid. Albumin is usually the plasma protein that is analysed in the BAL fluid as a measure of microvascular permeability. Evans blue dye conjugates with albumin and allows its detection after it enters the airway wall and lumen. Therefore, the extravasated albumin can be calculated by quantitated from the Evans blue in the sample such as BAL fluid or tissues after appropriate extraction. Although this is not the most sensitive technique it is a convenient one that provides a good estimate of microvascular leak when it is of substantial magnitude.

2.1.4 Isocapnic dry gas challenge

During the normal breathing, the tidal volume of awake rats (113-300g) is in the range of 0.87-2.08 ml or 6.83-7.67 ml/kg with a range of breathing frequencies from 85-103 breaths/min (81,82). When the rat is ventilated with a tidal volume of 8ml/kg and 150 breath/min, the minute ventilation is increased from 90-200ml/min to 240-300ml/min or 995-1151 ml/min/kg. Hyperventilation will cause a low P_{CO_2} and high blood pH, and subsequently acute respiratory alkalosis, hypokalaemia and an increase in plasma catecholamines. However, these changes can be prevented with hyperventilation with a gas composition (5% CO_2) that maintains isocapnic conditions.

2.2 Materials and methods

2.2.1 Animal preparation

Four rat strains, Fisher, BN, Lewis, and ACI (190-220g) were purchased from Harlan-Sprague Dawley. Rats were anaesthetized with xylazine (10mg/kg) and pentobarbital sodium (30mg/kg) intra-peritoneally. Intratracheal intubation was performed with a short piece of polyethylene tubing (PE-240; 6cm long; internal diameter: 0.037 inches, Dow Corning Corporation Medical Products, Midland, MI) was used to cannulate the internal jugular vein for fluid replacement, for Evans blue injection and for drug administration and. Supplemental anaesthesia equivalent to one-third of the initial dose of pentobarbital sodium was given 30 minutes after the first injection.

Inspiratory and expiratory tubes of a ventilator (Harvard rodent ventilator model 360, South Natick, MA) were attached to the tracheal tubing through a Y connector with a 3 cm

common segment (total dead space 0.03ml) to minimize conditioning of the inspired gas. The inspiratory port of the ventilator was connected to either a warm (35-37°C) humidifier through which was passed room air or dry gas. Rectal temperature was monitored by using an electronic thermometer and body temperature was maintained by a heating blanket.

All animals were mechanically ventilated for the purposes of hyperpnea challenge but breathed spontaneously for measurements of pulmonary responses to challenge.

The bile ducts of the rats were exposed through a small incision in the abdominal wall and were cannulated with polyethylene tubing (PE-60). Following surgery the animals were allowed to stabilize for a period of 120 min prior to challenge.

2.2.2 Experimental protocols

2.2.2.1 Hyperpnea challenge

Dry gas hyperpnea (BN; n=9, Fisher; n=7, Lewis; n=6, ACI; n=7) was performed by introducing a dry mixture of 5% CO₂-95% O₂ from a balloon into the inspiratory port of the mechanical ventilator. Ventilatory frequency was set at 150 breaths/min and the tidal volume was increased to 8 ml/kg; hyperpnea was continued for 5 min. Humidified gas hyperpnea challenge (BN; n=7, Fisher; n=7, Lewis; n=6, ACI; n=7) was performed in an identical fashion, except that the 5% CO₂-95% O₂ was bubbled through a warm water bath before it passed through the ventilator. Baseline ventilator settings and gas mixtures were resumed following hyperpnea challenge for a period which lasted until baseline R_L was reestablished. R_L measurements were obtained at 1 min intervals for the first 10 min into the recovery period, at 5 min intervals for the next 20 min, and at 15 min intervals thereafter up to 2

hours. The R_L was expressed as cm H₂O/ml/sec.

2.2.2.2 The effects of neurokinin antagonists on response to hyperpnea challenge

The role of tachykinins in hyperpnea-induced bronchoconstriction was evaluated using specific neurokinin receptor antagonists. The specific NK1 receptor antagonist CP-99994 and the NK2 receptor antagonist SR-48968 were dissolved in equal volumes of ethylene glycol and saline. The animals were administered either CP-99994 (1mg/kg, n=7) or SR-48968 (1mg/kg, n=7) into the jugular vein respectively and the same regimen was used in control animals to deliver an equivalent volume of vehicle to control animals. Hyperpnea challenge was performed 10 min after the administration of the NK antagonists.

2.2.2.3 The effects of a selective leukotriene D4 antagonist on responses to hyperpnea challenge

To evaluate the role of leukotriene D4 in hyperpnea-induced bronchoconstriction, a specific leukotriene D4 antagonist, pranlukast (30µg/kg, dissolved in a 0.9% saline), was administered to a group of BN rat (n=6) 10 minutes before the hyperpnea challenge.

2.2.2.4 Metabolism of tracheally instilled of 3H-LTC4

1 µCi of tritium labelled LTC4 was instilled into the trachea of 4 BN rats and bile was collected subsequently for 4 hours with 30 minute collection periods. 30% of each sample was added to 4 ml of scintillation fluid and the β emissions were counted directly whereas the rest of the sample was pooled to give 1 hour collections. 0.2 ml of bile was

analysed by RP-HPLC for the separation and quantitation of various metabolites of LTC₄.

2.2.2.5 Microvascular leakage

All rats, including the 75 that underwent hyperpnea challenge and an additional eighteen normal rats (BN; n=4, Fisher; n=4, Lewis; n=6, ACI; n=4) as negative control groups, were injected with 30 mg/kg of Evans blue dye in 1 ml of saline 5 minutes before hyperpnea challenge. Bronchoalveolar lavage was performed 2 hours after challenge.

2.3 Outcome Measurements

2.3.1 Pulmonary Resistance (R_L)

The tip of the intratracheal tube was inserted into a small Plexiglas chamber (volume, 265ml). A Fleisch no. O pneumotachograph coupled to a piezoresistive differential pressure transducer (Micro-Switch 163PC01D36; Honeywell, Scarborough, Ont., Canada) was attached to the other end of the chamber to measure airflow. The box was pushed with room air except while measurements of pulmonary mechanics were being made to avoid accumulation of carbon dioxide. Transpulmonary pressure (P_L) was measured using a liquid filled polyethylene tubing (consisting of a 9 cm PE-240, and 25 cm PE-160 terminal tip) placed in the lower third of the esophagus connected to one port a differential pressures transducer (Sanborn 268 BC; Sanborn, Waltham, MA); the other end was connected to the Plexiglas chamber. P_L and flow signals were amplified, passed through eight-pole Bessel filters (model 902lpf; Frequency Devices, Harverhill, MA) and record by a 12-bit analog-to-digital converter at a rate of 200 Hz (D7 2801-a, Data Translation, Marlborough, MA) for

final storage in an IBM compatible personal computer. The animals breathed spontaneously in resting conditions. R_L was calculated by multiple linear regression analysis by fitting the equation of motion of the lung: $P_{tp} = E_L V + R_L \dot{V} + k$ to 10 seconds segments of data with a commercial software package (RHT Infodat Inc, Montreal, QC, Canada) so as to obtain pulmonary resistance.

2.3.2 Airway microvascular leakage

Evans blue dye was prepared (3% solution saline) and was filtered by a 0.2 μ m filter. A dose of 30mg/kg Evans blue dye was injected into the jugular vein 5 min before hyperpnea challenge. A 2 ml saline bronchoalveolar lavage (BAL) was performed 2 hours after challenge. The Evans blue dye in the BAL fluid was quantitated by absorbance at a wavelength of 620 nm using a spectrophotometer (DU-64, Beckman Instruments Inc., Fullerton, CA) and comparison with a series of standards. The Evans blue in the BAL fluid was expressed as μ g/ml.

2.3.3 Measurement of biliary N-acetyl-leukotriene E4

In order to determine the extent of pulmonary cysteinyl-leukotriene synthesis we collected and analysed the bile of BN rats undergoing both dry gas and wet gas challenge; bile is the principal route of excretion of cysteinyl-leukotrienes in the rat. For 1 hour before and for 2 hours after hyperpnea challenge bile was collected under a stream of argon into Eppendorf tubes that were kept on ice. Bile was stored at -80°C prior to analysis by RP-HPLC and quantified by radioimmunoassay. After precipitation of protein by adding of

methanol, the bile samples were subjected to precolumn extraction / RP-HPLC. N-acetyl-leukotriene E4 (N-Ac-LTE4, the major cys-LT species in rat bile) was quantitated in the column fractions by radioimmunoassay as described previously (106).

2.3.4 Measurement of airway responsiveness to leukotriene D4 in ACI, BN and Fisher rats

To determine responsiveness to LTD4 animals underwent airway challenge by insufflation of progressively increasing doses of LTD4 from 1 to 100 ng in 100 μ L of saline in ACI and Fisher rats. In BN rats the starting dose was 0.5 ng. Insufflation of LTD4 was performed through a small catheter placed inside the endotracheal tube. Measurements of R_L were performed 30 secs following insufflation. Measurements were repeated at intervals until a stable value was achieved prior to proceeding to the next dose of LTD4.

2.4 Data Analysis

The response to hyperpnea challenge was quantitated as an increase in pulmonary resistance (R_L) above the baseline value.

The total amounts of biliary cysteinyl leukotrienes were calculated by adding the amounts (in pmol per time period) for LTC4, LTD4, LTE4 and N-acetyl-LTE4. The increased level of leukotrienes appearing in bile collected between 0 to 2 hours after challenge were considered to be related to the hyperpnea challenge.

The absorbance of Evans blue dye in BAL fluid was detected by spectrophotometer and the amount of Evans blue dye in the BAL fluid was calculated by comparing absorbance

values to those of a standard curve using various solutions of varying concentrations.

2.5 Statistical Analysis

All results are expressed as mean $\pm$ 1 standard error of the mean (SE). Comparison of two means was performed using paired or unpaired *t* tests as appropriate. For comparison of several means, an ANOVA followed by the Fisher-least significant difference test was used. Differences were considered to be statistically significant when *p* values were less than 0.05.

2.6 Chemicals and drugs

N-acetyl-LTE₄, monoclonal antibody to LTC₄, the NK1 receptor antagonist CP-99994 and the NK2 receptor antagonist SR-48968 were kindly provided by Dr. Ian Rodger (Merck-Frosst, Pointe Claire, QC, Canada). The LTD₄ antagonist, pranlukast were kindly provided by Dr. T Leonard (Smith Kline Beecham Pharmaceuticals, Collegeville, PA). Pentobarbital was supplied by MTC Pharmaceuticals, Cambridge, Ontario, Canada. Evans blue dye was purchased from A&C American Chemicals Ltd., Montreal, Quebec, Canada.

CHAPTER 3

RESULTS

3.1 Airway response to hyperpnea challenge in various rat strains

There were no differences in baseline values of R_L between dry gas and wet gas hyperpnea challenged BN rats. After hyperpnea challenge R_L gradually increased from a baseline of 0.22 ± 0.007 cmH₂O/ml/sec and reached significantly higher values at 60 min (0.33 ± 0.03) and at 120 min (0.41 ± 0.08) in the dry gas group of BN rats (ANOVA and Fisher's LSD $p < 0.01$; Figure 1). The value of R_L at these time points was also significantly higher than the corresponding values in animals exposed to wet gas (60 min; 0.22 ± 0.01 and 120 min; 0.19 ± 0.02 cmH₂O/ml/sec; t -test $p < 0.05$). There were no significant differences in the peak values of R_L between the dry gas and wet gas hyperpnea challenged groups of Lewis and Fisher rats. However, ACI rats demonstrated small responses after dry gas hyperpnea challenge which occurred earlier than in the BN rats; there were significant differences in R_L between the wet and dry gas challenged groups at 3 min (wet gas 0.21 ± 0.01 vs dry gas 0.29 ± 0.03 cmH₂O/ml/sec; t -test $p = 0.05$) and 10 min (wet gas 0.21 ± 0.01 vs dry gas 0.29 ± 0.02 cmH₂O/ml/sec; t -test $p < 0.05$) after challenge (Figure 1).

Figure 1. Time course of R_L in response to hyperpnea challenge

R_L was significantly increased from baseline at 60 min and 120 min in the BN rats after dry gas challenge ($n=9$, arrow indicating start of hyperpnea challenge. $*p<0.01$ unweighted means ANOVA, Fisher's LSD test). R_L in the animals challenged with dry gas was also significantly higher than the values at the same time point in animals exposed to wet gas hyperpnea challenge ($n=8$, $**p<0.05$ t -test). There were no significant differences in the peak values of R_L either in the dry gas hyperpnea challenged ($n=6$) compared to the wet gas hyperpnea challenged ($n=6$) Lewis rats nor in the dry gas ($n=7$) compared to the wet gas hyperpnea challenged ($n=7$) Fisher rats. There were significant increases in R_L in the ACI rats at 3 min (dry gas, $n=7$, vs wet gas, $n=7$, t -test $p=0.05$) and 10 min after dry gas hyperpnea challenge (dry gas vs wet gas, t -test $p=0.03$)

3.2 Airway microvascular leak caused by hyperpnea challenge in the rats

The effects of dry gas and wet gas hyperpnea challenge on Evans blue dye extravasation in the airways of various rat strains are illustrated in Figure 2. For all strains the concentration of Evans blue dye in BAL fluid after hyperpnea challenge with dry gas was significantly higher than in unchallenged animals (ANOVA and Fisher LSD; $p < 0.05$). ACI showed the greatest increase in Evans blue dye whereas the BN showed the smallest change. The Evans blue dye in BAL fluid following dry gas challenge was significantly higher than in the wet gas challenged controls for BN (17.5 ± 2.8 vs $9.4 \pm 1.1 \mu\text{g/ml}$; $p < 0.05$) and Lewis ($27.61 \pm 6.1 \mu\text{g/ml}$ vs $12.9 \pm 3.3 \mu\text{g/ml}$; $p < 0.05$) animals. Dry gas challenge also evoked an increase in Evans blue dye extravasation in Fisher ($27.11 \pm 4.7 \mu\text{g/ml}$) and ACI rats ($34.1 \pm 5.7 \mu\text{g/ml}$) but these response were not significantly different from these observed with wet gas challenge (Fisher; $18 \pm 6.9 \mu\text{g/ml}$ and ACI; $33.3 \pm 7.9 \mu\text{g/ml}$).

Figure 2. The concentration of Evans blue dye in BAL fluid

The concentrations of Evans blue dye in BAL fluid in all four rat strains with dry gas hyperpnea challenge (* $p < 0.05$ unweighted means ANOVA, Fisher's LSD test) were significantly higher than those without any challenge. The concentrations of Evans blue dye in BAL fluid in the dry gas challenged animals was significantly higher than that in wet gas challenged BN and Lewis rats (# $p < 0.05$). Although there appears to be an increase in the Evans blue dye extravasation in Fisher rats of the dry gas group above the wet gas group it did not reach statistical significance ($p > 0.05$). The Evans blue dye in BAL fluid in both dry and wet gas (34.64 ± 5.27 and 33.35 ± 7.28) hyperpnea challenged ACI rats were not significantly different.

3.3 Effects of receptor antagonists on the airway responses to hyperpnea challenge

The specific NK1 receptor antagonist, CP-99994, the NK2 receptor antagonist, SR-48968 and the LTD4 receptor antagonist, pranlukast effectively prevented the increase in R_L in BN rats in response to dry gas hyperpnea challenge; R_L at the 60 min and 120 min time points was higher for the dry gas-challenged animals compared to pranlukast, CP-99994 and SR-48968 (ANOVA and Fisher's LSD test; $p < 0.05$, Figure 3). Evans blue dye extravasation was significantly decreased in BN rats treated with pranlukast ($9.08 \pm 1.7 \mu\text{g/ml}$; $P < 0.05$) and SR-48968 ($9.7 \pm 1.2 \mu\text{g/ml}$; $P < 0.05$) compared to controls ($17.5 \pm 2.8 \mu\text{g/ml}$) but not in rats pretreated with CP-99994 ($13.95 \pm 1.4 \mu\text{g/ml}$; Figure 4).

Figure 3. The effects of NK and LTD4 antagonists on R_L in the BN rats
 Specific NK1, NK2 and LTD4 receptor antagonists, CP-99994 (n=7), SR-48968 (n=7) and pranlukast (n=6) respectively, blocked the increases in R_L in BN rats in response to dry gas hyperpnea challenge at the 60 minute and 120 minute time point (unweighted means ANOVA, Fisher's LSD test * $p < 0.05$).

Figure 4. The effects of NK and LTD4 receptor antagonists on Evans blue dye in BAL fluid in the BN rats

Evans blue dye extravasation was significantly increased after dry gas hyperpnea challenge. Pretreatment with pranlukast and SR-48968 reduced Evans blue leak to control values (unweighted means ANOVA, Fisher's LSD test * $p < 0.05$).

3.4 Biliary N-ac-LTE4 and hyperpnea challenge

The baseline concentration of biliary N-Ac-LTE4 in the group of BN rats challenged with wet gas hyperpnea was somewhat higher than the group challenged with dry gas and fell after challenge (Figure 5). The N-Ac-LTE4 level fell in six and rose in two of the control rats subjected to challenge with wet gas. In contrast, N-Ac-LTE4 rose in 5 of the rats challenged with dry gas and fell in only 3 of these rats. However, these differences were not statistically significant.

Figure 5. Cys-LTs synthesis in BN rats

The level of biliary N-Ac-LTE₄ fell from baseline to post challenge values in the wet gas challenged BN rats, presumably reflecting recovery of LT levels from the trauma of surgery. There was a slight increase in the level of N-Ac-LTE₄ in the dry gas challenged BN rats although the difference did not reach statistical significance.

3.5 Airway responsiveness of BN, ACI and Fisher rats to exogenous LTD4

To compare the responses to LTD4, rats were challenged with increasing doses of this substance by intratracheal insufflation. To correct for differences in the baseline values of R_L among rats, the results were expressed as percentages of the baseline values. The Fisher and ACI rats had comparable percent increases in R_L which reached a plateau at about 50% above the baseline. BN rats responded more to LTD4 with a maximal response of about 250% above baseline observed at the highest doses of LTD4 tested. However, the doses of LTD4 required to increase R_L by 50% of the maximal change (EC_{50}) were not significantly different among strains.

Figure 6. Airway response to instilled LTD4 in different rat strains

The peak pulmonary response measured as R_L was determined after each dose of LTD4 insufflated intratracheally. The maximal R_L of BN rats was 250% above the baseline, whereas those of Fisher (n=9) and ACI (n=7) rats were 165% and 210% above the baselines respectively.

3.6 Metabolism of intratracheally instilled ^3H -LTC₄

The total biliary recovery of radioactivity and the proportion of various cysteinyl-leukotrienes appearing in the bile derived from instilled ^3H -LTC₄ have been expressed as a cumulative curve in figure 7. Total biliary radioactivity recovered was 31% of that instilled intratracheally. RP-HPLC analysis showed that N-acetyl-LTE₄ was the major metabolite of LTC₄ in the BN rat and was about 12% of instilled LTC₄. The early peak in the chromatogram represents unspecified polar metabolites, including various ω - and β -oxidized derivatives as well as sulphoxides and was 5.2% of the radioactivity instilled intratracheally. LTC₄, LTD₄ and LTE₄ comprised 2.9, 0.7 and 0.5% respectively (Figure 7) .

Figure 7. Metabolism of intratracheally instilled 3H-LTC₄

Total biliary radioactivity recovered was 31% of that instilled intratracheally. RP-HPLC analysis showed that N-acetyl-LTE₄ is the major metabolite of LTC₄ in the BN rat and is about 12% of instilled LTC₄. Unspecified polar metabolites was 5.2%. LTC₄, LTD₄ and LTE₄ were 2.9, 0.7 and 0.5% respectively.

CHAPTER 4

DISCUSSION

4. Discussion

This study has shown that dry gas hyperpnea challenge causes bronchovascular leak in all studied strains of rat. It also causes an increase in R_L in BN rats. The NK1 receptor antagonists, CP-99994, NK2 receptor antagonists, SR-48968 and LTD4 receptor antagonist, pranlukast block the increase in R_L in the BN rat. However, the bronchovascular leak is only blocked by SR-48968 and pranlukast. These data suggest that NK1, NK2 and LTD4 receptors are involved in the development of HIB and hyperpnea-induced microvascular leakage. There is signal of increase in N-Ac-LTE4 in the BN rat with dry gas hyperpnea challenge but not in the BN rats with wet gas hyperpnea challenge. This data indicates that there is presumable an increase in cys-LT synthesis in the BN rat with dry gas hyperpnea challenge although the increase was not statistically significant. Furthermore, compared to the airway of Fisher and of the ACI rats, the airway of BN rats exhibits hyperresponsiveness to LTD4 application. This result suggests that synthesis of cys-LT may play an important role in the development of HIB and microvascular leak in response to dry gas hyperpnea challenge in the BN rat.

Exercise and hyperventilation cause a transient increase in pulmonary resistance in approximately 75-80% of asthmatics. Dry air hyperventilation can produce a similar phenomenon in human (14) as well as in various animal models, such as guinea pig (27), dog (52), rabbit (124), cat (72) and monkey (12) which has been termed hyperpnea-induced bronchoconstriction (HIB). One of the hypotheses offered to explain the development of HIB is that exercise and hyperventilation cause airway drying during hyperventilation and this increases airway fluid osmolarity, stimulates local mediator release, and results in airway

smooth muscle contraction. Airway drying has been deduced to be important as a cause of bronchoconstriction in many species, such as guinea pig, rabbit, dog and as well as human being. For example, increasing the humidity of the inspired gas effectively attenuates the severity of the bronchospasm in guinea pig, rabbit, dog, monkey and human asthmatics (5,45,51,70,134). Evaporative heat loss has also been proposed to be the stimulus for bronchoconstriction after hyperventilation and the effects of drying and cooling have not been easy to distinguish.

The results of the present study demonstrate that isocapnic hyperpnea challenge with dry gas evokes an airway response in the rat. However there are significant strain-related differences in the patterns of response. All four of the rat strains studied demonstrated airway microvascular leak but only two of them showed evidence of airway narrowing as determined by pulmonary resistance. The rank order of change in microvascular permeability and airway narrowing were not the same. BN rats showed the largest increases in pulmonary resistance after challenge but had the lowest values of Evans blue dye in the bronchoalveolar lavage fluid whereas the ACI strain showed the greatest changes in microvascular leak in response to either wet or dry gas challenge and had only modest changes in pulmonary resistance. BN rats have shown to be more responsive than the rest of the other rat strains to an equivalent dry gas hyperpnea challenge. After antigen immunization, BN rats reveal higher serum IgE production than other strains, such as Lewis, Fisher, ACI rats, although Fisher rats show hyperresponsiveness to non-specific bronchial challenge. Immunized BN rats which appear to be "atopic" manifest typical early and late airway response as well as elevation of biliary cysteinyl-leukotriene production which corresponds to the time course

of the response and local inflammatory cell infiltration when they exposed to antigen. The fact that the "atopic" BN rat is more easily provoked than other rat strains in response to various stimuli, such as antigen and hyperpnea suggests that they may have some unique genetic factor that may determine the mechanism and magnitude of both HIB and allergen-induced responses in rats. Furthermore, differences in airway responsiveness to leukotriene D₄ may account in part for some of the strain-related differences in response to dry gas challenge.

The water content of inspired gas has been shown to be an important determinant of airway responses to dry gas isocapnic hyperpnea in both human subjects and other animals (52,69,151). Consistent with these results dry gas hyperpnea challenge evoked airway responses in all of the strains studied. In contrast, humidified gas hyperpnea challenge has no effect on pulmonary resistance in any of the strains investigated. Many studies have demonstrated that inhalation of hypertonic aerosols produce a similar response to hyperpnea-induced bronchoconstriction in the most asthmatics as well as dogs (50,148) and these responses are significantly correlated with HIB. A hypertonic stimulus had been shown to leads mast cell mediator release *in vitro*. Recently, hypertonic, but not hypothermic stimulus has been shown to lead to SP release from ganglion sensory neuron (54). These data support the hypothesis that hyperpnea challenge may cause airway drying which changes osmolarity of airway lining fluid and triggers biochemical mediators release from the inflammatory cells and nerve endings in the airway epithelium. Hyperpnea with dry air or dry gas have been reported to cause bronchovascular leak in several animals models. In dogs, leakage occurs immediately after hyperpnea ceases and continue for at least 24 hours after the initial

challenge (125). Bronchovascular leakage also occurs in the guinea pigs with dry gas hyperpnea challenge (55) and this phenomenon is partially prevented by LTD4 and tachykinin receptor antagonists. In the present study, dry gas hyperpnea challenge significantly increased microvascular leak in all four strains of rats. However, humidification of the inspired gas also had this effect in Lewis and ACI rats. Only the BN and Lewis strains exhibited greater microvascular leak in response to dry gas compared to humidified gas. These data suggest that bronchovascular leak appears not to contribute to HIB in rats because increase in R_L only occurs in BN rats but not in the other strains. Presumably the failure of wet gas to prevent the airway responses in Fisher and ACI rats resulted from an incomplete conditioning of the inspired air. The design of the experimental apparatus was not such as to ensure that inspired gas was at 37 °C when it reached the airway opening.

The time course for the increase in pulmonary resistance following dry gas challenge in the BN rats was somewhat slower in onset than that previously reported for the guinea pig (118,134). The guinea pig shows an increase in resistance immediately following challenge and recovers somewhat by 60 minutes whereas the response in the BN rat was not observed until at least ten minutes after challenge and had not subsided by 120 minutes. The delay in the response of the BN rat may reflect the fact that the synthesis and secretion of several mediators appears to be required for the response to occur. However the change in pulmonary resistance after dry gas challenge in the ACI rat, even if less than in the BN rat, was closer in pattern to the guinea pig response with the occurrence of an earlier peak in resistance.

Tachykinins, which include NKA and substance P are located in the airway afferent C-fibre endings and have been demonstrated to play a very important role in the development

of HIB in guinea pigs (27,135,136). Both capsaicin pretreatment to deplete tachykinins and the NK1 (CP-99994) and NK2 (SR-48968) receptor antagonists blunt HIB in guinea pig (135,149,166). In the present study there is also clear evidence of tachykinin involvement in the hyperpnea-induced airway response; both NK1 and NK2 antagonists blocked the increased pulmonary resistance in dry gas challenged BN rats. An acute administration of capsaicin has been shown to cause bronchovascular leak in Fisher rats (58). However, pretreatment with capsaicin in a sufficient dose to deplete airway tachykinins had been demonstrated to reduce bronchovascular leakage induced by hyperpnea challenge in guinea pig (56). These observations suggest the involvement of endogenous tachykinins in the development of bronchovascular leak. However, in contrast to the guinea pig, NK receptors are absent from airway smooth muscle in the rat (49) so that any effects of tachykinins on airway responses must be mediated indirectly. Such indirect effects have been shown to occur. For example administration of substance P and NKA to the rat causes an increase in pulmonary resistance which is abolished by the 5-HT antagonist methysergide favouring airway mast cells as the intermediary cells in the response to exogenous tachykinin (75,76). In the present study, the effect of 5-HT antagonists, such as ketanserin or methysergide on the response to hyperpnea challenge in the rat have not been tested. However, mast cells are unlikely to contribute significantly to the airway responses because of the completeness of the blockade by NK and LTD4 receptor antagonists. Interestingly, an antihistamine does not alter the response to hyperpnea challenge in the guinea pig nor does the concentration of histamine increase in the bronchoalveolar lavage fluid after challenge (166), arguing that the mast cell is not triggered in the guinea pig despite evidence that tachykinins administered

exogenously can trigger these cells in this species (91). Perhaps the sites of action of endogenously released tachykinins are more limited than those of exogenously administered tachykinins.

Eicosanoids are important contributors to dry gas hyperpnea-induced bronchoconstriction in the guinea pig (55) and the dog (51,126). Dry gas-induced bronchoconstriction is inhibited by pre-treatment of the guinea pig with the cyclooxygenase inhibitor piroxicam, the 5-lipoxygenase inhibitor A-63162, the LTD₄ antagonist ICI-198,615 or the 5-lipoxygenase inhibitor MK-0591 in the dog (126). The mechanism of release of eicosanoids has not been established. However, a direct link between tachykinins and LTD₄ in hyperpnea-induced airway responses in the guinea pig has been established by the demonstration that NK antagonists inhibited the synthesis of cysteinyl-leukotrienes which are evoked by dry gas hyperpnea challenge. A similar relationship seems likely in the rat given the efficacy of the selective LTD₄ antagonist pranlukast in attenuating the effects of dry gas challenge. However, we were unable to evaluate the possible effect of NK antagonists on leukotriene synthesis in the current experiments because the changes in biliary N-acetyl-leukotriene E₄ after dry gas challenge were not significantly different from those observed in the wet gas control animals. The cells of origin of the LTD₄ responsible for the airway response require elucidation but macrophages are a possible source (18).

Exogenous LTD₄ administration cause a bronchovascular leak in guinea pigs and this LTD₄-induced bronchovascular leak is prevented by pranlukast. In this study, pranlukast and SR-48968, but not CP-99994 effectively prevent the elevation of Evans blue dye extravasation in BN rats. These results suggest that tachykinins and leukotrienes do play a

role in dry gas hyperpnea-induced bronchovascular leakage in different species.

It was anticipated that there might be a relationship between the magnitude of airway responses to hyperpnea challenge and the responsiveness to methacholine, supposedly a measure of airway responsiveness to all bronchoconstrictive mediators. However there was no correlation between the magnitude of the airway response to hyperpnea challenge and the reported airway responsiveness to inhaled methacholine in the four rat strains studied. Fisher rats are much more responsive to methacholine than the other three strains but did not show significant bronchoconstriction after dry gas challenge (41). However the relative airway responses of some rat strains to LTD4 administered by insufflation differed from those results reported for methacholine provocation testing. The BN rats were more responsive to LTD4 than either ACI or Fisher rats, which may account in part for the greater responses of this strain to hyperpnea challenge. Whether the atopic character of the BN rat has any relationship to its responsiveness to hyperpnea challenge remains uncertain but it is not implausible that the dependence on cys-LTs of both responses may be the link between hyperpnea and allergen induced airway responses.

In contrast to the modest changes in pulmonary resistance in four of the strains following hyperpnea challenge there was significant microvascular leak in all four rat strains. This indicates that the development of airway edema is not sufficient to cause detectable changes in lung mechanical properties. A further difference between changes in pulmonary resistance and microvascular leak is the finding that both NK1 and NK2 antagonists inhibited the former whereas only the NK2 antagonist inhibited the latter. Hyperpnea with dry gas has also been reported to cause bronchovascular leakage in other animals (56,93,99). In dogs,

leakage occurs immediately after hyperpnea ceases and continues for at least 24 hours after the initial challenge (125). Microvascular leakage also has been demonstrated to occur in the guinea pigs with dry gas hyperpnea challenge (55) but in contrast to the present results, the inhibition of the phenomenon is only partially prevented by LTD4 and tachykinin receptor antagonists. Joos and coworkers have demonstrated that capsaicin causes plasma extravasation in airways of Fisher rats which was effectively inhibited by a specific NK1 receptor antagonist RP-67580 (58). The difference between the present observation and their findings suggests that tachykinins released by hyperpnea challenge may also cause microvascular leak by indirect mechanisms. Therefore, the action of the NK2 antagonist may not be on vascular cells but rather on cells synthesizing LTD4 in BN rats is postulate based on these data.

In conclusion, dry gas hyperpnea challenge causes a complex airway reaction involving bronchoconstriction and airway microvascular leak in rats. The increase in R_L in response to dry gas hyperpnea challenge is strain related and is greatest in the atopic strain, the BN. Tachykinins and cys-LTs released after dry gas hyperpnea challenge cause an increase in R_L in BN rats. The effect of the tachykinins is presumably mediated by an indirect mechanism. Airway microvascular leak occurred in response to dry gas hyperpnea challenge in all four rat strains suggesting that bronchoconstriction, but not the increase in airway microvascular permeability plays a crucial role in elevation of pulmonary resistance in BN rats. This is also suggested by the fact that NK2 but not NK1 receptors are responsible for the dry gas induced airway microvascular leakage whereas both receptor subtypes participate in the increase in pulmonary resistance that is observed.

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