MECHANISMS OF HYPERRESPONSIVENESS IN THE HUMAN NASAL AIRWAY: ROLE OF KININS AND NITRIC OXIDE

A thesis submitted by

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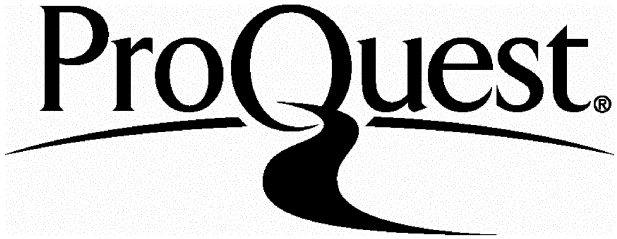

ABSTRACT

Allergic rhinitis is a condition which affects over 15% of the population in the United Kingdom. The pathological process involves two stages - nasal inflammation, and the development of a nasal hyperresponsiveness to allergen and a range of other non-specific stimuli. There is currently little information on the pathological process underlying hyperresponsiveness. This thesis presents an investigation into the potential role of kinins and nitric oxide in causing nasal hyperresponsiveness in man.

In the non-allergic subject, platelet activating factor (PAF) can be used to induce a nasal hyperresponsiveness which is similar to that observed in allergic rhinitis. The data obtained suggests that PAF-induced nasal hyperresponsiveness is mediated by an action of kinins at the bradykinin B_2 receptor. Experiments in subjects with seasonal allergic rhinitis also imply a similar role for kinins in allergen-induced hyperresponsiveness. Furthermore, the kinins may be involved in the recruitment of inflammatory cells which is seen in the hyperresponsive state. Data is presented which indicates that kinin generation can potentiate inflammatory cell recruitment, both *in vitro* and *in vivo* in the human nasal airway. However, application of exogenous bradykinin alone does not cause a nasal hyperresponsiveness, nor an influx of inflammatory cells. While suggestions are made as to the precise role of kinins, the mechanism does not appear to be dependent on subsequent neuropeptide release. Interestingly, modulating the degree of nitric oxide in the human nasal airway can also induce a hyperresponsiveness.

The data therefore imply that activation of the bradykinin B_2 receptor is necessary in both PAF- and allergen-induced nasal hyperresponsiveness in man. In addition, the generation of kinins may be important in the recruitment of inflammatory cells in allergic rhinitis.

CONTENTS

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PRESENTATIONS AND PUBLICATIONS

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ABBREVIATIONS

AAPVK	N-methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone
ACE	Angiotensin converting enzyme
[Ad]-BK	[1-adamantane acetyl-D-Arg^{0}, Hyp^{3}, Thi^{5,8}, D-Phe^{7}]-bradykinin
Ag	Antigen
AHR	Airway hyperresponsiveness
Amin.	Minimal cross-sectional area of the nasal airway
ANOVA	Analysis of variance
Antag	Antagonist
AUC	Area under curve
BALF	Bronchoalveolar lavage fluid
BK	Bradykinin
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CGRP	Calcitonin-gene related peptide
cNOS	Constitutive nitric oxide synthase
COX	Cyclooxygenase
Dil	Diluent
DMEM	Dulbecco's modified Eagle's medium
D-NAME	N^{G}-nitro-D-arginine methyl ester
ECP	Eosinophil cationic protein
EDHF	Epithelium-derived hyperpolarising factor
EDN	Eosinophil-derived neurotoxin
EDRF	Endothelium-derived relaxing factor
ELISA	Enzyme-linked immunosorbent assay
eNANC	Excitatory non-adrenergic, non-cholinergic
EpDRF	Epithelium-derived relaxing factor
EPO	Eosinophil peroxidase
FAD	Flavin adenine dinucleotide
FCS	Foetal calf serum
FMLP	N-formyl-met-leu-phe
GM-CSF	Granulocyte/macrophage-colony stimulating factor
HBSS	Hank's buffered salt solution
Hist	Histamine
HMWK	High molecular weight kininogen
HNE	Human neutrophil elastase
HSA	Human serum albumin
HUVECs	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
Icat	Icatibant
IFN	Interferon
IgA, IgE, IgG	Immunoglobulin A, Immunoglobulin E, Immunoglobulin G

IL-	Interleukin-
iNANC	Inhibitory non-adrenergic, non-cholinergic
iNOS	Inducible nitric oxide synthase
L-arg	L-arginine
LFA-1	Leukocyte function associated molecule -1
LMWK	Low molecular weight kininogen
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
LT	Leukotriene
LX	Lipoxin
MBP	Major basic protein
MGG	May-Grunwald-Giemsa
MIP-1α	Macrophage inflammatory protein-1α
mRNA	messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NANC	Non-adrenergic, non-cholinergic
NED	N-(L-naphthyl)-ethylene diamine
NEP	Neutral endopeptidase
NK	Neurokinin
NLF	Nasal lavage fluid / nasal lavage
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
%NSM	Percentage net stimulated migration
PAF	Platelet activating factor
PAR	Perennial allergic rhinitis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDE4	Phosphodiesterase IV
PG	Prostaglandin
PK	Plasma kallikrein
PLA_2	Phospholipase A_2
RIA	Radioimmunoassay
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute medium
Sal	Saline
SAR	Seasonal allergic rhinitis
Sub P	Substance P
TAME	N-α-*p*-tosyl-L-arginine methyl ester
TK	Tissue kallikrein
TNFα/β	Tumour necrosis factor α/β
Tx	Thromoboxane
VCAM-1	Vascular cell adhesion molecule-1
VIP	Vasoactive intestinal peptide
VLA-4	Very late antigen-4

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Allergic rhinitis and asthma are two of the most common immunological disorders producing chronic disease in man. Allergic rhinitis alone affects over 15% of the general population (BSACI, 1994). In both diseases, a local allergic reaction results in an inflammatory response, causing narrowing of the airway, wheezing and mucus secretion in asthma; and nasal congestion, rhinorrhoea ('runny nose'), pruritis (itching) of the nose and sneezing in allergic rhinitis. Continued exposure to allergen induces a state of chronic allergic inflammation. Contributing to this is the development of airway hyperresponsiveness (AHR), which results in the amplification of any subsequent allergic reaction.

While a variety of pharmacological agents can be used to treat the initial inflammatory response, only steroids are effective in preventing AHR. The process by which AHR occurs remains unclear. If the mechanism of AHR can be described, then this may provide new pharmacological targets for the treatment of chronic allergic airway disease. While allergic rhinitis is not life-threatening, the same is not true for chronic asthma, and the problem is compounded by the adverse effects associated with long-term steroid use.

Unfortunately, research into airway allergy using animal models and *in vitro* experiments are of limited value, since they are not representative of the situation in man (Pretolani & Vargaftig, 1993; Persson *et al.*, 1997). However, the human nasal airway is reasonably accessible for *in vivo* research into the development of nasal AHR, and may also yield some insight into the mechanism of AHR in asthma. The aim of this study was to investigate the mechanisms underlying AHR in the human nasal airway.

1.2 The human nose

The human nose has an important physiological role which is often under-appreciated. It consists of a cavity divided into two halves by the nasal septum. Each side of the cavity is also divided into two parts, the external and internal nasal cavity. The former projects from the surface of the face, includes the anterior nares or nostrils, and is continuous with the internal cavity which lies within the skull. Located on the lateral walls of the internal nasal cavity are three bony protrusions, referred to as the superior, middle and inferior turbinate bones (or conchae), as shown in Figure 1.1. All three bones are covered by a mucosal lining continuous with that of the nasal cavity. The inferior turbinate is the largest of the three turbinates, and protrudes significantly into the nasal cavity.

To prevent damage to the lungs, inhaled air must be cleaned, warmed and humidified before reaching the lower airways. The large surface area of the nasal cavity is increased by the turbinates, and provides a rich glandular and vascular supply which warms the air and raises its water content to 95% saturation. Filtration is achieved by nasal hair, cilia and a mucous layer which lines the epithelium of the mucosa. Fine particles are trapped in the sticky mucous layer, which is constantly regenerated by new glandular secretions. The older mucous is wafted by the cilia back towards the nasopharynx, thus giving rise to the term 'mucous escalator'. The system is so efficient that the human nose is able to filter particles as small as one micrometre in diameter.

The epithelium of the nasal mucosa consists of both ciliated and non-ciliated columnar epithelial cells, goblet secretory cells (which produce the mucous glycoproteins which constitute the bulk of the mucous layer) and basal cells. Underlying the epithelium is a submucosal layer, which is composed of mucous, seromucous and serous glands. The latter produce a number of proteins, many of which have bactericidal properties, including lysozyme, lactoferrin, neutral endopeptidase, various protease inhibitors and immunoglobulin A (Kaliner, 1994).

Figure 1.1: Internal structure of the human nasal airway (courtesy of Pharmacia & Upjohn).

The nasal mucosa is innervated by two different cranial nerves. The olfactory nerve (cranial nerve I) supplies the olfactory mucosa in the roof of the internal nasal cavity, and carries the sensation of smell. The remainder of the nasal mucosa, in particular that overlying the turbinates, is innervated by afferent fibres of the trigeminal nerve (cranial nerve V). The majority of these sensory nerves are unmyelinated C-fibres, and are responsible for the detection of both physical (e.g. abrasion) and noxious chemical stimuli (e.g. histamine). The nasal mucosa also receives an efferent nerve supply from the parasympathetic and sympathetic nervous systems, via the sphenopalatine ganglion and superior cervical ganglion respectively (Uddman & Sundler, 1986), as shown in Figure 1.2. In addition, a number of local nerve reflexes are present, which appear to modulate glandular secretion (Raphael *et al.*, 1991).

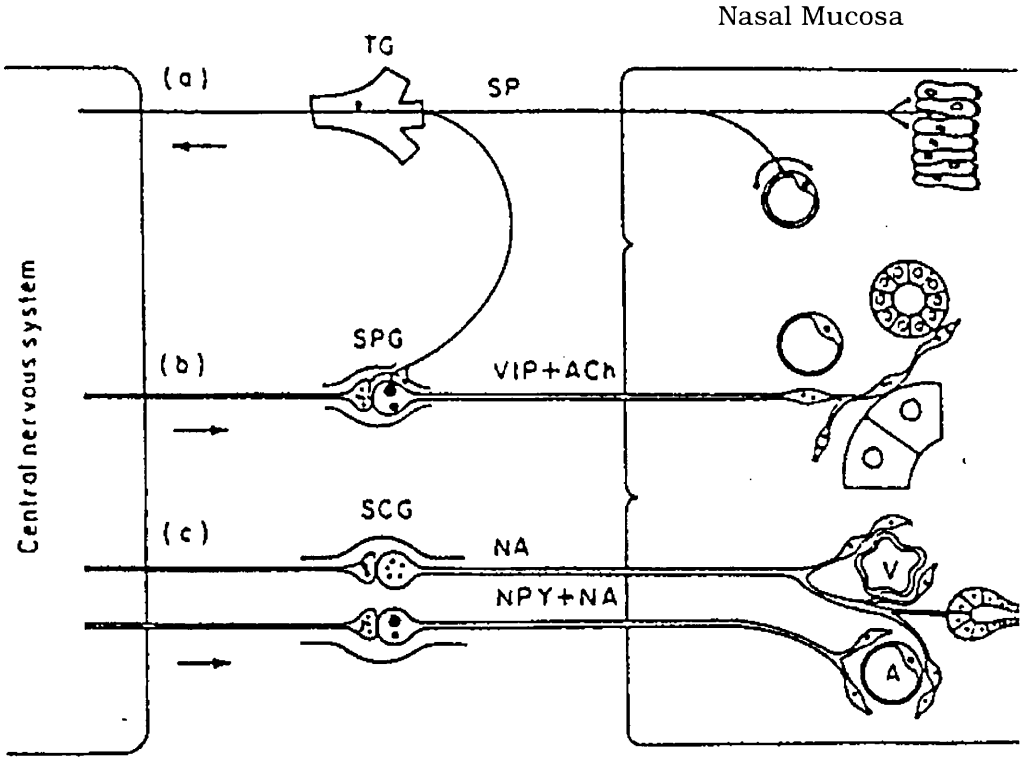

Figure 1.2: Diagrammatic representation of the nerve supply to the human nasal mucosa. (a) Trigeminal nerve; (b) Parasympathetic nerves; (c) Sympathetic nerves. TG = trigeminal ganglion; SPG = sphenopalatine ganglion; SCG = superior cervical ganglion. SP = substance P; VIP = vasoactive intestinal peptide; ACh = acetylcholine; NA = noradrenaline; NPY = neuropeptide Y; A = arteriole; V = venule. (adapted from Uddman & Sundler, 1986).

Nasal congestion occurs as a result of increased blood flow to the nasal mucosa. Located between the capillaries and venules of the nasal mucosa are cavernous sinusoids, analogous to erectile tissue. These are usually in a contracted state and empty. However, if sympathetic tone to the blood vessels is reduced, the sinusoids rapidly fill with blood. This causes the nasal mucosa to swell, decreasing the diameter of the nasal airway and resulting in nasal blockage. The mucosa of the middle and inferior turbinates has the highest concentration of sinusoids, so these bones constitute the major site of resistance to airflow through the nasal airways. In about 80% of the population, the sympathetic tone to the blood vessels of the nasal mucosa of one nostril is greater than to the other, alternating approximately every four hours. This results in a phenomenon known as nasal cycling, where one side of the nasal cavity is more blocked than the other: alternating in a cyclic manner.

Nasal blockage can also be induced by exposure of the nasal mucosa to a vasodilator, either experimentally or as part of an allergic response. Many inflammatory mediators, such as histamine and bradykinin, cause nasal blockage in this way. In addition, the nasal airway can be further occluded by the extravasation of plasma into the nasal mucosa causing tissue oedema, together with an associated increase in glandular secretion.

Assuming that the nose is a simple tube through which air flows in a non-turbulent manner, one can apply Poiseuille's law:

$$I = \frac{\pi \Delta P . r^4}{8 \eta l} \qquad (1.1)$$

I = rate of airflow through the nasal airway
ΔP = pressure difference across the nasal airway
r = radius of tube (airway)
η = viscosity of air
l = length of nasal airway

Ohm's law can be used to describe the resistance to airflow (R) through the nasal cavities:

$$R = \frac{\Delta P}{I} \qquad (1.2)$$

ΔP = pressure difference across the nasal airway
I = rate of airflow through the nasal cavity

Substituting l for equation 1.1:

$$R = \frac{8\eta l}{\pi . r^4} \qquad (1.3)$$

Thus, the resistance to airflow through the tube is inversely proportional to the fourth power of the radius of the tube. In other words, a small decrease in the diameter of the tube (such as that caused by vasodilatation of the blood vessels on the nasal mucosa of the turbinates) results in very large increase in resistance to airflow. In reality, the nasal airway is not a simple tube, as it has a number of internal structures protruding into it, so the airflow is turbulent and not streamlined. Nonetheless, Poiseuille's law provides a reasonable approximation, demonstrating how a minor change in blood flow to the nasal mucosa will cause large changes in resistance to airflow i.e. nasal congestion.

1.3 Experimental techniques used in the investigation of allergic rhinitis

A number of different techniques have been employed to study the pharmacology of the human nasal airway, and the development of allergic rhinitis.

1.3.1 Induction of a physiological response in the nasal airway

In order to assess the mediators and inflammatory cells involved in allergic rhinitis, it is first necessary to administer allergen to the nasal mucosa. A number of different techniques have been developed to achieve this. These include using a nasal spray, instilling the allergen using a dropper, or applying paper discs containing the allergen on to a suitable location on the nasal mucosa, such as the inferior turbinate bone. A new method which has been developed is called the "nasal-pool" device, and involves sealing the base of the external nasal cavity with a Foley catheter, through which a solution of the allergen can be passed, and "pooled" in the cavity (Svensson *et al.*, 1998).

An alternative approach is to apply not the allergen but a putative inflammatory mediator (in solution) to the nasal mucosa, and then monitor the response, in order to assess whether the mediator might be responsible for some of the symptoms of allergic rhinitis. Such a procedure is called a nasal challenge.

1.3.2 Methods used to monitor the response of a stimulus in the nasal airway

(1) Assessment of the patency of the nasal airway:

The nasal patency, or resistance to airflow in the nasal airway, can be determined by a number of methods. Rhinomanometry is the direct measurement of the resistance to airflow, which assesses the airflow through the nasal cavity and the pressure difference across the nasal airway. It can be used to calculate the resistance to airflow using Ohm's law (Kern, 1973). However, this technique requires some expertise on the part of the subject, and is, therefore, not suitable for use in a clinical setting. An alternative method is acoustic rhinometry, which uses the sonar principle to make various anatomical measurements of the nasal cavity (Jackson *et al.*, 1977). Changes in the physical dimensions of the nasal airway, determined by acoustic rhinometry, have been found to reflect the resistance to airflow measured directly by rhinomanometry (Austin & Foreman, 1994a). Acoustic rhinometry provides a quick method by which nasal obstruction can be accurately quantified, with minimal subject training. A simpler, but less precise, method is the measurement of nasal inspiratory peak flow (Holmstrom *et al.*, 1990).

(2) Lavage of the nasal cavity to collect released substances and superficial cells:

A number of different techniques have been developed to assess mediator release into the nasal cavity. These include using a saline aerosol to wash the cavity, and then recovering the fluid by suction (Druce *et al.*, 1985) or by the subject expelling the fluid into a collection vessel (Linder *et al.*, 1988). Another method uses a modified Foley catheter to form a seal between the posterior nares and the nasopharynx. By asking the subject to extend their neck, fluid can then be instilled into the nose and recovered using the catheter. In a further modification, using the "nasal-pool" device described above, following challenge, the liquid can be used to lavage the same area of the nasal mucosa which was exposed to the challenge agent (Svensson *et al.*,

1998). In the method of Naclerio *et al.* (1983), sterile saline is instilled into the nasal cavity, with the head extended and the posterior nasal cavity sealed from the nasopharynx by the subject pressing their tongue against the upper palate. The head is then brought forwards, and the subject expels the fluid into a collection vessel. Another method involves using a latex plug to form a seal around the anterior nares, and with the neck flexed forwards, lavaging each side of the nasal cavity separately (Wihl *et al.*, 1995). This technique allows for the lavage of each nostril independently, thus permitting the assessment of contralateral differences in mediator release.

By using these techniques before and after a nasal challenge, it is possible to measure the release of a number of inflammatory mediators, including histamine, bradykinin and various eicosanoids, using an appropriate assay. Albumin levels, in nasal lavage fluid, are commonly used as a measure of plasma extravasation (Naclerio *et al.*, 1983). Furthermore, by detecting and quantifying cells isolated in the lavage fluid, it is possible to relate cell-derived mediators to the different cell types observed. The lavage fluid may also contain enzymes, which have been released following the nasal challenge. For example, the ability of the lavage fluid to release methanol from N-α-*p*-tosyl-L-arginine methyl ester (known as TAME esterase activity) can be measured. This is used as an index of the amount of kinin-forming enzymes, known as kallikreins, and mast cell tryptase present (Naclerio *et al.*, 1983).

There are a number of problems associated with the use of nasal lavage. The levels of a mediator in initial nasal washings is often very high, so it is necessary to wash the nose twice prior to a third washing which is then used as a baseline (Naclerio *et al.*, 1983). Nasal lavage has an unpredictable effect on nasal patency, so the two cannot be measured at the same time. Finally, the concentration of a substance in nasal lavage fluid does not necessarily reflect its concentration in the fluid lining the nasal mucosa. In fact, the proportion of lavage fluid consisting of nasal secretions can range from 4% to 48% (Balfour-Lynn, 1996). Nonetheless, studies using markers of dilution, such as inulin and radiolabelled albumin, have found no correlation between the proportion of nasal secretions and the concentration of inflammatory mediators in lavage fluid, implying that the degree of dilution is not a confounding variable (Bisgaard *et al.*, 1988 & 1990).

(3) Collection of nasal secretions:

Nasal secretion can be collected by asking the subject to blow the secretions into a piece of preweighed material. Alternatively, preweighed absorbent material (such as discs of filter paper) can be placed into the nasal cavity, and reweighed after a fixed time (Riccio and Proud, 1996). This latter technique also permits the secretions to be eluted from the material into a suitable buffer, and then measured using an appropriate assay. Secretions, such as mucus glycoprotein, lactoferrin and lysozyme, can also be assayed in nasal lavage fluid (Greiff *et al.*, 1993).

(4) Sampling of nasal tissue and cells:

In order to assess the histological and cytological changes in the nasal airway, a number of techniques have been developed to obtain cells from a deeper compartment than is obtained with nasal lavage. This include brushing the nasal mucosa (Pipkorn *et al.*, 1988), taking a scraping of the nasal epithelia with a special probe (Rhinoprobe) (Meltzer *et al.*, 1994), or nasal biopsy.

(5) Subjective assessment of nasal symptoms

The symptoms of allergic rhinitis, such as rhinorrhoea, pain, nasal obstruction, can be assessed subjectively using a visual analogue scale (Lebel *et al.*, 1988).

1.3.3 In vitro techniques

Recently, a number of *in vitro* techniques have been developed to investigate the pathology of allergic rhinitis using *ex vivo* human nasal tissue. For example, tissue obtained from nasal biopsy has been used to obtain nasal epithelial cells, which can then be cultured (Calderon *et al.*, 1997). Such an experimental model can be used to investigate the role of the epithelium in allergic rhinitis. Alternatively, tissue excised during surgery can be used to prepare membrane preparations for receptor binding studies (Dear *et al.*, 1996b), to obtain cells by tissue dispersion (Austin *et al.*, 1996), or for the preparation of explants to measure nasal mucous secretion (Mullol *et al.*, 1993). It is also possible to use the endothelium of nasal polyps to investigate the adhesion and subsequent accumulation of inflammatory cells into the nasal mucosa (Symon *et al.*, 1994).

1.4 Pathogenesis of allergic rhinitis

There are two distinct forms of allergic rhinitis, depending on the allergen to which the subject is sensitive. In perennial allergic rhinitis (PAR), the subject is exposed to the allergen throughout the year, while in subjects with seasonal allergic rhinitis (SAR), or hayfever, allergen exposure is limited to certain times during the year. Subjects with PAR are sensitive to allergens including those from the house-dust mite (*Dermatophagoides pteronyssinus*), other mites, animal danders and certain foods. In contrast, the allergens involved in SAR are tree and grass pollens, moulds and certain fungal spores.

Nonetheless, the general pathology of PAR and SAR are similar, and can be divided into two stages: nasal inflammation, and nasal hyperresponsiveness.

1.4.1 Nasal inflammation

When particles of allergen are first inhaled into the nasal cavity, they cross the nasal mucosa and are exposed to the immune system; in susceptible individuals, this results in sensitisation, as follows: The allergen is processed by Langerhan cells and other antigen presenting cells (Holm *et al.*, 1995) and presented to both T- and B-lymphocytes. The former release a range of cytokines, which activate the B-lymphocytes and stimulate them to synthesise immunoglobulin E (IgE). The IgE becomes fixed to cells by binding IgE receptors (known as FcεR1 receptors) on the surface of inflammatory cells, including eosinophils and mast cells.

If these cells subsequently encounter the same allergen, crosslinking occurs between IgE molecules on the cell surface, causing aggregation of the FcεR1 receptors. This activates the cells, releasing inflammatory mediators, including histamine, kinins and eicosanoids such as prostaglandin D_2 (PGD_2), as shown in Table 1.1. These mediators are responsible for the symptoms of allergic rhinitis. Nasal blockage results from a combination of increased blood flow to the nose, plasma extravasation and tissue oedema. Rhinorrhoea is a consequence of the stimulation of nasal secretory glands which produce a mixture of watery or serous secretion and viscous mucous

secretion, mixed together with extravasated plasma. Stimulation of sensory nerves in the nasal cavity cause sneezing and pruritis. In addition, there is a corresponding increase in TAME-esterase activity; this represents approximately 75% plasma kallikrein, 20% mast cell tryptase and 5% glandular kallikrein activity, and an increase in this is strongly associated with the symptoms of allergic rhinitis (Naclerio *et al.*, 1983).

The response of subjects with allergic rhinitis to intranasal challenge can often be divided into an immediate phase, occurring during the first 2 hours following exposure to antigen, and, in about 30-40% of subjects, a late phase occurring 6-12 hours later. The late phase is associated with the infiltration of a number of different inflammatory cells into the nasal airway, including eosinophils, neutrophils and T-lymphocytes. These cells release a variety of mediators including leukotrienes, cationic proteins such as eosinophil cationic protein (ECP), and cytokines.

Mediator	Immediate Phase	Late Phase
Histamine	✓✓	✓✓
TAME-esterase	✓✓	✓✓
Prostaglandin D_2	✓✓	×
Leukotrienes $LTB_4/C_4/D_4$	✓	✓
Kinins	✓✓	✓
Platelet Activating Factor (PAF)	✓	×
Albumin	✓✓	✓✓
Neuropeptides e.g. CGRP and VIP	✓	×
Substance P	✓	✓
Eosinophil-derived mediators e.g. ECP, MBP	×	✓✓
Interleukin-1 (IL-1)	✓	✓
IL-3, IL-4, IL-5, IL-6, TNFα	×	✓

Table 1.1: Mediators found in nasal lavage fluid collected during the immediate and late phase response, in atopic individuals challenged intranasally with allergen (Naclerio *et al.*, 1983).

Interestingly, experimental induction of allergic rhinitis in susceptible individuals, using exogenous antigen under controlled conditions, has demonstrated a number of differences between the two different forms of allergic rhinitis. In PAR, the predominant symptom is nasal blockage, while patients with SAR also present with marked rhinorrhoea and sneezing (G. Scadding, personal communication). Histamine H_1 antagonists inhibit sneezing and reduce the rhinorrhoea associated with allergic rhinitis, but has little effect on nasal blockage (Naclerio *et al.*, 1990), indicating that other mediators are involved in the allergic reaction. This may explain why histamine H_1 antagonists are useful in treating SAR, but are ineffective in reducing the immediate allergic response of PAR. In PAR, the kinins may be the major mediator causing symptoms (Dear, 1996). Icatibant, an antagonist at the bradykinin B_2 receptor, inhibits the nasal blockage in PAR (Austin *et al.*, 1994), but has little effect on the acute phase in SAR (Dear, 1996). This implies a major role for bradykinin in PAR, but not in SAR. In SAR, histamine appears to be the major mediator involved in the acute reaction. Interestingly, there is also evidence that the allergen (house dust mite) in PAR can generate kinin independent of the inflammatory response, either by the direct activation of kallikrein, the enzyme which generates kinins (Maruo *et al.*, 1991), or by possessing such biochemical activity itself (Stewart *et al.*, 1993).

1.4.2 Nasal airway hyperresponsiveness

Nasal hyperresponsiveness is a hallmark of allergic rhinitis (Druce *et al.*, 1985; Mullins *et al.*, 1989). Subjects with allergic rhinitis show an increased response to nasal challenge with a variety of stimuli, including histamine and bradykinin, both of which are released following allergen challenge. The nasal airway effectively becomes more sensitive to the allergen, contributing to the chronic allergic state. AHR is usually associated with the late phase reaction, but can continue well beyond this stage. In fact, it is induced irrespective of whether a late phase of inflammation occurs (Van Wijk *et al.*, 1992).

One important consideration is the type of response involved in AHR. In asthma, the hyperresponsiveness is manifested as an increase in the contractile response of the airway smooth muscle. However, there is little smooth muscle present in the human

nasal airway. The increased response in nasal AHR is probably caused by increased blood flow to the nasal mucosa. However, the same mediators may be involved in the development of AHR in both the upper and lower airways, though acting on different targets. Therefore, although one cannot simply extrapolate data on the mechanisms of AHR from the nasal airway to the lower airways, and vice versa, it is useful to compare the two.

AHR is also associated with increased mucus production and oedema following allergen challenge, in both the upper and lower airways. Therefore, AHR would appear to result from a direct or indirect potentiation of the overall receptor activation, and not simply from an action on a particular cell type alone. With this in mind, there are a number of potential mechanisms by which AHR might occur (Figure 1.2):

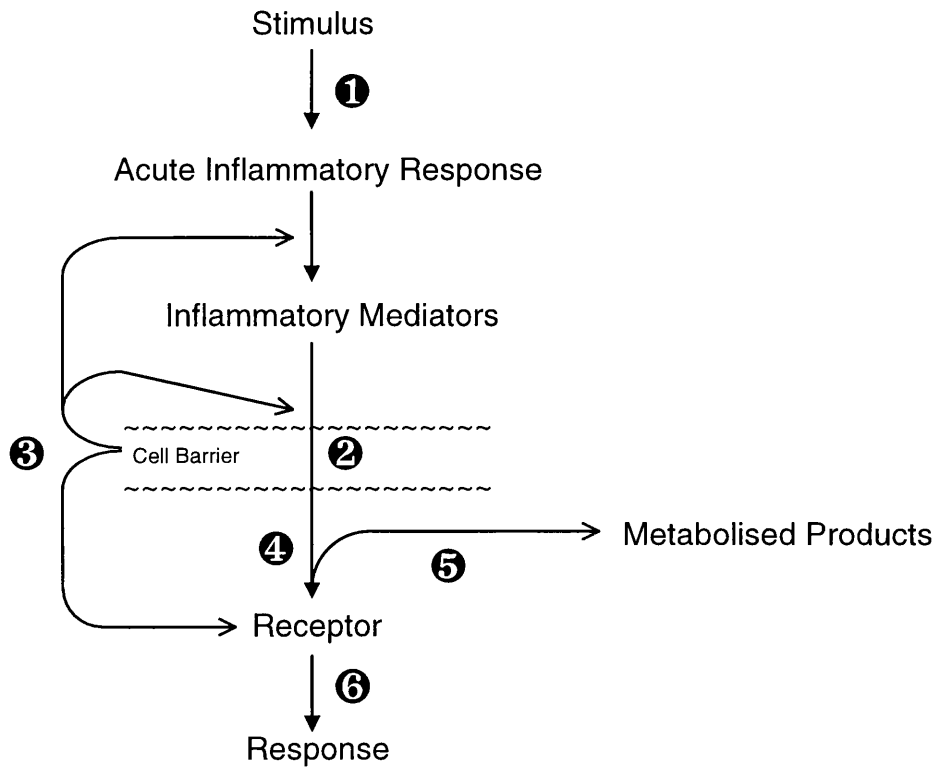

Figure 1.3: Potential mechanisms in the development of airway hyperresponsiveness.
❶ Inflammatory Cascade; ❷ Altered exposure of the receptor to the stimulus; ❸ Release of modulating agent by other cells; ❹ Change in the receptor properties; ❺ Altered metabolism of the mediator; ❻ Altered regulation of the intracellular signalling resulting in the response.

(1) The inflammatory cascade:

The initial exposure to allergen might trigger a cascade reaction, causing increased mediator release and, therefore, greater receptor activation. For instance, subjects with SAR experience increasingly severe symptoms as the pollen season continues. However, a number of inflammatory mediators can induce AHR in the absence of any other mediator. Furthermore, the development of AHR can occur independently of a chronic inflammatory response (Van Wijk *et al.*, 1992). It is likely, therefore, that AHR potentiates the effect of the inflammatory cascade seen in chronic allergic rhinitis, rather than being caused by it.

(2) Increased exposure of receptors on the nasal mucosa to the stimulus:

A common histological finding in chronic airway allergy is the damage and shedding of epithelial cells lining the airway. The airway epithelium can act as a physical barrier between the airway lumen and the underlying mucosa. Therefore, removal of these cells would increase the exposure of the receptors on the mucosa to any stimulus present, resulting in greater receptor activation and potentially, AHR. In the lower airways, removal of the airway epithelium increases the response of smooth muscle in both animal models and human bronchi *ex vivo* (Aizawa *et al.*, 1988). While there may be a link between epithelial shedding and the development of AHR in allergic individuals (Laitinen *et al.*, 1985), mediators which induce AHR on their own (e.g. platelet activating factor) do not cause epithelial shedding in the time taken for AHR to develop (Ohashi *et al.*, 1997). In a study by Riccio & Proud (1996), subjects with PAR showed an increased secretory response to bradykinin compared to non-allergic controls. Interestingly, bradykinin induced the same degree of albumin release, irrespective of the allergic status of the subject, implying that the availability of bradykinin to vascular receptors was not altered in the allergic state, even though AHR was observed. Furthermore, there is no evidence for increased epithelial permeability in subjects with allergic rhinitis (Svensson *et al.*, 1998). Alternatively, the loss of airway epithelial cell function, but not necessarily barrier function, may be sufficient to induce AHR (Hulsmann *et al.*, 1994). Mediators such as PAF cause AHR at doses which are toxic to airway epithelial cells, and can also damage the mucociliary clearance system associated with the airway epithelium, which may contribute to AHR (Ohashi *et al.*, 1997).

(3) Modulating agents:

The ability of acetylcholine to cause vasodilatation is dependent upon the release of nitric oxide from the endothelium (and possibly other mediators, collectively known as endothelium-derived relaxing factors). It has been proposed that airway epithelial cells may generate mediators, such as arachidonic acid metabolites, nitric oxide and the putative epithelium-derived relaxing factor (EpDRF) (also known as EDHF - epithelium-derived hyperpolarising factor) which modulate the responsiveness of the airways. However, the evidence for such a factor is equivocal (Holroyde, 1986; Aizawa *et al.*, 1988; Devillier *et al.*, 1988a; Sparrow *et al.*, 1995). Lactoferrin, a protein present in serous secretions in both the upper and lower airways, may have a protective function in the airways, since it prevents antigen-induced AHR in the lower airways of sheep by inhibiting mast cell-derived tryptase (Elrod *et al.* 1997). Though the precise role of tryptase in airway allergy is not fully known, it can induce AHR in the lower airways of sheep (Molinari *et al.*, 1996) and in human bronchi *ex vivo* (Johnson *et al.*, 1997), so a reduction in the secretion of lactoferrin may result in the susceptibility of the airways to tryptase-induced AHR.

(4) Receptors:

AHR may result from a change in the characteristics of receptors located on the nasal mucosa, for example, an increase in the receptor density or in affinity of a receptor for its agonist. It has been reported that cholinergic agonists, such as methacholine, cause more secretion in allergic subjects than in non-allergic controls, and this could be explained by an increase in the density of cholinergic receptors (White, 1993). However, a study by Van Megen *et al.* (1991a) demonstrated that the density of muscarinic receptors on nasal tissue from allergic subjects actually decreases slightly, possibly a result of adaptation due to overstimulation, although the authors also found that the remaining receptors exhibit an increased affinity for muscarinic agonists, which might contribute to AHR. No significant differences in receptor density or kinetics were identified in α- or ß-adrenoceptors (Van Megen *et al.*, 1991b). Therefore, it is likely that any change in receptor density or affinity in allergy are probably a consequence, rather than a cause, of AHR.

(5) Metabolism:

It has also been proposed that AHR may be a consequence of prolonged activity of a variety of mediators, as a result of a change in their metabolism. The epithelium may be important in the metabolism of various stimuli, particularly adenosine (Advenier *et al.*, 1988), tachykinins (Devillier *et al.*, 1988a) and acetylcholine (Koga *et al.*, 1992). Loss of epithelial cell function might reduce the metabolism of these mediators, enhancing their ability to activate receptors and effect a response. For example, the tachykinins are degraded by neutral endopeptidase-24.11 (NEP), and the activity of NEP is significantly lower in subjects with nasal AHR (Lacroix *et al.*, 1995). However, epithelial damage can apparently occur without the presence of AHR, and vice versa (Butterfield & Leiferman, 1993).

(6) Altered intracellular signalling:

Finally, the intracellular pathways which are activated by the receptor/agonist complex may be altered in the hyperresponsive state. For example, a number of mediators modulate the generation of cyclic guanosine monophosphate (cGMP) in cells. cGMP is an important stimulus of airway smooth muscle relaxation in animals (Sadeghi-Hashjin *et al.*, 1996a), so a decrease in cGMP may precipitate AHR. Inhibitors of phosphodiesterase IV (PDE4), an enzyme which metabolises cGMP, abolished antigen-induced AHR in some animal models (Teixeira *et al.*, 1997), though this was not observed in the lower airways of human subjects (Harbinson *et al.*, 1997). The role of PDE4 in the human nasal airway remains uninvestigated. Interestingly, inhibitors of nitric oxide synthase may induce AHR by decreasing cGMP production (Sadeghi-Hashjin *et al.*, 1996a), and it has been proposed that the generation of free-radicals in allergic inflammation causes a decrease in cGMP, resulting in AHR (Kips *et al.*, 1995). In the chronic allergic state, AHR may result, in part, from a change in gene transcription within cells (e.g. the NF-κB transcription system). This would alter intracellular signalling pathways which might be involved in the development of AHR, though the timescale of onset of hyperresponsiveness makes this process unlikely in the initial induction of AHR.

1.5 Mediators implicated in the development of AHR

In 1933, Sir Henry Dale described a number of criteria which should be used to identify potential inflammatory mediators. Applying these to the development of AHR:

- administration of the potential mediator should cause AHR *in vitro* and *in vivo.*
- the mediator should be present in subjects with AHR, at an appropriate concentration.
- The mechanisms involved in the generation of the mediator should be present and increased in AHR.
- A mechanism should exist to terminate the action of the mediator (important, because AHR is a reversible phenomenon).
- Antagonists of the mediator should reduce the induction of AHR.
- Receptors / signalling pathways should be present in the nasal mucosa, and be activated by the mediator.

The process by which AHR occurs remains unclear, no doubt due, in part, to the complexity of the mechanism and the difficulty in regulating the process under controlled experimental conditions. Nonetheless, because it is associated with the influx of cells to the site of inflammation, a number of hypotheses have been proposed involving mediators released from these cells.

One particular cell type, the eosinophil, has been implicated in AHR. In both SAR and PAR, antigen challenge results in an increase of eosinophils in the nasal mucosa and the release of various eosinophil-derived mediators (Bascom *et al.*, 1989; Svensson *et al.*, 1990; Knani *et al.*, 1992; Kato *et al.*, 1995). Eosinophils contain distinct preformed proteins, stored within their granules, which are released upon activation, but can also synthesise many other mediators. The latter can be divided into three sub-groups, namely lipids derived from arachidonic acid, peptides and cytokines, as shown in Table 1.2. Many of these mediators have been implicated in the pathology of allergic rhinitis and the induction of AHR. However, the majority of these mediators can also be generated by other cells, including epithelial cells and neurones. Therefore, the remainder of this introduction will review the possible role of these mediators in the development of AHR.

Mediator	Generation / Localisation	Potential role in allergic airway disease
1) Lipid Mediators		
Platelet Activating Factor (PAF)	Acetylation of lysophospho-lipid, following stimulation with A23187, various chemo-tactants (C5a, FMLP or ECF-A) or via IgG pathway.	• Platelet aggregation • Increases vascular permeability • Bronchoconstriction • Migration and activation of eosinophils and neutrophils, causing mediator release, superoxide generation, and degranulation.
Leukotrienes	Metabolism of arachidonic acid via 5-lipoxygenase pathway, producing LTA_4 which is hydrolysed to LTC_4. Induced by A23187, PAF, IgG and IgE pathways. LTC_4 is the main product in eosinophils.	• LTC_4, and its derivatives LTD_4 and LTE_4, are potent stimulators of smooth muscle contraction, resulting in broncho-constriction and mucous secretion. • LTB_4 is not generally synthesised by eosinophils, since they lack the enzyme to convert LTA_4 to LTB_4.
Lipoxins	Action of 15-lipoxygenase pathway on arachidonate following stimulation with A23187, generating the lipoxins LXA_4 and LXC_4.	• LXA_4 antagonises many actions of LTB_4, in particular plasma extravasation and leukocyte migration; causes vasodilation. *In vitro*, inhibits LTB_4- and FMLP-induced neutrophil chemotaxis and activation. • LXA_4 is a potent activator of protein kinase C, more so than diacylglyceride. • Role of other lipoxins are not yet known.
Prostaglandins, Prostacyclins and Thromboxanes	Metabolism of arachidonic acid via the cyclooxygenase pathway. Main products in eosinophils are PGE_2 and TBX_2, though others are also formed. Production *in vitro* is stimulated by A23187.	• Not clear, but exogenous PGE_2 can inhibit eosinophil degranulation. • PGD_2, PGE_2 and PGI_2 are powerful vasodilators, acting in synergy with other vasodilators (e.g. histamine, bradykinin). Potentiate the actions of histamine and bradykinin in causing plasma leakage. • Probable autocrine and paracrine actions.
2) Peptide Mediators		
Substance P	Found in secretory granules within eosinophils; mRNA localised to granules suggests local synthesis. NB: Sensory nerves are the main source of neuropeptides in the human airway.	• Enhances proliferation of smooth muscle cells, lymphocytes, fibroblasts and endothelial cells. • Induces vasodilation, smooth muscle contraction and plasma extravasation. • Stimulates mast cell degranulation, with subsequent histamine release.
Vasoactive Intestinal Peptide (VIP)	As for substance P.	• Causes vasodilation and contraction of smooth muscle cells, stimulates secretion from mainly serous cells. • Stimulate cytokine release from T-cells.
3) Cytokines		
TGFα	mRNA detected in eosinophils by PCR and northern blotting.	Stimulate epithelial cell proliferation in airways
TGFß		Promotes ECM formation, IgA secretion
IL-1		General proinflammatory cytokine
IL-3		Required to promote eosinophil survival and inhibit apoptosis i.e. autocrine action
GM-CSF		
IL-5, IL-6, IL-8	Currently under investigation	IL-5 regulates eosinophil function.

Table 1.2: Eosinophil-derived mediators (adapted from Weller, 1993)

1.5.1 Lipid-derived mediators

1.5.1.1 Platelet activating factor

Platelet activating factor (PAF) is a naturally occurring phospholipid, and is the only endogenous compound known to induce AHR in both animals and man (Barnes *et al.*, 1988).

PAF was first isolated in 1972, when Benveniste *et al.* described a soluble factor, released from basophils stimulated by IgE, which caused platelet activation; the substance was therefore called platelet activating factor. Five years later, PAF was identified as an acetyl-glycerol-ether-phosphorylcholine (Benveniste *et al.*, 1979).

1.5.1.1.1 Synthesis of PAF

PAF is synthesised by a number of different cell types, including neutrophils, eosinophils, monocytes, alveolar macrophage, platelets, lymphocytes, epithelial cells and fibroblasts (Braquet & Rola-Pleszczynski, 1987), in response to a variety of stimuli including activated complement factor V, calcium ionophore A23187, thrombin, and possibly, via an IgE mediated pathway in macrophages (Barnes *et al.*, 1988). Although PAF was first identified as a product from rat basophils, human basophils fail to release PAF by either an IgE-dependent or independent pathway *in vitro* (Betz *et al.*, 1980). While human lung mast cells produce PAF, the very small quantities generated are not secreted (Schleimer *et al.*, 1986), and it is unclear whether mast cell-derived PAF is involved in the inflammatory response (Harvima & Schwatz, 1993).

PAF is not a single molecule, but rather, a group of ether-linked phospholipids which share a similar structure and are biologically active (McManus *et al.*, 1993). The most common form of PAF is 1-*O*-alkyl-2-acetyl-s*n*-glycero-3-phosphocholine, with alkyl groups of size C_{12}, C_{16} or C_{18}. PAF is most active as the C_{16} alkyl ether (Hanahan *et al.*, 1981).

There are two separate pathways for the synthesis of PAF, as shown in Figure 1.4. In inflammation and allergy, PAF is generated by the remodelling pathway, in which lyso-PAF is both the inactive precursor and metabolite (Page, 1994). In this pathway, activation of the enzyme phospholipase A_2 (PLA_2) cleaves a free fatty acid from an ether-lipid intermediate to form lyso-PAF. This is then acetylated by a second, rate-limiting enzyme called acetyl transferase. Acetyl transferase is located in the cytoplasm of a number of inflammatory cells (Barnes *et al.*, 1988). In patients with eosinophilia, this enzyme becomes unregulated, resulting in the high production of PAF (Snyder, 1985). It has been proposed that a similar process may occur in allergic individuals, causing a predisposition for abnormal eosinophil activation (Page & Coyle, 1990). Alternatively, PAF may be produced from the action of phosphocholine transferase on alkyl-lyso-glycerophosphate. This is known as the *de novo* pathway, and is thought to be responsible for producing endogenous PAF which is required for normal physiological functions (Snyder, 1990). Lyso-PAF is also the metabolite of PAF, formed as a result of the action of acetyl hydrolase on PAF.

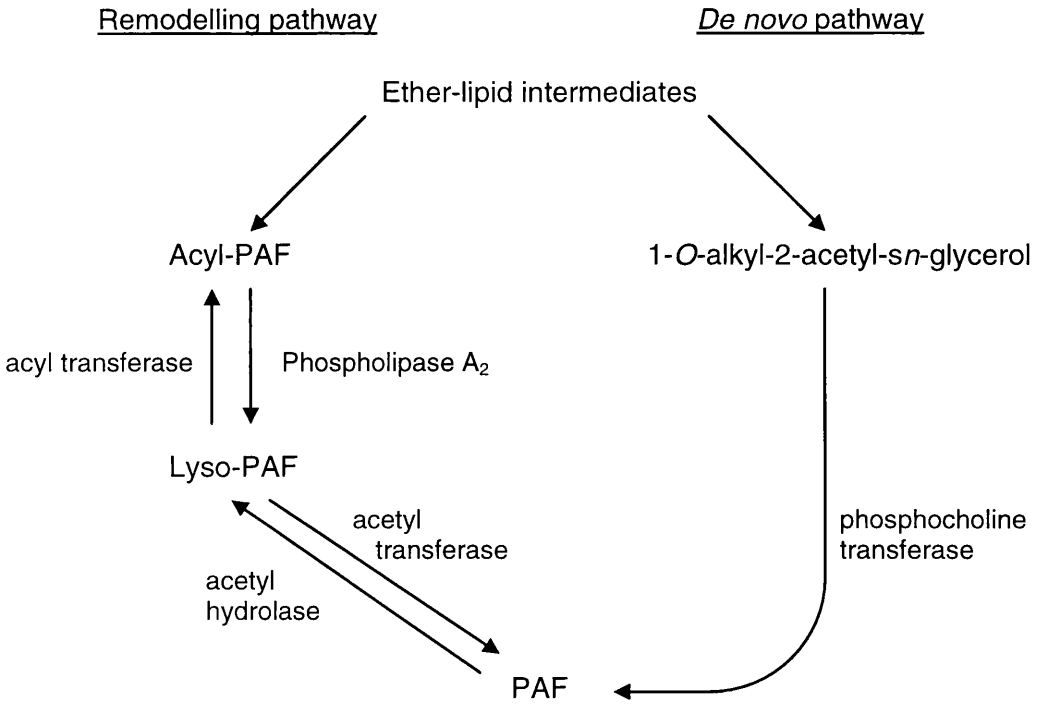

Figure 1.4: Synthesis of platelet activating factor.

1.5.1.1.2 PAF receptors

Originally, it was thought that there might more than one type of human PAF receptor, because two binding sites, of different affinities, were identified for PAF on human platelets (Valone *et al.*, 1982) and on guinea pig eosinophils (Kroegel *et al.*, 1989). However, it is now thought that there is a single human PAF receptor, with seven transmembrane domains and coupled to the $G_{q/11}$ and $G_{i/o}$ types of G-proteins (Honda *et al.*, 1994). PAF receptors have also been identified on neutrophils, eosinophils, macrophages, monocytes, smooth muscle cells and nerves (Chou & Olson, 1993). Activation of the receptor causes phosphoinositide metabolism, by activating phospholipases A_2 and C, increasing intracellular inositol triphosphate, diacylglycerol and $[Ca^{2+}]$. PAF can also induce the expression of a number of genes which modulate the cell cycle (Mazer *et al.*, 1991) and activate the transcription factor AP-1 in human bronchial epithelial cells (Le Van *et al.*, 1998). Interestingly, only a small proportion of the PAF generated by cells is released extracellularly, and the majority of the PAF remains within the cell. It has, therefore, been proposed that PAF may also have an important function as an intracellular messenger (Stewart & Harris, 1991).

1.5.1.1.3 Action of PAF in the human nasal airway

Application of exogenous PAF, at a dose of 157 µg or above, causes a dose-dependent increase in nasal obstruction, as well as rhinorrhoea, sneezing, pruritis and a burning sensation (Leggieri *et al.*, 1991; Tedeschi *et al.*, 1994b). The nasal obstruction occurs via a different mechanism to PAF-induced bronchoconstriction, since the latter involves the contraction of airway smooth muscle (Barnes *et al.*, 1988), while the nasal airway does not possess significant amounts of airway smooth muscle. PAF causes an eosinophilia in the human nasal airway (Klementsson & Andersson, 1992), and is a powerful chemotactant for neutrophils and eosinophils (Barnes *et al.*, 1988). It can also induce free radical production and leukotriene synthesis in these cells (O'Flaherty & Wykle, 1983).

1.5.1.1.4 Role of PAF in nasal airway hyperresponsiveness

In normal, non-atopic subjects, PAF induces a nasal hyperresponsiveness to histamine and bradykinin (Andersson & Pipkorn, 1988; Austin & Foreman, 1993) and causes an increased response to pollen in subjects with SAR (Andersson & Pipkorn, 1988). The resulting AHR is similar, in many ways, to that observed in allergic rhinitis, as PAF also causes a significant nasal neutrophilia and eosinophilia, together with raised ECP levels in nasal lavage fluid (Austin & Foreman, 1993; Tedeschi *et al.*, 1994a & 1994b).

Although PAF can induce AHR in the human nasal airway, its role in allergen-induced AHR is less well-defined. Antigen challenge with grass pollen in atopic individuals with SAR causes the release of lyso-PAF and PAF (Miadonna *et al.*, 1989; Shin *et al.*, 1994), but it is unclear whether PAF or lyso-PAF are released in PAR (Tsai *et al.*, 1995). One possibility is that any PAF generated is converted to lyso-PAF by the enzyme acetylhydrolase present in the lavage fluid. The activity of this enzyme in lavage fluid is significantly raised following grass pollen challenge in sensitive subjects (Shin *et al.*, 1994; Touqui *et al.*, 1994).

PAF may act directly on the nasal mucosa, but while radioligand binding studies indicate the presence of specific binding sites for PAF in human lung tissue (Hwang *et al.*, 1985), no such studies have yet been performed using tissue obtained from the human nose. One of the main features of PAF is its ability to attract and activate a variety of inflammatory cells, including eosinophils, neutrophils, monocytes, macrophage and platelets (Barnes *et al.*, 1988). PAF may also release from airway epithelial cells a range of chemotactic factors for neutrophils and eosinophils, including the cytokine GM-CSF (Arima *et al.*, 1995). The subsequent activation of these cells will cause the release of a range of mediators that act on other cells in the airway. Administration of PAF into the nasal airway also causes ECP release, which could contribute to the AHR (Austin & Foreman, 1993; Tedeschi *et al.*, 1994a).

If PAF is an important mediator of nasal AHR, one would expect PAF-antagonists to reduce leukocyte infiltration and inhibit antigen-induced AHR. In animal models of

allergic airway disease, PAF antagonists have been found to have this effect in some cases (Soler *et al.*, 1989; Coyle *et al.*, 1989; Ishida *et al.*, 1990), but not in others (Underwood *et al.*, 1992). Studies in human subjects have been equivocal. Preliminary data from Shin *et al.* (1994) indicate that the PAF antagonist WEB 2086, administered orally, did not affect antigen-induced AHR in the human nasal airway, and another study using the antagonist UK 74,505 produced similar results (Kuitert *et al.*, 1993). However, both these antagonists exhibit a low potency for PAF receptors, and therefore may have had only a weak inhibitory action at the doses used.

PAF causes mucosal dysfunction and damage, inhibiting ciliary action and increasing exfoliation of the epithelial lining of the airway, both *in vivo* in the lower airways of the rabbit (Ohashi *et al.*, 1997), and *in vitro* using explants from human nasal mucosa (Ganbo *et al.*, 1990). Interestingly, the latter study found that PAF is itself cytotoxic to epithelial cells, without requiring the production of other cytotoxic mediators. PAF-induced AHR in the human nasal airway was almost abolished by pretreatment with the antioxidant, vitamin E (Austin & Foreman, 1993); thus implying a mechanism which is dependent on the generation of free radicals. This supports the hypothesis that PAF causes tissue damage, perhaps via the generation of free radicals, which is independent of the PAF-induced release of other cytotoxic mediators. The source of the free radicals could be epithelial cells, so in effect, the epithelium may cause its own destruction (Webber *et al.*, 1992).

It is unclear, however, whether sufficient quantities of PAF are produced in the antigen-induced allergic response to cause AHR, and there are probably differences in the underlying mechanisms of AHR induced by PAF, and by antigen.

1.5.1.2 Leukotrienes

1.5.1.2.1 Synthesis and action of leukotrienes in the human nasal airway

The action of PLA_2 on membrane phospholipids results in the production of arachidonic acid. This can then be converted by the enzyme 5-lipoxygenase to leukotriene A_4 (LTA_4), a short-lived intermediate, which is further metabolised to LTB_4

(by LTA_4 hydrolase) or to LTC_4 (via LTC_4 synthase), and released into the extracellular environment. Cleavage of the peptide groups of LTC_4 results in the formation of the other peptidyl-cysteinyl leukotrienes, LTD_4 and LTE_4. Two classes of leukotriene receptors have been identified in man, namely the BLT receptor, which are activated by LTB_4, and $CysLT_1$ and $CysLT_2$ receptors, through which the cysteinyl leukotrienes act. In the human lower airways, the cysteinyl leukotrienes are thought to act solely through the $CysLT_1$ receptor (Coleman *et al.*, 1995).

Although the cysteinyl leukotrienes are produced by a number different cell types, including mast cells, macrophages, neutrophils and airway epithelial cells, eosinophils are the main source of LTC_4 in the human airway (Butterfield & Leiferman, 1993). It causes smooth muscle contraction and vasodilatation. In the human nasal airway, LTD_4 induces an increase in nasal blood flow (Bisgaard *et al.*, 1986), resulting in nasal obstruction (Okuda *et al.*, 1988). Although human eosinophils cannot produce LTB_4 because they lack the enzyme needed for its synthesis, it is the main lipoxygenase product in neutrophils, and acts as a potent stimulus for leukocyte infiltration and subsequent degranulation (Weller, 1993).

1.5.1.2.2 Role of leukotrienes in nasal airway hyperresponsiveness

The leukotrienes are generated in both the immediate and late phases following antigen challenge in subjects with SAR (Naclerio *et al.*, 1985), and during the immediate phase in PAR (De Graaf-in't Veld *et al.*, 1996). The cysteinyl leukotrienes cause a long-lasting eosinophil infiltration, and have been associated with AHR in the lower airways in rats (Wang *et al.*, 1993) and in man (Christie *et al.*, 1992a); both these actions appear to be dependent on eosinophil activation.

Although inhibitors of leukotriene synthesis reduce the nasal blockage experienced following challenge with grass pollen in allergic subjects (Knapp, 1990; Howarth, 1995), no studies have investigated the effect of such drugs on AHR in the human nasal airway, though zileuton, a lipoxygenase inhibitor, reduced antigen-induced AHR in the lower airways of asthmatics (Fischer *et al.*, 1995). Pharmacological intervention can also be achieved at the level of the receptor, and a number of leukotriene

receptor antagonists have been developed which inhibit AHR in animal models (Obata *et al.*, 1994). Many of these are currently under study in man, including pranlukast, which may reduce AHR in asthmatics (Hamilton *et al.*, 1998), and montelukast, which has recently been approved for use in the USA. The role of LTB_4 in AHR is not clear, but LTB_4 antagonists inhibit antigen-induced AHR in the lower airways of primates (Turner *et al.*, 1996) and the guinea pig (Seeds *et al.*, 1995).

1.5.1.2.3 Lipoxins

The lipoxins are acyclic eicosanoids, formed as a result of the action of 5-, 15- and possibly 12-lipoxygenase on arachidonic acid. The main two products are lipoxin A_4 (LXA_4) and LXB_4. The physiological role of the lipoxins is not fully known (Serhan, 1991). LXA_4 appears to antagonise many of the actions of LTB_4, including plasma extravasation and leukocyte migration (Hedqvist *et al.*, 1989). The lipoxins remain under-investigated with respect to their role in allergic airway disease. One study in asthmatic subjects found that although LXA_4 did not alter the response to allergen challenge, it did inhibit the AHR induced by LTC_4 (Christie *et al.*, 1992b).

1.5.1.3 Prostaglandins and Thromboxanes

Prostaglandins and thromboxanes together constitute a group of mediators known as the prostanoids.

1.5.1.3.1 Synthesis and action of the prostanoids in the human nasal airway

The prostanoids are generated from arachidonic acid, by the action of cyclooxygenase (COX). There are two known isoforms of this enzyme: COX-1 is a constitutive enzyme, thought to be responsible for the basal release of prostanoids which are required to maintain normal physiological function. COX-2 is an inducible isoform, the expression of which is caused by a variety of other inflammatory mediators such as cytokines (Mitchell *et al.*, 1995). The expression of COX-2 is modulated by the transcription factor NF-κB, and this may explain the inhibitory action of corticosteroids on COX-2 expression. The prostanoids acts through specific cellular

receptors which have been classified (Coleman *et al.*, 1994). The prostaglandins PGD_2 and PGE_2, and PGI_2 (prostacyclin) are powerful vasodilators, and can act in synergy with other vasodilators (e.g. histamine, bradykinin) to increase blood flow and cause plasma extravasation.

1.5.1.3.2 Role of the prostanoids in nasal airway hyperresponsiveness

The prostaglandins PGD_2 and PGE_2 are detected at increased levels in nasal lavage fluid following allergen challenge in subjects with SAR (Sugimoto *et al.*, 1994; Wagenmann *et al.*, 1996) and PAR (Ramis *et al.*, 1991), but only in the early response and not the late phase of inflammation (Naclerio *et al.*, 1985). Inhibitors of COX do not affect the response to antigen in the human nasal airway (Naclerio *et al.*, 1985). PGE_2 is synthesised by the airway epithelium and has been proposed as a possible EpDRF (Folkerts & Nijkamp, 1998). In the lower airways, AHR may result from epithelial damage, reducing PGE_2 generation by epithelial cells and, therefore, decreasing its relaxant effect on airway smooth muscle (Folkerts & Nijkamp, 1998). However, in the human nose, any action of PGE_2 would presumably have to be on blood vessels (since there is little airway smooth muscle in the human nose), and one would also expect a decrease in PGE_2 release if it was involved in AHR, yet the opposite is true. Thromboxane A_2 (TxA_2) may mediate AHR in animal models (Chung *et al.*, 1986; Lanes *et al.*, 1986), and might do likewise in man (Jones *et al.*, 1992). Inhibition of thromboxane synthesis reduces AHR and also inhibits airway eosinophilia after allergen challenge (Itoh *et al.*, 1996). The thromboxanes may, therefore, have an important role in upregulating the eosinophil-associated response (Weller, 1993). The contribution of thromboxanes to nasal allergy remains undefined, and more research is needed to investigate their potential role in the development of AHR.

1.5.2 Eosinophil granule-derived proteins

Eosinophils contain granules composed of four basic proteins. The core of these granules is major basic protein (MBP), while the matrix surrounding the core is composed of eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) (Gleich *et al.*, 1994). The granules themselves

appear to be divided into two populations, those which contain EPO, and a less dense sub-group which is peroxidase-negative (Venge, 1993). It has been reported that *in vitro*, the release of these proteins may be selective, depending on the stimulus used. For example, stimulation with IgG caused ECP but not EPO release, while IgA caused the release of all the eosinophil proteins (Tomassini *et al.*, 1991). However, there is no evidence for the selective release of eosinophil proteins *in vivo* (Venge, 1993), and other research groups have failed to reproduce the results of Tomassini *et al.* (G.Gleich, personal communication). The possible roles of these proteins in allergic airway disease are described in Table 1.2.

The levels of ECP, EPO and MBP are raised following antigen challenge in allergic rhinitis (Knani *et al.*, 1992; Kato *et al.*, 1995; Nishioka *et al.*, 1995; Shin *et al.*, 1994), and these increases often correspond with the presence of AHR. MBP and other cationic proteins, including the synthetic protein poly-L-lysine, cause AHR in the lower airways of rats (Coyle *et al.*, 1993), which appears to be dependent on their cationic charge. However, no study has yet been conducted to investigate whether these cationic proteins can themselves induce AHR in the human nasal airway.

Protein	Cell content ($\mu g/10^6$ cells)	Role in allergic airway disease
MBP	9	• causes histamine release from basophils and mast cells • cytotoxic for human epithelial cells • causes bronchoconstriction and induces airway hyper-responsiveness in animals. • activates neutrophils
ECP	5	• causes histamine release from mast cells • cytotoxic to a variety of cells
EDN	3	• undefined
EPO	12	• cytotoxic to airway epithelium • provokes bronchoconstriction in animals • in the presence of H_2O_2 and halide, causes mast cell degranulation and histamine release and inactivates leukotrienes

Table 1.3: Cationic proteins found in eosinophil granules (Gleich *et al.*, 1994)

The mechanism of MBP-induced AHR is unknown, though it may be dependent on causing epithelial damage (Gleich *et al.*, 1988), since MBP only induces AHR in guinea pig tracheal preparations with an intact epithelium (Flavahan *et al.*, 1988). However, Coyle *et al.* (1993) observed that cationic proteins induce AHR in the rat lower airway without causing any epithelial damage. Furthermore, although all the eosinophilic cationic proteins are cytotoxic to the airway epithelium (Gleich, 1989), only MBP caused AHR in a study on primates (Gundel *et al.*, 1991). In the lower airway of the rat, AHR induced by MBP or poly-L-lysine is abolished by both neurokinin NK_1 receptor (Coyle *et al.*, 1994) and bradykinin B_2 receptor antagonists (Coyle *et al.*, 1995), indicating a role for the tachykinins and kinins in the development of AHR. It is also possible that eosinophil cationic proteins act on other inflammatory cells to generate the conditions required for AHR (Butterfield & Leiferman, 1993). While there is evidence for a relationship between ECP levels in nasal lavage and nasal AHR (Linder *et al.*, 1987), this has not always been reproduced in other studies (Andersson *et al.*, 1989; Klementsson *et al.*, 1991), so ECP is unlikely to be solely responsible for the induction of nasal AHR.

1.5.3 Neuropeptides

Neuropeptides are a group of peptides involved in neurotransmission. They include calcitonin-gene related peptide (CGRP), and the tachykinins substance P (sub P) and neurokinin A (NK-A).

1.5.3.1 Synthesis, metabolism and action of neuropeptides

The tachykinins are derived from precursor molecules, called preprotachykinins, which are expressed in nodose and jugular ganglion cells. However, some inflammatory cells can also generate tachykinins; these include macrophages (Ho *et al.*, 1997) and eosinophils (Aliakbari *et al.*, 1987). The tachykinins are mainly metabolised by neutral endopeptidase-24.11 (NEP) although a number of other enzymes, including angiotensin converting enzyme (ACE, kininase II) also have this action (Nadel, 1991). Three tachykinin receptor subtypes have been identified in man,

which are coupled to a G-protein that activates phospholipase C. The receptors have been characterised by the agonist potency of the tachykinins at the receptors:

NK_1 receptor:	Sub P	>	NK-A	>	NK-B
NK_2 receptor:	NK-A	>	NK-B	>>	Sub P
NK_3 receptor:	NK-B	>	NK-A	>	Sub P

CGRP has been found to be colocalised with sub P in nerves, and acts on specific G-protein linked receptors, that activate adenylate cyclase and raise intracellular cyclic AMP. The metabolism of CGRP is not fully known, although NEP may be involved (Katayama *et al.*, 1991).

1.5.3.2 Effect of neuropeptides in the human nasal airway

The nasal mucosa contains sensory nerves derived from branches of the trigeminal nerve, which form part of the non-adrenergic, non-cholinergic (NANC) nervous system. The release of neuropeptides from these nerves can generate a local axon reflex, which causes an increase in vascular permeability, plasma leakage and subsequent tissue oedema (Geppetti, 1993). This response is known as neurogenic inflammation, and is mediated by the tachykinin NK_1 and NK_2 receptors (Barnes, 1998).

Sub P, NK-A, CGRP, and gastrin-releasing peptide have all been identified within nerves in the nasal mucosa, by immunohistochemistry (Kaliner, 1994). In addition, the nasal mucosa is also innervated by parasympathetic nerves containing vasoactive intestinal peptide (VIP) (Said & Mutt, 1988), while sympathetic nerves contain neuropeptide Y (Baraniuk *et al.*, 1992). The submucosal glands and blood vessels are also innervated by nerves containing neuropeptides. Although nerves are the main source of neuropeptides in the nasal mucosa, neuroendocrine cells around the submucosal glands can also release neuropeptides (Fang & Shen, 1998). In addition, eosinophils are capable of producing VIP and sub P (Metwali *et al.*, 1994).

Application of exogenous tachykinins to the nasal mucosa causes nasal obstruction, plasma extravasation and, in some subjects, the recruitment of inflammatory cells (Braunstein *et al.*, 1991; Lurie *et al.*, 1994; Chatelain *et al.*, 1995). The data obtained from these studies imply that nasal blockage is probably mediated through the NK_1 receptor, while plasma extravasation and inflammatory cell recruitment are dependent on the activation of NK_2 receptors (Braunstein *et al.*, 1991). This is in agreement with the observation that in the human nasal airway, NK_1 receptors are localised to the epithelium, glands and blood vessels, while NK_2 receptors are limited to arterial vessels (Baraniuk *et al.*, 1991). CGRP causes nasal blockage, but does not affect vascular permeability (Guarnaccia *et al.*, 1994). Raphael *et al.* (1991) observed that VIP caused significant glandular secretion from explants of nasal tissue, while sub P and NK-A only had a weak effect; this is probably due to the innervation of the glands by VIP-containing parasympathetic nerves. Interestingly, sub P causes the release of histamine from human nasal mast cells *in vitro* (Schierhorn *et al.*, 1995). However, there is no evidence that the action of sub P *in vivo*, in the human nasal airway, is mediated by histamine (Braunstein *et al.*, 1991 & 1994). Exogenous tachykinins, applied to the nasal mucosa, appear to be almost exclusively metabolised by NEP, as phosphoramidon, an NEP inhibitor, significantly potentiates the action of the exogenous tachykinins, while the ACE inhibitor captopril has no effect (Chatelain *et al.*, 1995).

1.5.3.3 Role of the neuropeptides in nasal airway hyperresponsiveness

The levels of sub P, CGRP and VIP are increased in nasal lavage fluid following nasal challenge with grass pollen in atopic subjects (Mosimann *et al.*, 1993), but their role in AHR remains unclear. However, there is an inverse correlation between the presence of AHR and the activity of NEP in the human nose (Lacroix *et al.*, 1995). Furthermore, in both PAR and SAR, AHR to bradykinin appears to be mediated by neural reflexes (Riccio & Proud, 1996), which could conceivably include a role for neuropeptides.

Neuropeptides appear to be important in AHR in a variety of animal models. In the lower airway of the rat, AHR induced by eosinophil-derived cationic proteins is inhibited by neurokinin NK_1 receptor antagonists and capsaicin (Coyle *et al.*, 1994). In

the guinea pig, application of capsaicin to the lower airway (an action which depletes sensory nerves of neuropeptides) also abolishes antigen-induced AHR (Ladenius & Biggs, 1989; Matsuse *et al.*, 1991). The tachykinins, particularly sub P, may enhance eosinophil recruitment (Numao & Agrawal, 1992; Van Oosterhout, 1996) and, therefore, cause AHR via an eosinophil-dependent mechanism. However, the inhibition of AHR by capsaicin does not affect lipoxygenase activity or eosinophil infiltration, suggesting that neuropeptides cause AHR independently of, or after, eosinophil activation and leukotriene synthesis (Matsuse *et al.*, 1991).

Neurogenic inflammation may be a phenomenon only found in animals, since one study found no evidence of capsaicin-induced neurogenic inflammation in the human nasal airway (Greiff *et al.*, 1995). However, other studies, some utilising higher doses of capsaicin, have confirmed that neurogenic inflammation does occur in allergic rhinitis (Philip *et al.*, 1996; Sanico *et al.*, 1997). Furthermore, application of capsaicin reduces the symptoms caused by antigen challenge in SAR (Stjarne *et al.*, 1998) and PAR (Zhang *et al.*, 1995), though neither of these studies investigated AHR.

The development of AHR may result, at least in part, from an upregulation of neurogenic inflammation, possibly as a result of epithelial damage increasing the exposure of sensory nerves. A number of stimuli, including antigen (Mosimann *et al.*, 1993) and bradykinin (Baumgarten *et al.*, 1997), cause neuropeptide release in the human nasal airway, while histamine has a similar effect on human lung tissue *ex vivo* (Saria *et al.*, 1988). Increased exposure of sensory nerves to these stimuli may, in turn, increase the release of neuropeptides. This may directly cause an increase in neuropeptide-mediated symptoms such as nasal obstruction and rhinorrhoea: in effect the 'hyper'-response observed in AHR. However, there is no evidence for a reduction in epithelial barrier function, which could increase the exposure of sensory nerves, in allergic rhinitis (Riccio & Proud, 1996; Svensson *et al.*, 1998). Alternatively, the neuropeptides could potentiate AHR by acting on inflammatory cells (e.g. eosinophils) or by stimulating cytokine release from cells in the nasal mucosa, a process which occurs in the human nasal airway (Okamoto *et al.*, 1993).

There is also evidence to suggest that an inhibition of tachykinin metabolism may also contribute to AHR. Phosphoramidon, a NEP inhibitor, can cause AHR to antigen in the lower airways in guinea pigs (Kohrogi *et al.*, 1991), and to bradykinin in human subjects (Crimi *et al.*, 1995). NEP is present in the human nasal mucosa (Ohkubo *et al.*, 1994b), and application of phosphoramidon potentiates the activity of neuropeptides in the human nasal airway (Chatelain *et al.*, 1995). Therefore, epithelial damage may cause AHR by reducing the activity of NEP on airway epithelial cells. Lacroix *et al.* (1995) found that chronic inflammation and hyperreactivity in non-allergic rhinitis is associated with a decrease in NEP activity. However, other immunohistochemical studies have found no evidence for defective NEP activity in the airways of subjects with asthma or allergic rhinitis (Barnes, 1998), so it is doubtful whether a reduction in NEP activity actually contributes to AHR.

It is unknown which particular neuropeptides may be involved in the development of AHR. Sub P is the most potent endogenous mediator at the NK_1 receptor, but it does not induce AHR in the lower airways of sheep, but NK-A does (Abraham *et al.*, 1991). Differences in the location of the tachykinin receptor sub-types between the upper and lower airways, and also between species, may explain the inconsistency that NK_1 or NK_2 receptor antagonists prevent AHR in some studies but not in others. In chronic asthma, both receptor subtypes are upregulated (Bertrand & Geppetti, 1996), and this may happen in chronic allergic rhinitis as well. There are no reports investigating the role of CGRP in AHR.

1.5.4 Cytokines

1.5.4.1 The Th1/Th2 paradigm of cytokine release

Cytokines are intercellular messenger peptides which are released by a variety of cells to influence the activity of other cells. It is thought that they have a vital role in the development of chronic airway allergy (Barnes *et al.*, 1998). A particular profile of cytokine release is observed in allergic airway diseases, and these cytokines appear to be produced by CD4-positive T-lymphocytes of a particular phenotype. These cells have been classified as Th2 cells (T-helper 2 cells), in contrast to Th1 cells which

release a different range of cytokines, as shown in Table 1.4. This observation led to development of the Th1/Th2 paradigm, which proposes that atopy may be a result of an excessive Th2-type response compared to Th1. In atopic subjects, antigen appears to cause a Th2-like response, releasing IL-4 and IL-13 which expand the Th2 cell population, while inhibiting the differentiation of Th0 cells into Th1 cells. This local cytokine environment also stimulates the generation of IgE, which contributes to the chronic allergic state (Humbert & Durham, 1998). Indeed, it has been proposed that immunotherapy may cause a switch from Th2-like response to a Th1-type response, and this may explain the ability of immunotherapy to desensitise atopic subjects (S. Durham, personal communication).

A full review of the cytokines is beyond the scope of this introduction. However, three cytokines are of vital importance in the development and regulation of eosinophil function: the interleukins IL-3 and IL-5, and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF). These cytokines are essential for the accumulation of eosinophils, and to inhibit apoptosis and prolong the survival of eosinophils (Teixeira *et al.*, 1995). In particular, IL-5 is essential for the differentiation of progenitor cells into eosinophils (Sanderson, 1993). The Th2-like response may generate the environment needed to maintain the eosinophilic inflammation which is observed in chronic allergy, in both the upper and lower airways, as shown in Figure 1.5.

	Th1-type response	Th2-type response
Interferon-γ (IFNγ)	+++	-
Tumour necrosis factor-β (TNFβ)	+++	-
Interleukin-2 (IL-2)	+++	+
TNFα	+++	+
GM-CSF	++	++
IL-3	++	++
IL-10	+	+++
IL-13	+	+++
IL-4	-	+++
IL-5	-	+++

Table 1.4: Profile of cytokines release by Th1- and Th2-like T-lymphocytes (adapted from Humbert & Durham, 1998)

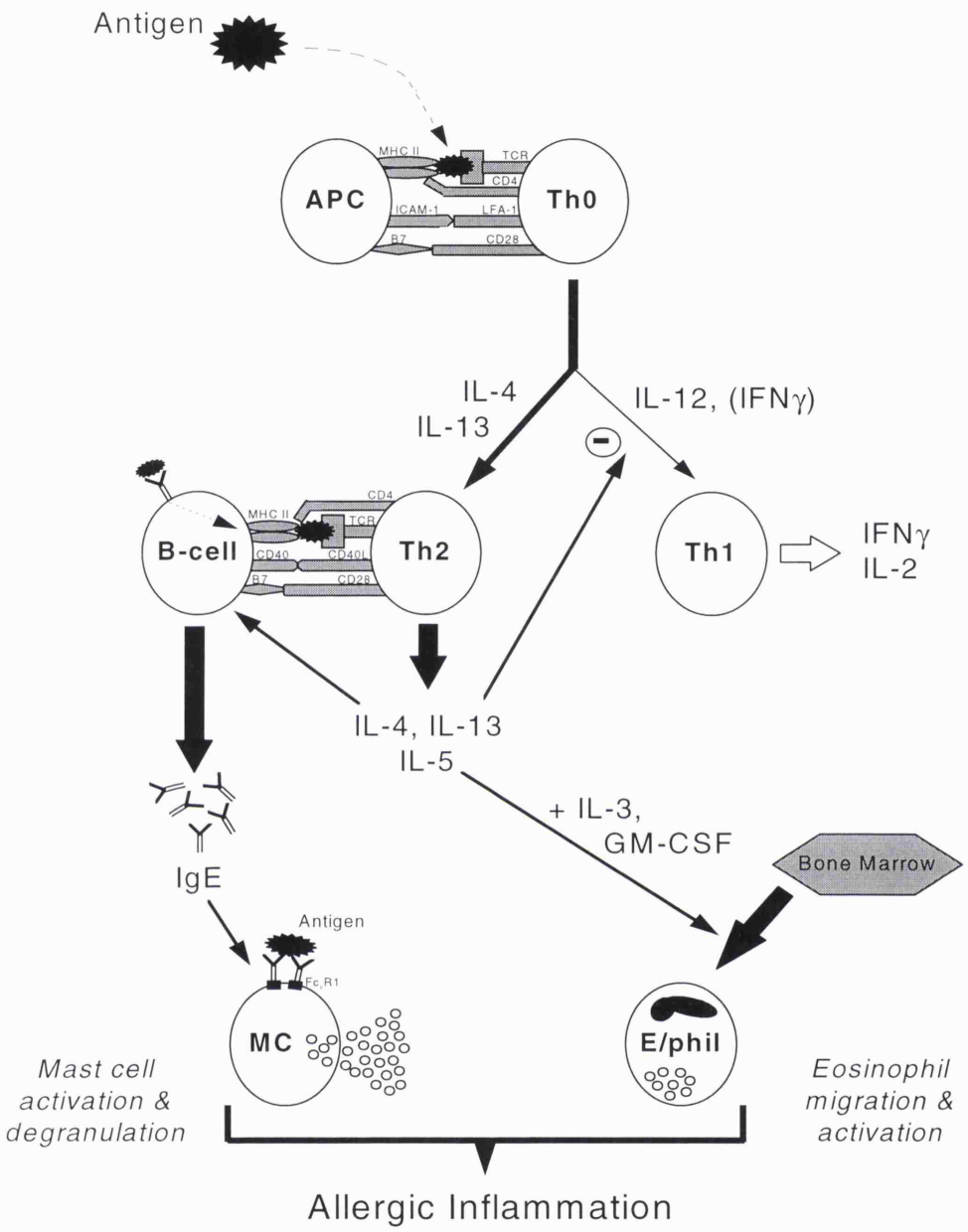

Figure 1.5: The role of cytokines in the Th2-like response in allergic inflammation. (MC = mast cell; E/phil = Eosinophil; TCR = T cell receptor). Antigen is processed by antigen presenting cells (APC) and presented to naive T-helper cells (Th0). In sensitive subjects, this stimulates the release a particular range of cytokines, particularly IL-4 and IL-13, which cause the differentiation of Th0 cells into Th2 cells. The Th2 cells interact with B-cells, causing the further release of IL-4 and IL-13, which act on B-cells to stimulate the production of IgE antibodies. The IgE becomes bound to mast cells which degranulate upon subsequent exposure to antigen. The Th2 cells also generate IL-5, which, in conjunction with IL-3 and GM-CSF, stimulate the recruitment and activation of eosinophils.

A number of other cells present on the nasal mucosa also release cytokines. These include epithelial cells (IL-1, IL-8, GM-CSF), mast cells (IL-4, IL-5, TNFα), eosinophils (IL-1, IL-3, IL-6, IL-8, GM-CSF) and neutrophils (IL-1, IL-6, IL-8, G-CSF, IFNα, TNFα, TGFβ) (Weller, 1993; McColl & Showell, 1994; Howarth, 1995). Human nasal mast cells may have an important contributory role in eosinophilic inflammation, since they release cytokines that are required for eosinophil recruitment and survival (Ying *et al.*, 1994). While cytokines may not be able to initiate an inflammatory response *per se*, it is thought that they enhance the recruitment of inflammatory cells by creating an environment which favours inflammatory cell accumulation and survival (Bachert *et al.*, 1998).

1.5.4.2 Role of cytokines in nasal hyperresponsiveness

Following nasal allergen challenge, the levels of IL-1α, IL-1ß, IL-5, IL-6, IL-8 and GM-CSF are raised in nasal secretions (Gosset *et al.*, 1993; Sim *et al.*, 1994 & 1995; Lantero *et al.*, 1996), and human eosinophils are potential sources of these cytokines (Galli *et al.*, 1994). Epithelial cells, isolated from allergic rhinitics, show increased immunostaining for GM-CSF, IL-8, the receptors for IL-1 and TNF-α (Nonaka *et al.*, 1996), and they also release more IL-1ß, IL-8, GM-CSF and TNF-α compared to epithelial cells from non-allergic subjects (Calderon *et al.*, 1997). Similar increases in IL-4, IL-5 and GM-CSF positive cells are observed in biopsies from the nasal mucosa of atopics (Durham *et al.*, 1992). Both interferon-γ and TNF-α (and possibly other cytokines) cause an upregulation of ICAM-1 on human nasal epithelial cells (Altman *et al.*, 1993), while IL-4 upregulates the expression of VCAM-1 (Bradding *et al.*, 1993). Both these adhesion molecule are upregulated in allergic rhinitis (Bradding *et al.*, 1993).

Few studies have specifically investigated the role of cytokines in the AHR of allergic rhinitis, although IL-4 and IL-5 have been implicated (Hogan *et al.*, 1997). In animals, IL-5 causes a marked eosinophilia, eosinophil activation and AHR (Van Oosterhout *et al.*, 1996). Monoclonal antibodies to IL-5 abolish antigen-induced eosinophilia and AHR in the lower airway of the guinea pig (Mauser *et al.*, 1993). IL-4 regulates the

activity of CD4+ T-lymphocytes, which release a range of cytokines capable of priming and activating eosinophils (Howarth, 1995), and can also activate neutrophils (Boey *et al.*, 1989). Patients with SAR or PAR have a raised number of CD4+ T-lymphocytes (Bradding *et al.*, 1993). Furthermore, memory T-lymphocytes in the nasal mucosa of patients with nasal allergy can produce IL-4 during allergen exposure (Hellquist & Karlsson, 1992), which would potentiate the inflammatory response by inducing further inflammatory cell recruitment and activation.

Cytokines may promote AHR by upregulating the recruitment and activation of eosinophils (Figure 1.5) and neutrophils. For example, there are close correlations between the number of eosinophils and GM-CSF levels in bronchoalveolar fluid (BALF) from subjects with asthma (Wooley *et al.*, 1995), while the survival of eosinophils in BALF from subjects with allergic rhinitis is associated with an increase in IL-5 and GM-CSF (Ohnishi *et al.*, 1993). Neurokinin NK_2 receptor antagonists inhibit AHR induced by IL-5 in the guinea pig, but not the associated eosinophilia (Kraneveld *et al.*, 1997b), indicating that cytokines are involved in cell recruitment, following which various mediators are released such as neuropeptides which may cause the development of AHR. IL-1 may also be involved in the development of AHR (Selig & Tocker, 1992), but it has a wide range of cellular actions, so it is difficult to suggest a precise role for it in the pathogenesis of AHR. Interestingly, it may inhibit the activity of NEP (Tsukagoshi *et al.*, 1995), or induce the generation of oxygen-based free radicals from macrophages and neutrophils (Tsukagoshi *et al.*, 1994).

Finally, glucocorticoids downregulate the production of IL-3 and IL-5 following allergen challenge; this effect may contribute to the action of steroids in abolishing AHR (Lantero *et al.*, 1996), although glucocorticoids have other actions too.

1.5.4.3 Chemokines

Chemokines are cytokines which possess chemotactic activity, and are divided into groups depending on their structure. The two main groups are the CC chemokines, where two cysteine residues are adjacent to each other (e.g. RANTES, MIP-1α, eotaxin), and the CXC chemokines, in which the two cysteine residues are separated

by a third amino acid (e.g. IL-8). Some chemokines (RANTES, eotaxin) appear to be selective for eosinophils, while IL-8 may have chemotactic activity only for neutrophils (although there are reports that IL-8 also acts as a chemoattractant for primed human eosinophils) (Barnes *et al.*, 1998).

The levels of RANTES (Sim *et al.*, 1995; Rajakulasingam *et al.*, 1997), MIP-1α (Sim *et al.*, 1995), eotaxin (Minshall *et al.*, 1997), and IL-8 (Sim *et al.*, 1995; Gosset *et al.*, 1997) detected in nasal lavage are raised following nasal allergen challenge in man. Treatment with glucocorticoids, which inhibits inflammatory cell recruitment, abrogates these increases (Sim *et al.*, 1995; Meltzer, 1997). Interestingly, mucosal cells obtained from the noses of subjects with allergic rhinitis show increased expression of mRNA for RANTES (Rajakulasingam *et al.*, 1997) and eotaxin (Minshall *et al.*, 1997). It is now generally accepted that RANTES and eotaxin are important in IL-5-mediated eosinophilia, where the latter causes the mobilisation of eosinophils into the circulation while the local release of chemokines provides a 'homing' mechanism for the migration of eosinophils into the tissues (Barnes *et al.*, 1998). However, no study has yet investigated the specific role of chemokines in nasal AHR. Notwithstanding, administration of RANTES into the nasal airway of subjects with allergic rhinitis causes an eosinophilia but not an influx of other inflammatory cells (Kuna *et al.*, 1998). However, the same study also found that after allergen challenge, administration of RANTES also caused an influx of basophils, neutrophils, lymphocytes and monocytes, as well as causing epithelial shedding, a response similar to that observed in nasal AHR. It is therefore likely that chemokines have an important role in the recruitment of inflammatory cells that is observed during the development of nasal AHR.

1.5.5 Nitric Oxide

In 1980, Furchgott and Zawadski demonstrated that the ability of acetylcholine to cause vasodilatation was dependent upon the release of a mediator from intact endothelial cells. The mediator was termed "endothelium derived relaxing factor" (EDRF), and although a number of substances possess EDRF activity, the most well

known is nitric oxide (NO) (Moncada *et al.*, 1991). It is now apparent that NO has a role in the pathophysiology of a number of diseases (Moncada *et al.*, 1991).

1.5.5.1 Synthesis of nitric oxide

Nitric oxide is produced by the action of nitric oxide synthase (NOS) on the amino acid L-arginine. The reaction is dependent on the presence of a number of different cofactors, as shown in Figure 1.6. The process is also stereospecific, since addition of D-arginine reduces NO production, which can then be restored by L-arginine (Palmer *et al.*, 1988). Similarly, a number of arginine analogues which inhibit NOS have been described, and these are only active as the L-isomer (reviewed by Griffith & Gross, 1996). The inhibitors include N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA). Both of these inhibitors compete with L-arginine for the active site of NOS, and so their inhibition can be reversed by adding excess L-arginine. In addition, L-NMMA, but not L-NAME, reduces entry of L-arginine into the cell, by competing with L-arginine at the amino acid transporter in the cell membrane (Bogle *et al.*, 1992).

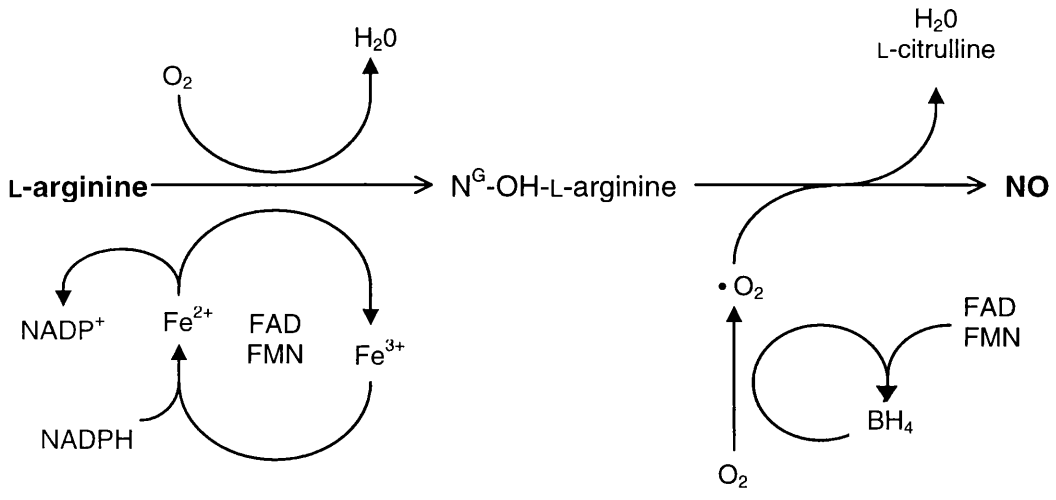

Figure 1.6: Synthesis of nitric oxide (NO) from L-arginine. L-arginine is first hydroxylated, and then oxidised to generate NO. The reaction converts L-arginine and reduced nicotinamide adenine dinucleotide phosphate (NADPH) into NO and L-citrulline, and requires the presence of haem, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobioterin (BH_4) as cofactors (Knowles & Moncada, 1994).

Three different isoforms of NOS have been identified (reviewed by Knowles & Moncada, 1994), of which two are expressed constitutively (known as cNOS). Neuronal NOS (nNOS) (also known as Type I NOS) has been found on neural tissue in the brain, spinal cord, and in peripheral nerves (Moncada & Higgs, 1995). Endothelial NOS (eNOS, Type III NOS) is localised to endothelial cells lining blood vessels, though it is now clear that it is also present on airway epithelial cells (Shaul *et al.*, 1994). Both these enzymes are calcium-dependent, and produce the low levels of NO that are thought to be important in normal physiology. However, there is also a third, inducible isoform (iNOS, Type II NOS), which is not normally expressed in cells, but can be induced by cytokines or bacterial toxins. It generates large amounts of NO, which may contribute to the inflammatory response. The expression of iNOS is upregulated by the transcription factor NF-κB (Barnes & Karin, 1997). The three isoforms are described further in Table 1.5.

	nNOS	**eNOS**	**iNOS**
Expression	Constitutive	Constitutive	Inducible
Location (in man)	Neural tissue in the brain, spinal cord, skeletal muscle.	Endothelium of blood vessels	Inducible in a variety of cells, including vascular smooth muscle, immune cells, cardiac muscle, gut cells, hepatocytes and macrophages.
Calcium/ calmodulin dependent?	Yes	Yes	No
Cellular location	Cytosolic	Usually associated with the cell membrane, and is translocated to the cytosol following cell stimulation	Cytosolic
Gene location	chromosome 12	chromosome 7	chromosome 17
Physiological effects	NO produced by nNOS may mediate NANC transmission. This may also modulate vascular tone.	Causes vasodilatation, inhibits platelet activation.	Cytotoxic, may potentiate the inflammatory response and prolong eosinophil survival.

Table 1.5: Properties of the different isoforms of NOS (Knowles & Moncada, 1994).

Nitric oxide exerts its effects is a number of ways. It causes smooth muscle relaxation, by activating guanylate cyclase and producing cyclic GMP, resulting in dilatation of blood vessels and also bronchodilation (Ward *et al.*, 1995). The NO produced by nNOS may have a role as an inhibitory mediator of NANC nerve transmission in some tissues: inhibiting the effects of other neurotransmitters (Belvisi *et al.*, 1992). NO is also a highly reactive free radical, combining with reactive oxygen species to form a variety of species, including peroxynitrite, and these may account for some of the biological actions of NO (Stamler & Feelisch, 1996).

1.5.5.2 Action of nitric oxide in the human nasal airway

All three isoforms of NOS have been identified in the human nasal airway, using immunohistochemical markers. nNOS was found to be localised to the nerves of the nasal mucosa (Kulkarni *et al.*, 1994), while eNOS was identified on the vascular endothelium, submucosal glands and the surface epithelium (Furukawa *et al.*, 1996). Interestingly, a NOS isoform, most similar in structure to nNOS, has been identified in the secretory granules of human nasal mast cells isolated from non-allergic subjects (Bacci *et al.*, 1994). Furukawa *et al.* (1996) demonstrated the presence of mRNA for iNOS primarily on inflammatory cells, while the enzyme itself was found on endothelial, epithelial, glandular and inflammatory cells in nasal specimens in which there was an underlying inflammation.

Although nitric oxide is important in the control of vascular tone, NOS inhibitors do not alter the resting patency of the human nasal airway (Dear *et al.*, 1996a). The plasma extravasation induced by histamine or bradykinin in the human nasal airway is reduced following administration of L-NAME or L-NMMA, but interestingly, only the reduction in nasal patency induced by bradykinin was inhibited. This implies that the nasal obstruction induced by bradykinin, but not with histamine, is mediated by nitric oxide production (Dear *et al.*, 1996a).

1.5.5.3 Role of nitric oxide in nasal airway hyperresponsiveness

There is evidence that NOS activity is increased in PAR (Garrelds *et al.*, 1995) and in SAR (Martin *et al.*, 1996; Kharitonov *et al.*, 1997b), and it is thought that this is due to an upregulation of iNOS (Barnes & Liew, 1995). This would increase the amount of nitric oxide available to react with superoxide, generating other free radicals that may contribute to the development of AHR (Sadeghi-Hashjin *et al.*, 1996b). Nitric oxide may also have a role in the production of cytokines necessary for eosinophil survival, such as IL-4 and IL-5 (Barnes & Liew, 1995). Therefore, one might expect inhibitors of NOS to prevent AHR, either by reducing free radical production or inhibiting eosinophil survival. Paradoxically, NOS inhibitors actually induce AHR to histamine and bradykinin in the lower airways of human asthmatic subjects (Ricciardolo *et al.*, 1996) and the guinea pig (Nijkamp *et al.*, 1993). It is possible that in the normal, non-inflammed airway, nitric oxide is protective, so inhibitors of NOS would cause AHR, but in chronic airway inflammation, an inappropriate degree of nitric oxide production may be harmful and potentiate the allergic response.

Interestingly, NO is thought to be the central mediator of inhibitory NANC transmission. Therefore, inhibition of NOS may cause a reduction in the activity of inhibitory NANC nerves, potentiating neurogenic inflammation mediated by excitatory NANC nerves that might be involved in AHR. In chronic allergy, excessive NO production could cause AHR by the formation of the peroxynitrite free radical, which causes AHR in the guinea pig airway, possibly by inhibiting cGMP production (Sadeghi-Hashjin *et al.*, 1996a & 1996b). Furthermore, other NO metabolites, such as nitryl chloride, can be synthesised by neutrophils. This particular metabolite inactivates endothelial cell angiotensin converting enzyme (Eiserich *et al.*, 1998), which is involved in the degradation of kinins and possibly tachykinins in allergic rhinitis (Lurie *et al.*, 1994; Chatelain *et al.*, 1995). Thus, inhibition of this enzyme may potentiate the action of these mediators, causing AHR. Inhibition of NOS may also cause a shift in arachidonic acid metabolism, from COX products to an increase in leukotriene synthesis, and it has been suggested that this may account for the ability of NOS inhibitors to induce AHR (Folkerts *et al.*, 1995).

Interestingly, in a murine model of allergic asthma, airway inflammation is significantly reduced in mice lacking iNOS. However, the degree of AHR following allergen was not affected, so the development of AHR may be independent of an increase in iNOS activity following exposure to allergen (Xiong *et al.*, 1999).

1.5.6 Kinins

The kinins are a group of vasoactive peptides, the first member of which was discovered in 1937. Previously, in 1926, a substance was isolated from human urine and other tissues which caused a reduction in blood pressure in dogs (Frey & Kraut, 1926). This substance was later called kallikrein, since it was thought that the pancreas (Greek: kallikreas) was the main source of the substance in the body. In 1936, Werle observed that kallikrein only caused hypotension and contraction of the guinea pig ileum when mixed with serum, and isolated a product of the reaction of kallikrein on serum, which was named kallidin (Werle *et al.*, 1937). In 1949, a substance, similar to kallidin, was discovered when snake venom (*sp. Bothrops jararaca*) was incubated with plasma from a dog (Roche e Silva *et al.*, 1949). This new substance caused a slow contraction of the guinea pig ileum, and was therefore called bradykinin (meaning 'slow movement').

1.5.6.1 Synthesis and metabolism of the kinins

Bradykinin and kallidin are the most important members of the kinin group of peptides, consisting of 9 and 10 amino acids respectively. Kallidin has the same structure as bradykinin, with an added lysine residue:

Bradykinin: H - Arg - Pro - Pro - Gly - Phe - Ser - Pro - Phe - Arg - OH
Kallidin: H- Lys - Arg - Pro - Pro - Gly - Phe - Ser - Pro - Phe - Arg - OH

In man, the kinins are formed from two precursor molecules - high molecular weight kininogen (HMWK) and low molecular weight kininogen (LMWK) - by the action of kininogenases: the main two members of which are plasma kallikrein (PK) and tissue kallikrein (TK) (Bhoola *et al.*, 1992). PK is produced by the liver as an inactive pre-

enzyme called plasma prekallikrein, and secreted into the plasma. The conversion of plasma prekallikrein to PK occurs via a process dependent on the activation of coagulation factor XII (Hageman factor), by contact of the factor with a negatively charged surface, for example basement membrane components, glass or proteoglycans such as heparin released from mast cells (Reddigari *et al.*, 1997). Once activated, PK preferentially uses HMWK as its substrate, hydrolysing Lys-Arg and Arg-Ser groups of HMWK to produce bradykinin. However, in the presence of neutrophil elastase, LMWK can also be metabolised to bradykinin (Sato & Nagasawa, 1988).

TK releases kallidin from both LMWK and HMWK, and has been identified in a number of human tissues, including the pancreas, kidney, salivary glands and airway and gut mucosa. TK also exists as an inactive prekallikrein, which is activated by the cleavage of seven amino acids from the N-terminal by proteolytic enzymes, including trypsin, plasmin and PK (Proud & Kaplan, 1988). TK lacks the ability to hydrolyse the Lys-Arg bond of kininogen, and can therefore only generate kallidin, and not bradykinin. However, kallidin is rapidly converted to bradykinin by aminopeptidase-N (Proud & Kaplan, 1988).

A number of other proteases have also been reported to generate kinins from kininogen. These include mast cell tryptase (Proud *et al.*, 1988b), calpains (Higashiyama *et al.*, 1986) and a number of other allergen-associated proteases (Takahashi *et al.*, 1990). In addition, ECP can also activate plasma kallikrein (Venge *et al.*, 1979). The formation of the kinins is summarised in Figure 1.7.

The kininogens are synthesised in the liver and then secreted; LMWK is found in both plasma and in tissues, while HMWK is found only in plasma, where it circulates as a dimer, bound to PK. Interestingly, it has been reported that neutrophils can release TK, and also bind PK, LMWK and HMWK on their cell membrane (Gustafson *et al.*, 1989; Henderson *et al.*, 1994). It has been proposed that kinin generation by neutrophils may be involved in the diapedesis of inflammatory cells out of blood vessels and into the surrounding tissue (Naidoo & Bhoola, 1997).

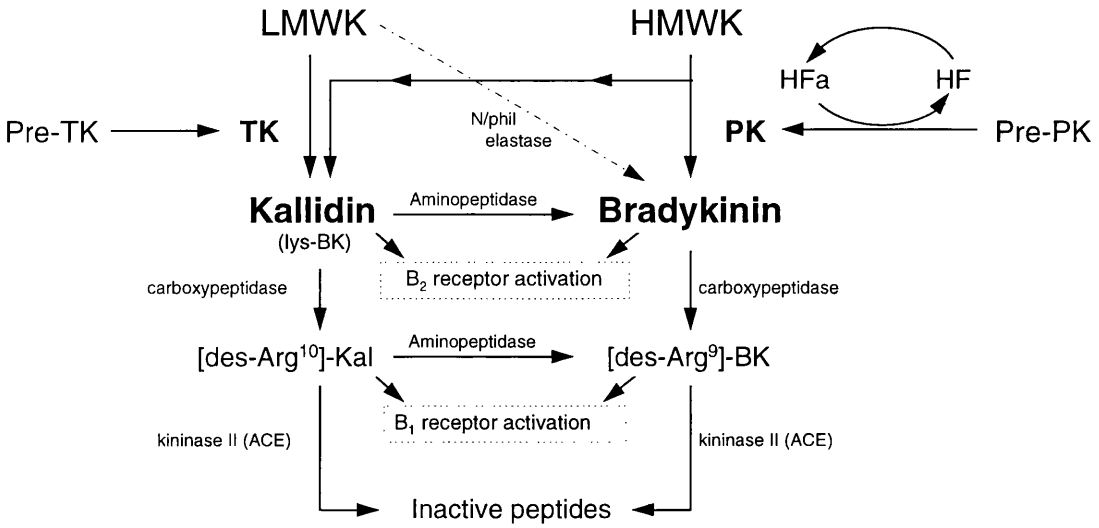

Figure 1.7: Synthesis and metabolism of the kinins. LMWK and HMWK = low and high molecular weight kininogen, respectively; TK = tissue kallikrein; PK = plasma kallikrein; HF and Hfa = unactivated and activated Hageman factor; BK = bradykinin; ACE = angiotensin converting enzyme II.

The kinins are rapidly metabolised in the plasma, and have a half-life of under 30 seconds. A number of kinin-metabolising enzymes or kininases have been identified in man. The majority of kininase activity is thought to be due to angiotensin converting enzyme (ACE, kininase II), which is localised to endothelial cells (particularly in the lung) and also epithelial cells in the upper and lower airways (Erdos & Skidgel, 1997). NEP can also metabolise the kinins, but has a much lower affinity for bradykinin compared with ACE. Carboxypeptidase N (kininase I) metabolises bradykinin and kallidin, releasing active metabolites such as [des-Arg^9]-bradykinin which are agonists at the bradykinin B_1 receptors.

1.5.6.2 Kinin receptors

The effects of bradykinin are often, but not exclusively, mediated by specific receptors on the cell surface. Two types of bradykinin receptors have been characterised, according to the potency of various kinin agonists at the receptors. Both receptors are

G-protein-coupled receptors with seven transmembrane domains (McEachern *et al.*, 1991).

The bradykinin B_1 receptor is stimulated by agonists as follows (with decreasing potency):

[des-Arg^{10}]-kallidin > [des-Arg^{9}]-bradykinin ≥ kallidin > bradykinin

This receptor has been identified in the aorta and mesenteric vein of the rabbit. In man, it is only found in a few tissues, but its expression is upregulated in an inflammatory state (Marceau, 1995).

The bradykinin B_2 receptor was first observed in the smooth muscle of blood vessels, but now appears to be the receptor through which most of the actions of the kinins are mediated *in vivo.* The order of agonist potency at this receptor is:

kallidin ≥ bradykinin > [des-Arg^{10}]-kallidin > [des-Arg^{9}]-bradykinin

Investigations into the bradykinin B_2 receptor have been possible as a result of the development of a variety of antagonists at this receptor. The first such antagonists were analogues of [D-Phe^{7}]-bradykinin, formed by a substitution of the Pro residue in position 7 by D-Phe (Vavrek & Stewart, 1985). Newer antagonists have modifications of both the Pro residue in position 7 and the Phe residue in position 8. One of the most potent B_2 receptor antagonists of this class is icatibant (Hoe 140), D-Arg-[Hyp^{3},Thi^{5},D-Tic^{7},Oic^{8}]-bradykinin (Hock *et al.*, 1991; Wirth *et al.*, 1991). The high affinity of this antagonist for the B_2 receptor (K_i = 0.48 nM in human nasal membrane preparations) (Dear *et al.*, 1996b) may be due to the lipophilic region resulting from the [D-Tic^{7},Oic^{8}] group which causes a prolonged occupation of the receptor (Rhaleb *et al.*, 1992). Icatibant also is resistant to degradation by kininase I and II, and this may also contribute to its activity (Hock *et al.*, 1991). However, their peptide structure renders these antagonists susceptible to proteases, and this limits their duration of action and imposes constraints on the route of administration. Consequently, a number of non-peptide B_2 antagonists are under development, the most promising of

which is FR 165649 (Asano *et al.*, 1997). Using these antagonists, B_2 receptors have been identified in the gastrointestinal tract, genitourinary system, cardiovascular system, respiratory tract, and in neural tissue of most species (Hall & Morton, 1997).

It has been suggested that there may also other bradykinin receptor subtypes, in addition to the two described above. In a number of isolated tissues, bradykinin receptors have been identified which have a different agonist potency to that described for the B_1 or B_2 receptor, and from which B_2 receptor antagonists were unable to displace radiolabelled bradykinin (Hall & Morton, 1997). However, the evidence for other receptor subtype is limited, and many of the discrepancies may be due to differences in agonist/antagonist binding between species, so no definite conclusions can be made (reviewed by Hall & Morton, 1997). Although a bradykinin B_3 receptor has been described in the lower airways of the guinea pig (Farmer *et al.*, 1989), other studies have failed to confirm this (Trifilieff *et al.*, 1994; Scherrer *et al.*, 1995).

Bradykinin (and kinin analogues) can also release histamine from mast cells, via a process which appears to be independent of receptor activation (Devillier *et al.*, 1988b). This may occur by the direct stimulation of pertussis toxin-sensitive G-proteins in mast cells (Mousli *et al.*, 1991).

1.5.6.3 Action of bradykinin in the human nasal airway

The kinins appear to be important mediators of inflammation, and therefore their action in the human airways have been extensively investigated. The administration of exogenous bradykinin into the human nasal airway causes nasal obstruction, rhinorrhoea due to plasma extravasation and nasal secretion, and nasal pain (Proud *et al.*, 1988a; Austin & Foreman, 1994b). These effects appear to be mediated by the bradykinin B_2 receptor, since icatibant, a bradykinin B_2 receptor antagonist, abolished the bradykinin-induced nasal obstruction and plasma extravasation, while agonists at the bradykinin B_1 receptor do not cause any nasal symptoms (Rajakulasingam *et al.*, 1991; Austin & Foreman, 1994b). Bradykinin binding sites have been identified on small muscular arteries, venous sinusoids and submucosal nerves in the human

nasal airway (Baraniuk *et al.*, 1990), and radioligand binding studies have demonstrated the presence of the B_2 receptor in human nasal tissue (Dear *et al.*, 1996b).

Bradykinin causes the release of sensory neuropeptides from sensory nerves in a number of different tissues (reviewed by Geppetti, 1993), including in the human nasal airway (Baumgarten *et al.*, 1997). The bradykinin-induced release of neuropeptides from airway sensory nerves *in vitro* is mediated by the bradykinin B_2 receptor (Miura *et al.*, 1992). While the release of tachykinins may mediate bradykinin-induced increases in plasma extravasation in the rat and guinea pig nasal mucosa (Bertrand *et al.*, 1993; Ricciardolo *et al.*, 1994a), there is no evidence that the actions of bradykinin involves nerve activation in the human nasal airway (Dear *et al.*, 1996a).

The nasal obstruction and rhinorrhoea induced by higher doses of bradykinin (300-1000 µg) may be mediated, in part, by the release of histamine, since cetirizine and terfenadine, both histamine H_1 antagonists, reduced these effects (Austin *et al.*, 1996). Although high concentrations of bradykinin cause histamine release from nasal tissue *ex vivo*, no histamine was detected in nasal lavage fluid following bradykinin challenge *in vivo*, possibly as a result of the rapid clearance of histamine into the venous circulation (Austin *et al.*, 1996).

1.5.6.4 Role of kinins in nasal airway hyperresponsiveness

Bradykinin and kallidin are released following nasal antigen challenge in sensitive subjects, during both the early and late phase responses (Naclerio *et al.*, 1985; Proud *et al.*, 1983). Similarly, antigen also causes the release of other components of the kallikrein-kinin system, including kininogen (Baumgarten *et al.*, 1995), PK and TK (Baumgarten *et al.*, 1986a & 1986b) and various kininases (Proud *et al.*, 1987).

A number of studies have identified a role for kinins in the development of AHR in the lower airways of both the guinea pig (Farmer *et al.*, 1992) and sheep (Soler *et al.*, 1990). Furthermore, the ability of MBP and synthetic cationic proteins to induce AHR

in the lower airways of rats is dependent on the generation of kinins (Coyle *et al.*, 1995). In all three studies, administration of a bradykinin B_2 receptor antagonist inhibited the development of AHR, and, where investigated, also appeared to have an effect on the recruitment of eosinophils into the airway. ECP can stimulate kallikrein activity *in vitro* resulting in kinin production (Venge *et al.*, 1979), so eosinophil-derived cationic proteins may generate kinins which result in AHR.

The mechanism by which kinins may induce AHR remains unknown. Exogenous kinins applied to the lower airways of the rat do not cause AHR (Coyle *et al.*, 1995), although another study found that bradykinin can produce AHR to acetylcholine in the guinea pig airway (Omini *et al.*, 1989). Interestingly, the eosinophilia induced by antigen challenge in the lower airway of the guinea pig is reduced by bradykinin receptor antagonists (Farmer *et al.*, 1992) or an inhibitor of tissue kallikrein (Evans *et al.*, 1996), thus providing evidence for the involvement of kinins in the recruitment of eosinophils following exposure to allergen. The role of kinins in the development of AHR in the human nasal airway will be discussed further in chapters 3, 4 and 5.

1.6 The role of inflammatory cells in hyperresponsiveness

From the evidence presented, it might appear that eosinophils have a vital role in the development of AHR. However, the relationship between eosinophil activation and AHR remains controversial. Certainly, eosinophils are involved in the late-phase allergic response, but the development of AHR may not be dependent on the presence of eosinophils. Studies conducted in animal models indicate that AHR can occur without a detectable eosinophilia, and vice versa (Spina *et al.*, 1991). Data obtained from studies in the human nose similarly imply that AHR does not necessarily occur together with eosinophil activation (Andersson *et al.*, 1989; Klementsson *et al.*, 1991), while antigen can induce an eosinophilia without causing AHR in the guinea pig lower airways (Seeds *et al.*, 1991). There are a range of airway inflammatory conditions which feature an eosinophilic infiltration, but no associated AHR (Butterfield & Leiferman, 1993). Nonetheless, the close association between eosinophil recruitment, activation and AHR in many studies (Bascom *et al.*, 1989;

Austin & Foreman, 1993; De Graaf-in't Veld *et al.*, 1996) implies, at the very least, that eosinophils may contribute to the development of AHR.

It is now generally accepted that eosinophils, isolated from peripheral blood, demonstrate a functional heterogeneity, and can be divided into normodense and hypodense (<1.082 g/ml) populations (reviewed by Owen, 1993). The hypodense population is increased in atopic disease, including allergic rhinitis (Frick *et al.*, 1988; Takagi *et al.*, 1995). Hypodense eosinophils release higher amounts of PAF, lipoxins and cytokines, and also have an increased capacity for generating superoxide (Owen, 1993). However, many of these effects may be dependent on the presence of different priming stimuli in atopic subjects, and so the increase numbers of hypodense eosinophils in atopy may not contribute significantly to the underlying eosinophilic inflammation (Butterfield & Leiferman, 1993).

Although eosinophils are the main cells which have been implicated in the mechanism underlying AHR, it is likely that other cells are involved, including neutrophils, T-lymphocytes, macrophages, B-cells and basophils (Howarth, 1995). In particular, cytokines released from T-lymphocytes probably maintain the local environment necessary for the chronic inflammatory state, as described above. Basophils, which may be involved in the late allergic response, are capable of generating histamine, bradykinin, MBP, LTC_4, IL-4 and IL-8 (Howarth, 1995; Costa *et al.*, 1997), which may contribute to AHR. Although a correlation has been found between basophil infiltration and bronchial AHR in asthmatics (Koshino *et al.*, 1996), no similar association has been identified in the human nasal airway. Macrophages may also be involved in the development of nasal AHR, possibly by generating cytokines and free radicals (Bachert *et al.*, 1991). In a canine model of airway allergy, pulmonary alveolar macrophages may increase smooth muscle contraction *in vitro*, by releasing metabolites of arachidonic acid (Tamaoki *et al.*, 1990).

The involvement of the neutrophil in AHR is unclear, although there are many potential mechanisms by which they could have an active role. Not only may they contribute to the generation of kinins and cause tissue damage via the superoxide burst, but they also generate PAF, LTB_4 and a variety of cytokines (McColl & Showell,

1994). Interestingly, ECP has now been detected in neutrophils (Sur *et al.*, 1998). Neutrophils isolated from atopic subjects have an increased capacity for myeloperoxidase release, and this enzyme can generate nitric oxide-derived oxidants, that can induce AHR and inhibit kininase II (Eiserich *et al.*, 1998). Neutrophil elastase may also contribute to epithelial damage in chronic allergic inflammation (Fujisawa *et al.*, 1990). Stimulated neutrophils induce AHR in human bronchial tissue *ex vivo*, by a mechanism which may be dependent on arachidonic acid metabolites (Hughes *et al.*, 1994; Anticevich *et al.*, 1996). Neutrophils may also be involved in the development of AHR by free radicals, such as ozone (O'Byrne *et al.*, 1984). In the human nasal airway, PAF-induced AHR is associated with both a neutrophilia and the release of neutrophil myeloperoxidase (Miadonna *et al.*, 1996). However, other studies have concluded that neutrophils do not appear to have an important role in the AHR induced by PAF or antigen, in the lower airways of human subjects (Wardlaw *et al.*, 1990; Frangova *et al.*, 1996). The accumulation and activation of neutrophils may be a parallel event to the eosinophilia in allergic inflammation, and could, therefore, contribute to the chronic inflammatory state (Frangova *et al.*, 1996).

The upregulation of ICAM-1 and VCAM-1 by cytokines in allergic rhinitis has already been mentioned. ICAM-1 and VCAM-1, present on endothelial cells, bind respectively to integrins LFA-1 ($\alpha_L\beta_2$ integrin) and VLA-4 ($\alpha_4\beta_1$ integrin) on leukocytes, allowing leukocytes to adhere to the endothelium prior to migration. The interaction between VCAM-1 and VLA-4 appears to be particularly important in eosinophil migration, as neutrophils do not have the VLA-4 receptor on their surface (Texeira *et al.*, 1995). The preferential recruitment of eosinophils in airway allergy may result from the release of mediators which selectively upregulate VCAM-1/VLA-4 expression, such as IL-4 (Walsh *et al.*, 1993). Interfering with the VCAM-1/VLA-4 pathway may, therefore, prevent eosinophil-dependent AHR. Monoclonal antibodies (mAbs) to the integrin sub-unit α_4 have been developed which abolish antigen-induced AHR in the lower airways of a number of animal models of airway allergy (Laberge *et al.*, 1995; Abraham *et al.*, 1997; Kraneveld *et al.*, 1997c). Interestingly, this effect was not always associated with a decrease in the airway eosinophilia (Laberge *et al.*, 1995; Abraham *et al.*, 1994), possibly because other mechanisms exist (e.g. via ICAM-1)

whereby eosinophil migration may occur. Further development of these mAbs may provide a useful therapeutic intervention for airway allergy in man.

1.7 Summary

It would appear that a number of mediators are capable of inducing AHR in the human nasal airway, perhaps acting via different mechanisms, which would help explain the conflicting evidence regarding a role for eosinophils or airway epithelial damage in this process. However, the fact that inhibitors of leukotrienes, PAF, kinins and tachykinins can all inhibit antigen-induced AHR implies that there may be a central common pathway in the pathogenesis of AHR. Since neurogenic inflammation can potentially be modulated by all of these mediators, neuropeptides may be involved in the final stage of AHR induction, perhaps as shown in Figure 1.8.

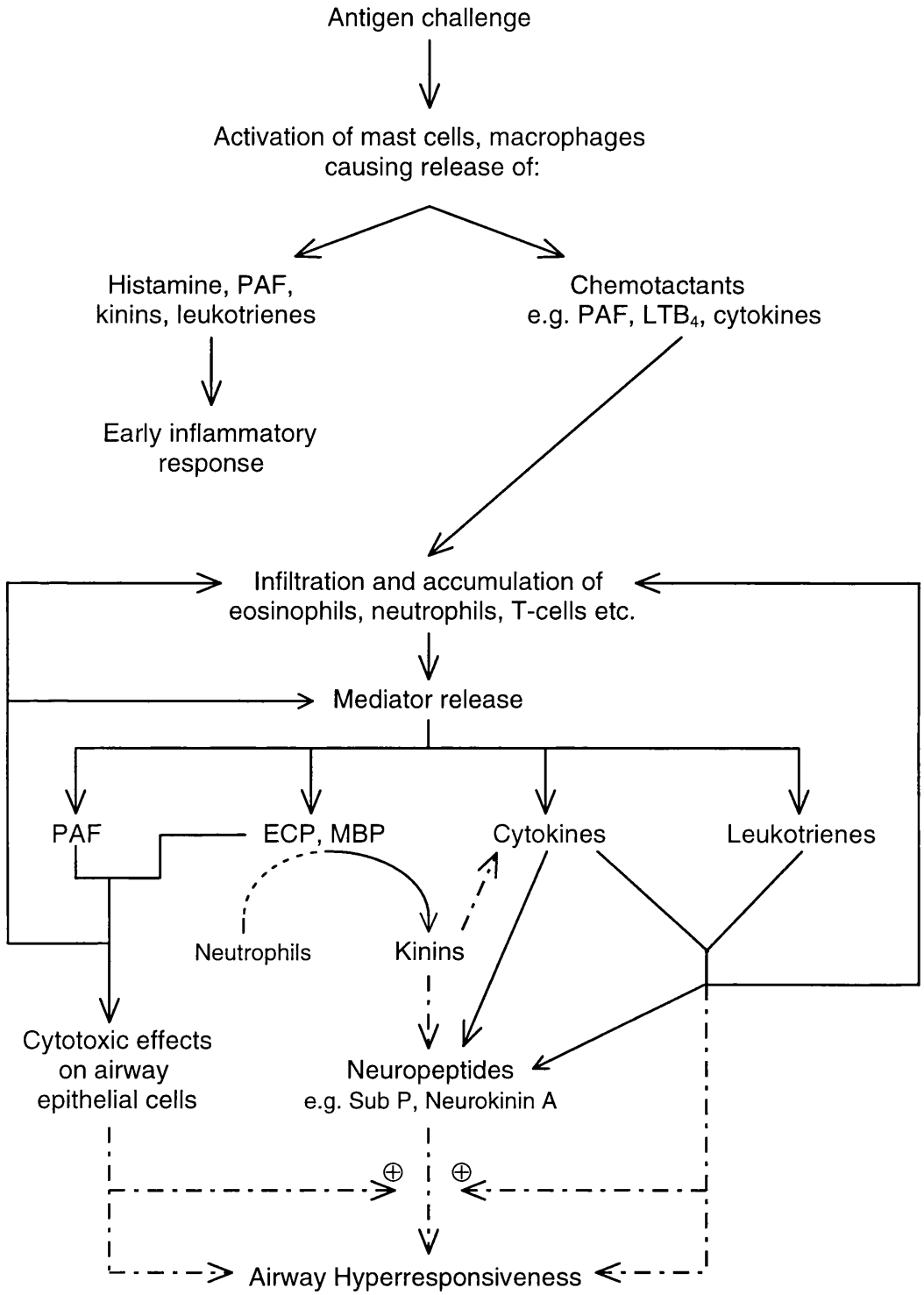

Figure 1.8: Putative mechanisms for the development of airway hyperresponsiveness

1.8 Aims of the project

The aim of this study was to investigate the role of kinins and nitric oxide in the development of hyperresponsiveness in the human nasal airway. In addition, the role of the eosinophil in this process was evaluated. Specifically, the project investigated the following:

- The role of kinins in the development of AHR in the nasal airways of normal, non-atopic subjects challenged with PAF, and patients with seasonal allergic rhinitis.

- The involvement of kinins in the recruitment of neutrophils and eosinophils, both *in vivo* in the human nasal airway, and *in vitro* using a model of transmigration.

- The potential role of neuropeptides in the process underlying AHR. Also, the role of NEP in AHR was also investigated, using phosphoramidon, an inhibitor of NEP.

- Finally, agents which modulate nitric oxide generation were investigated, with respect to their ability to induce AHR in the normal, human nasal airway.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The materials used in this study are shown below, along with their sources.

MATERIAL	SOURCE
Pharmacological Agents	
Aprotinin	Sigma, Poole, UK.
L-arginine	Sigma, Poole, UK.
Bradykinin	Calbiochem, Nottingham, UK.
[Des-Arg^9]-bradykinin	Bachem, Saffron Walden, UK.
[1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7] -bradykinin	Bachem, Saffron Walden, UK.
Lys[Des-Arg^9, Leu^8]-bradykinin	Bachem, Saffron Walden, UK.
Calcium ionophore, A23187	Sigma, Poole, UK.
Eosinophil cationic protein (ECP)	Pharmacia, Uppsala, Sweden.
N-formyl-met-leu-phe (FMLP)	Sigma, Poole, UK.
Grass pollen mixed antigen	Allerayde, Nottingham, UK.
High molecular weight kininogen (HMWK)	Sigma, Poole, UK.
Histamine	Sigma, Poole, UK.
Icatibant (Hoe 140)	Hoechst, Frankfurt, Germany
N-methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone (AAPVK)	Sigma, Poole, UK.
N^G-monomethyl-L-arginine methyl ester (L-NMMA)	Calbiochem, Nottingham, UK.
Neurokinin A	Calbiochem, Nottingham, UK.
N^G-nitro-D-arginine methyl ester (D-NAME)	Sigma, Poole, UK.
N^G-nitro-L-arginine methyl ester (L-NAME)	Sigma, Poole, UK.
Phosphoramidon	Alexis Corporation, Nottingham, UK
Plasma kallikrein (PK)	Sigma, Poole UK.
Platelet activating factor (PAF) (C_{16})	Calbiochem, Nottingham, UK.

Lyso-PAF (C_{16})	Calbiochem, Nottingham, UK.
Substance P	Calbiochem, Nottingham, UK.
Assay materials	
Albumin radial immunodiffusion plates	Behring, Marburg, Germany.
Albumin standard, control serum	Behring, Marburg, Germany.
Bradykinin radioimmunoassay kit	Peninsula, St. Helens, UK.
Eosinophil cationic protein radioimmunoassay kit	Pharmacia, Uppsala, Sweden.
Flavin adenine dinucleotide (FAD)	Sigma, Poole, UK.
Griess reagents	Promega, Southampton, UK.
Interleukin-5 assay	Cytimmune Science Inc., USA
Interleukin-8 assay	HyCult biotechnology b.v., Netherlands
Lactate dehydrogenase	Sigma, Poole, UK.
Nicotinamide adenine dinucleotide phosphate (NADPH)	Sigma, Poole, UK
Nitrate reductase	Boehringer Mannheim, Germany.
Cell Separation/Tissue Culture	
anti-CD3 magnetic microbeads	Miltenyi Biotec, Bisley, UK.
anti-CD14 magnetic microbeads	Miltenyi Biotec, Bisley, UK.
anti-CD16 magnetic microbeads	Miltenyi Biotec, Bisley, UK.
Biocoat fibronectin-coated cell inserts	Becton Dickinson, Oxford, UK.
Cell dissociation fluid	Sigma, Poole, UK.
Dextran (MW 110,000)	Fluka, Poole, UK.
DMSO	Sigma, Poole, UK.
Dulbecco's Modified Eagle's Medium (DMEM) with F-12 HAM nutrient mixture	Sigma, Poole, UK.
Falcon cell culture flasks / 6- and 96- well plates	Marathon Laboratories, London, UK.
Fibronectin (human plasma)	Sigma, Poole, UK.
L-glutamine	Sigma, Poole, UK.
Granulocyte-macrophage colony stimulating factor (GM-CSF)	R & D Systems, Abingdon, UK.
Hanks' balanced salt solution (HBSS)	Sigma, Poole, UK.
Heat-inactivated foetal calf serum (FCS)	Gibco, Paisley, UK.
Histopaque	Sigma, Poole, UK.
MACS cell separation system	Miltenyi Biotec, Bisley, UK.

Penicillin/Streptomycin	Sigma, Poole, UK.
Phosphate buffered saline (PBS) pH 7.4	Gibco, Paisley, UK.
Roswell Park Memorial Institute medium (RPMI-1640)	Sigma, Poole, UK.
Transwells	Corning Costar, High Wycombe, UK
Miscellaneous	
Bovine serum albumin (BSA) fraction V	ICN Biochemicals, Thame, UK.
Chromotrope 2R	Gurr Lab. Products, Poole, UK.
Ethylene diamine tetraacetic acid (EDTA)	BDH, Poole, UK.
Haematoxylin stain	Sigma, Poole, UK.
Human serum albumin (HSA) fraction V	Miles Laboratories, Naperville, USA.
May-Grunwald-Giemsa stain	Raymond A. Lamb, London, UK.
Medical grade silicone seals	Kapitex Healthcare, Wetherby, UK.
Methylene blue	Gurr Laboratory Products, Poole, UK.
Phenol (detached crystals)	BDH, Poole, UK.
Shandon disposable cytofunnels	Life Sciences, Basingstoke, UK.
Xylene	Genta Medical, York, UK.

All other substances were of Analar or similar quality.

2.2 Nasal challenge experiments

2.2.1 Dilution of nasal challenge agents

All compounds used for nasal challenge were dissolved or suspended in sterile saline solution (NaCl, 154 mM) for both stock concentrations, and subsequent dilution to working concentrations. Stock solutions or suspensions were divided into appropriate aliquots and stored at -20°C unless otherwise stated, to avoid thawing and refreezing cycles. Bradykinin was dissolved to give a final concentration between 2 mg/ml and 5 mg/ml, icatibant and [1-adamantane acetyl-D-Arg^{0}, Hyp^{3}, Thi^{5,8}, D-Phe^{7}]-bradykinin to 2 mg/ml, and histamine (as a diphosphate salt) to 0.3-3 mg/ml. Platelet activating factor (PAF) was suspended in saline and stored at -70°C in vials filled with nitrogen gas; this was to prevent the conversion of PAF to lyso-PAF. Both PAF and lyso-PAF

were suspended to a final concentration of 0.6 mg/ml, immediately prior to use. Substance P and neurokinin A were diluted from stock to 100 µg/ml, and phosphoramidon was dissolved from solid stock to 920 µM. L-NAME was dissolved to give final concentrations of 3-100 mM, D-NAME and L-NMMA to 10 mM, and L-arginine to 300 mM. Stock solutions were diluted to their final concentrations immediately prior to use, and allowed to equilibrate with room temperature prior to administration.

The grass pollen antigen mixture was composed of *Argostis stolonifera, Anthoxanthum odoratum, Dactylis glomerata, Lolium perenne, Arrhenatherum elatius, Festuca rubra, Poa pratensis, Secale cereale, Phleum pratense* and *Holcus lanatus* in equal concentrations. The antigen stock solution, of concentration 10,000 units/ml (standardised to the FDA approved ID_{50} EAL method) was diluted, where necessary, with sterile saline to 2000 units/ml, immediately prior to use.

2.2.2 Subjects

For all studies, except those using antigen challenge in subjects with allergic rhinitis, normal healthy non-atopic volunteers were recruited, with an age range of 19-48 years. Subjects with symptoms of nasal infection or allergy, or who were taking medication within the previous 4 weeks, were excluded. All subjects gave their informed consent, and the studies were approved by the Joint Ethics Committee of University College London and University College London Hospitals NHS Trust. Experiments were performed in a laboratory at a controlled temperature (21°C) and humidity.

In the study using nasal antigen challenge in subjects with seasonal allergic rhinitis, subjects were included only if they had a clinical history of seasonal allergic rhinitis and a positive skin prick response to grass pollen antigen. Subjects who also had a positive response to house-dust (*Dermatophagoides farinae*) or house-dust mite (*Dermatophagoides pteronyssinus*) were excluded. All subjects participating in the study had not been using any oral, intranasal or systemic therapies for the 4 weeks prior to the study, nor presented with any evidence of nasal polyposis or upper

respiratory tract infection. Patients with asthma were recruited only if their asthma was very mild and not exacerbated during the pollen season. Furthermore, such patients were used under close medical supervision and regular expiratory peak flow measurements were taken. Subjects were aged 21-54 years, and gave their informed consent prior to participating. The studies were approved by the Ethics Committee at the Royal National Throat Nose and Ear Hospital (Royal Free Hospital NHS Trust), London.

2.2.3 Acoustic rhinometry

The patency of the nasal airways was measured using an acoustic rhinometer supplied by GM Instruments (Kilwinning, UK). This apparatus consists of a hollow wave tube, approximately 1 metre long, with a spark generator at the lower end. This produces an audible sound pulse which is transmitted up the tube and into the anterior nares of the subject. The sound wave is then reflected back down the tube by structures inside the subject's nose, and received by a microphone located at the top of the tube. The signal is amplified and then transmitted to a computer, where it is analysed and displayed as a graph showing nasal cross-sectional area (cm^2) against distance into the nasal cavity. Each single measurement takes approximately 10 ms.

A plastic tube is inserted into the anterior nares and this fits on to the end of the wave tube. In order to ensure a good seal between this and the subject's nostril, three different sizes of plastic tube were available. Furthermore, the position of each subject's head and angle of the wave tube was kept constant by careful positioning during measurements.

A number of parameters can be measured using acoustic rhinometry, including volume of the nasal cavity. The minimal cross-sectional area of the nasal airway (Amin.) often corresponds to the area between the inferior turbinate bone (located on the lateral side of the nasal cavity) and the nasal septum, and can be used as an anatomical measure of nasal congestion. Changes in Amin. correlate well with resistance to airflow through the nasal airways, measured using rhinomanometry and other measures of nasal congestion. In addition, acoustic rhinometry is quick and

simple to perform, requires little subject training and gives consistent results (the coefficient of variation for mean Amin. measurements is 5.7% compared to 9.6% for nasal airway resistance measurements using rhinomanometry; n=10) (Austin & Foreman, 1994a). Measurement of Amin. using acoustic rhinometry is, therefore, the method of choice in assessing the patency of the nasal airways in subjects.

2.2.4 Nasal lavage

This involves washing the nasal cavity with fluid to collect any chemical mediators which have been released. The technique can also be used to collect cells which are lining the nasal mucosa. The fluid is subsequently recovered and the mediators can be measured using an appropriate assay. In addition, it is also possible to assess any cytological changes, by quantifying the different cell types found in the lavage fluid.

The method used in this study was developed from two other methods for nasal lavage: one described by Naclerio *et al.* (1983); the other by Wihl *et al.* (1995). The modified method involves the gentle syringing of each nasal cavity with 5 ml sterile saline, using a syringe to which a nasal olive (made of medical grade silicone) is attached. Subjects were sitting forwards with the neck flexed and the head at an angle of about 50° to the vertical, to prevent fluid from reaching the nasopharynx. To ensure adequate washing, the lavage fluid was passed slowly into the nasal cavity and then back into the syringe twice. Finally, the fluid was instilled back into the nasal cavity, and collected by the subject expelling the fluid into a collection vessel.

This technique has the advantage of permitting lavage of each side separately with no spill-over into the contralateral nasal cavity. Furthermore, it requires minimal subject training and is well tolerated. Recovery of lavage fluid by this method is approximately 85% (range 60-95%). The method was validated by constructing a dose-response curve to histamine, using albumin levels in the lavage fluid (measured by radial immunodiffusion assay) as the response. The results were similar to those obtained by Naclerio *et al.* (1983).

In each experiment, three initial washes were performed to remove any pre-existing mediators, and the third wash was retained and used as a baseline. Lavage samples were centrifuged at 1000g for 10 min at 4°C, after which the supernatants were separated and stored at -70°C until analysis. For certain experiments, the lavage sample was treated after collection, to stabilise it and prevent metabolism of the mediator of interest.

2.2.5 Cytological studies

In order to assess cytological changes during the nasal challenge experiments, cytological slides were prepared. The lavage sample was centrifuged, as described in the previous section, and the supernatant aspirated. The residual pellet was immediately resuspended in 0.5 ml of phosphate buffered saline (PBS) with 0.1% human serum albumin (HSA), and stored at 4°C until processing.

Two cytospin slides for each sample were prepared using 100 µl aliquots (centrifugation at 500 rpm for 10 minutes in a Shandon II cytocentrifuge; Shandon Southern Ltd, Runcorn, UK). Slides were then stained using the May-Grunwald-Giemsa (MGG) method, to estimate the cellularity of the sample. If necessary, the cell suspension was diluted or concentrated further and new slides prepared. Slides were then stained with carbol chromotrope for 30 min, washed and counter-stained using methylene blue, following which they were cleared in xylene and examined under light microscopy (Figure 2.1). At least 200 cells were counted on each slide, and cells were classified as eosinophils, neutrophils or other. Furthermore, for all samples, the total number of eosinophils present on the slide was counted to obtain an absolute number of eosinophils. The reader was blinded as to which treatment subjects had received.

2.2.6 Nasal challenge

Compounds were administered to the nasal cavity using a hand-held nasal pump spray (Perfect-Valois UK Ltd). This delivers 100 µl per actuation with an accuracy of 98%. Nasal antigen challenge was carried out using a pump spray supplied by the

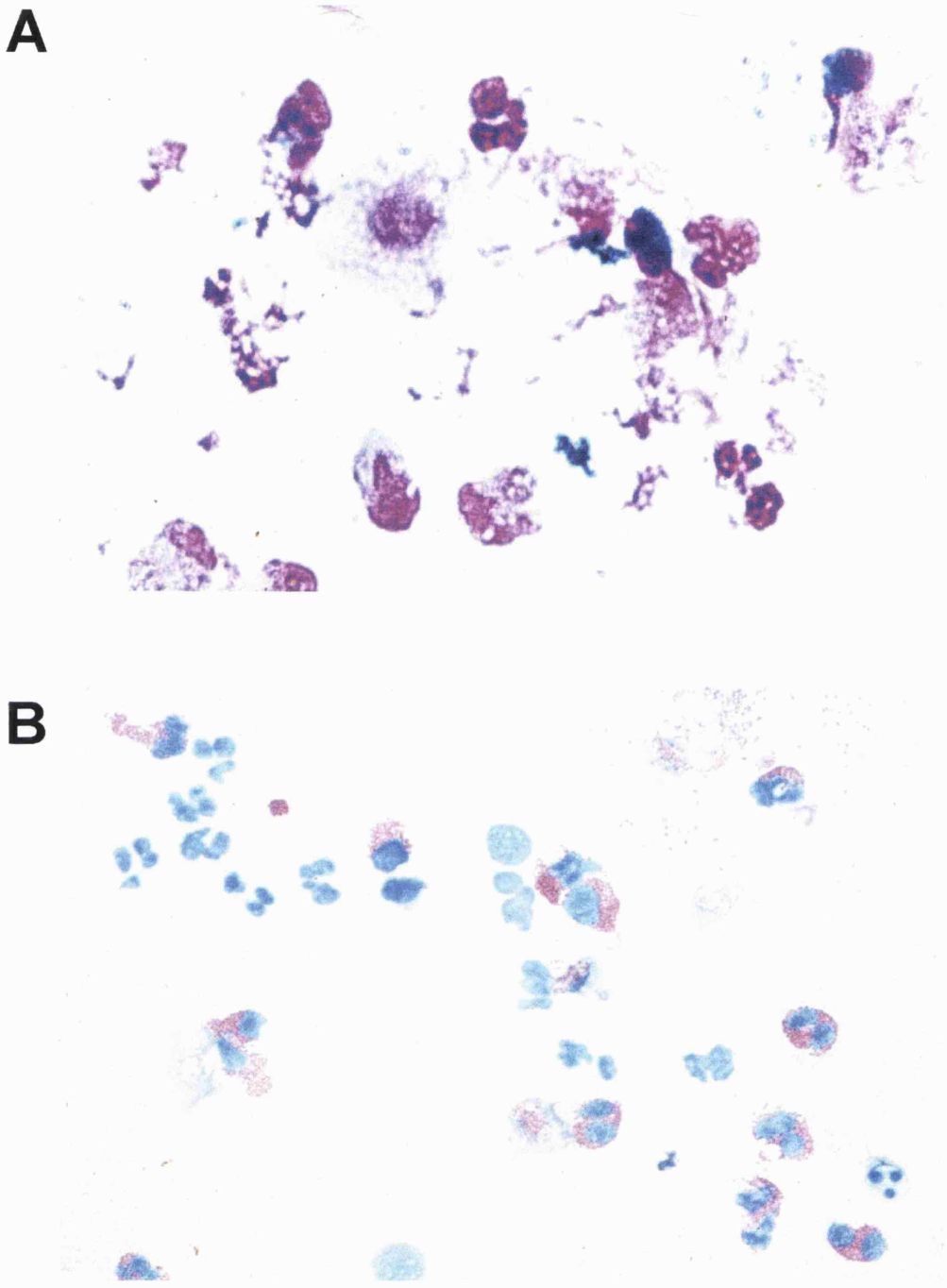

Figure 2.1: Typical photomicrograph of a cytocentrifuged lavage sample, obtained two hours after intranasal administration of platelet activating factor, 60 µg. The specimens were prepared using May-Grunwald-Giemsa (micrograph A) or carbol chromotrope (micrograph B), as described in the text. In micrograph B, eosinophils can be identified by the presence of a granular region, stained red, surrounding the nucleus. Magnification is ×400.

manufacturer, delivering 50 µl per spray. The dose administered was controlled by varying the concentration of the solution in the pump spray. In all experiments, compounds were delivered to both nostrils, and the doses stated are the amounts delivered into each nostril.

2.2.7 General protocol for nasal challenge experiments

Although protocols varied from one experiment to another, the same general procedure was used throughout the study. Where acoustic rhinometry was used to assess patency of the nasal airways, three readings of Amin. were taken in each nostril 2, 5 and 10 minutes after challenge, during which time the maximal response to the challenge agent occurs (Austin, 1994). For nasal lavage, each side of the nasal cavity was initially rinsed three times, the third sample being kept to serve as a baseline. Subsequently, each side of the nose was rinsed once only. All experiments were designed to follow a double-blind, randomised, placebo-controlled, cross-over protocol. This is essential, since parameters such as Amin. vary considerably between subjects under similar conditions. Therefore, each subject acted as their own control, with a suitable wash-out period between treatments. The placebo control used was the vehicle in which the active treatment had been dissolved. The doses of bradykinin, histamine and PAF used were based upon the findings of previous studies (Austin & Foreman, 1994a; Austin, 1994).

2.2.8 Data analysis

The dimensions of the nasal airway vary between subjects, due to differences in the anatomical structure of the nasal cavity between individuals, and also within subjects from day to day. In order to accommodate this, the data obtained from measurements of nasal patency were normalised by expressing changes in Amin. as a percentage decrease in Amin. from the baseline value. Measurements of Amin. were expressed as the mean value for both nostrils, to minimise any effect due to nasal cycling, a phenomenon where one side of the nasal airway alternates in becoming more congested than the other. In all experiments, the mean baseline value for Amin. is given, together with the standard error of the mean (s.e.mean).

For each determination of nasal patency in a subject, a response-time curve was plotted of the percentage decrease in Amin. (from baseline) against time, and the area under the curve (AUC) calculated. Thus, a decrease in Amin. corresponds to an increase in AUC and the degree of nasal obstruction. Data from the determination of nasal patency are expressed as the mean AUC (for all subjects) ± s.e.mean. The advantage of this method is that the response of the nose was assessed over a period of time, and not just at a single timepoint.

2.2.9 Statistical analysis

The data obtained from acoustic rhinometry and nasal lavage were analysed using non-parametric statistical analysis. In all studies, the null hypothesis was that there was no difference in the nasal response between control and active treatments.

For parametric analysis to be valid, data must be normally distributed and the data sets to be compared should have similar standard deviations (Altman, 1991). Although data obtained from acoustic rhinometry generally follows a normal distribution, the standard deviations of the groups being compared were often significantly different. Therefore, data from acoustic rhinometry are expressed as mean values (together with standard errors), but analysed using non-parametric techniques. Data from nasal lavage studies did not, apparently, fit a normal distribution, and the sample sizes used were too small to determine reliably whether the data might be normally distributed within the population. Consequently, these data are given as median values, together with the interquartile ranges and 80% central range, and analysed using non-parametric statistical tests.

Where two groups of data are compared, for example, active treatment with control, the Wilcoxon sign-rank test was used. If a number of treatments were used, either a range of doses or different pre-treatment protocols, the data was first analysed using the Friedman's test. This is the non-parametric equivalent of the parametric two-way analysis of variance (ANOVA) test for paired data. Comparisons between two sets of variables were analysed using the Spearman's rank test. In all experiments, a statistical test was used to determine significant differences between baseline values, as a measure of variation, and not for comparative purposes with results obtained after treatment (Senn, 1989). A probability value, p, of less than 0.05 was considered statistically significant.

2.3. Tissue culture and isolation of human peripheral blood cells

2.3.1 Safety and sterility

The preparation and culturing of cell lines or human cells was performed in an Astecair BHA 48 Class 2 safety cabinet, using aseptic techniques. Cells were prepared for culture under sterile conditions, and incubated at 37°C in an atmosphere containing 95% air and 5% carbon dioxide, with 100% humidity.

2.3.2 Purification of human peripheral blood neutrophils

50 ml of venous blood was obtained from healthy volunteers and 0.8 ml of 0.5 M EDTA in PBS was added and mixed immediately to prevent coagulation. The blood was then mixed with 10 ml of 6% w/v Dextran (mol. wt. 110,000) in Hank's balanced salt solution (HBSS), and left to stand for 30 minutes, or until 25 ml of supernatant had separated out. This was carefully layered onto 25 ml of ficoll-hypaque (histopaque) with a specific gravity of 1.077 g/ml, and centrifuged at 850g for 20 minutes, at room temperature (20°C).

Following centrifugation, the top layers of dextran/plasma, the PBMC (peripheral blood mononuclear cell) layer (visible as a "buffy coat" between the plasma and histopaque) and histopaque were carefully removed. The residual pellet was resuspended in PBS and transferred to a new tube. The cells were then washed once in PBS, and centrifuged at 10°C. Subsequent "washing" of cells involved the suspension of cells in 25-50 ml of buffer/media, centrifugation at 300g for 8 minutes unless otherwise stated, followed by aspiration of the supernatant.

Contaminating red blood cells were then removed by hypotonic lysis. 27 ml of ice-cold distilled water was added and the cell pellet resuspended. After 30 seconds, 3 ml of ×10 PBS was added and the suspension mixed, to restore iso-osmotic balance. Cells were centrifuged (300g, 8 minutes, 4°C) and the supernatant (containing lysed red blood cells) was removed. The remaining leukocytes were washed twice in Ca/Mg-free HBSS, and counted by mixing a 20 µl aliquot of the suspension with an equal volume of trypan blue dye (0.4% w/v) and counting in an improved Neubauer

haemocytometer, using phase-contrast light microscopy. Trypan blue is excluded by live cells, so this technique also allowed for an assessment of cell viability. Samples were only used if 95% or more cells were viable.

Typical yields obtained by this method were 1.4×10^8 neutrophils from 50 ml of whole blood. Typical cell viability was >99%. The percentage of eosinophils present was determined by mixing an aliquot of the cell suspension with an equal volume of carbol chromotrope stain, leaving for 30 minutes, and counting the aliquot in an improved Neubauer haemocytometer (Hakansson *et al.*, 1987). Differential cell counts were also made from cytocentrifuged slides, stained with carbol chromotrope (as described in section 2.2.5). Typically, the eosinophil differential count in samples obtained from normal healthy volunteers was 0.5-4% of total leukocytes.

For the eosinophil/neutrophil transmigration studies, approximately 20×10^6 neutrophils were suspended in 20 ml of Roswell Park Memorial Institute medium-1640 (RPMI-1640) supplemented with 10% v/v heat inactivated foetal calf serum (FCS) and 1% v/v penicillin-streptomycin. Cells were then incubated for 48 hours at 37°C, in an atmosphere of 95% air/5% CO_2 and 100% humidity.

2.3.3 Purification and culture of eosinophils isolated from human peripheral blood

100 ml of venous blood was obtained from volunteers, predetermined to have an eosinophil count of greater than $0.3 \times 10^9/dm^3$ blood. Eosinophils were then isolated using a modified technique to that described by Hansel *et al.* (1991). The leukocyte fraction was prepared as described above for neutrophils. Following the penultimate washing stage, cells were washed with 50 ml of PBS containing 0.5% w/v bovine serum albumin (BSA) and 5 mM EDTA, and counted. Cells were resuspended in 1 ml PBS/BSA/EDTA buffer and mixed with a cocktail of MACS magnetic microbeads (Miltenyi Biotech, Bisley, UK) at the following concentrations: 50 µl of anti-CD16 microbeads per 50×10^6 cells, plus 20 µl of anti-CD3 and 20 µl of anti-CD14 microbeads. The cells were then incubated at 4°C for 30 minutes.

The suspension was then diluted with PBS/BSA/EDTA buffer to a density of 1×10^8 cells/ml, and passed through a magnetic cell separation column (Type CS depletion column, Miltenyi Biotech, Bisley, UK) following the manufacturer's recommended instructions. The negative-labelled fraction, in this case eosinophils, passes through the column and can be collected, while the positive-labelled fraction of CD16-positive neutrophils and CD3- and CD14-positive PBMCs is retained within the magnetic field induced in the depletion column.

The eosinophil-rich fraction was collected on ice, washed twice in Ca/Mg-free HBSS, and resuspended in 20 ml of RPMI-1640 supplemented with 10% v/v FCS and 1% v/v penicillin-streptomycin. Granulocyte-macrophage colony stimulating factor (GM-CSF) was added to a final concentration of 5 ng/ml, and the eosinophils were incubated for 48 hours at 37°C as described above. Typical yields obtained were 14×10^6 eosinophils from 100 ml of whole blood, with a cell viability of >98% and a population purity of >96%.

2.3.4 Cell culture of human umbilical vein endothelial cells (HUVECS)

EA.hy926 cells were kindly provided by Dr M. Perretti (William Harvey Research Institute, London, UK). This cell population is a hybridoma between human umbilical vein endothelial cells (HUVECs) and the epithelioma A549, and is an immortalised cell line which retains the majority of the functional characteristics of HUVECs (Edgell *et al.*, 1983; Thornhill *et al.*, 1993). In this thesis, cells from this cell line will be termed HUVECs. The HUVECs were cultured in Dulbecco's modified Eagle's medium (DMEM) with F-12 HAM nutrient additive, supplemented with 10% v/v FCS, antibiotic solution (100 U/ml penicillin, 100 U/ml streptomycin) and 1% v/v L-glutamine. Cell populations were sub-cultured twice a week (cells reached confluence every 48-72 hours). In brief, confluent cells were washed twice in PBS and dislodged by incubating with 10 ml of cell dissociation fluid (Sigma, Poole, UK) for 20 minutes at 37°C. The cell suspension was then centrifuged at 300g for 5 minutes, resuspended in medium and seeded in a ratio of 1:3 for further growth. The medium was replaced with fresh medium every 36-48 hours, without passaging the cells. Cells were used for the transmigration assay between passages 35 and 48.

2.4 Generation of kinin in human cells *in vitro*

2.4.1 Generation of kinin from kinin moieties on neutrophils

Neutrophils were isolated from human peripheral blood as described, resuspended in HBSS at a density of 2×10^7 cells/ml, and divided into 450 µl aliquots in reaction tubes. The cells were pre-incubated in a water bath set to 37°C for 5 minutes, following which 50 µl of stimulus or control (HBSS) was added. The reaction was allowed to proceed for 30 minutes in a shaking incubator (G24 Environmental Incubator Shaker, New Brunswick Scientific, Edison, New Jersey, USA) at 37°C and 300 strokes/min, before being halted by the addition of 300 µl ice-cold HBSS containing 100 kIU Aprotinin and EDTA to a final concentration of 40 mM (to inhibit the breakdown of kinin by peptidases). The tubes were then centrifuged (400g, 10 minutes, 4°C), the supernatants removed and stored at -70°C until assay.

2.4.2 Generation of kinin from kinin moieties on HUVECs

In order to investigate whether HUVECs can bind high-molecular weight kininogen (HMWK) in a form available for cleavage by kallikrein to generate kinin, the method of Nishikawa *et al.* (1992) was modified as follows. HUVECs were seeded into 6 well plates at a density of 1×10^6 cells per well and grown to confluence. Cells were washed twice with a buffer solution (buffer 1), consisting of 137 mM NaCl, 4 mM KCl, 11 mM glucose, 10 mM HEPES, 1 mM $CaCl_2$ and 1% v/v FCS, and then pre-incubated for 20 minutes with 2 ml of binding buffer (consisting of buffer 1 with the addition of 50 µM $ZnCl_2$). The supernatants were aspirated, and incubated with 2 ml of binding buffer containing 1 µg/ml HMWK for 0, 30, 60 or 120 minutes at 37°C. The cells were then washed three times with binding buffer (to remove excess HMWK), and further incubated with 2 ml of binding buffer containing 100 ng/ml plasma kallikrein. At 0, 15, 30 and 60 minutes after the start of the incubation, 250 µl aliquots of supernatants were removed, peptidase inhibitors added (aprotinin, 100 kIU, and EDTA to a final concentration of 40 mM), and stored at -70°C until assay. At the end of the experiment, the number of cells in each well was counted and recorded.

2.4.3 Data and statistical analysis

The data generated was expressed as kinin production per 10^6 cells. Statistical analysis was carried out using the Student's paired t-test or two-way analysis of variance (ANOVA). A probability value, p, of less than 0.05 was considered statistically significant.

2.5 Cell migration assay

2.5.1 Preparation of HUVEC monolayers

For the investigations of neutrophil transmigration across a HUVEC monolayer, 5×10^4 HUVECs in 100 µl of HUVEC culture medium were seeded into Biocoat fibronectin-coated cell culture inserts (Becton Dickinson, Oxford, UK) of pore size 3 µm and diameter 6.4 mm. The fibronectin coat aids adherence of the HUVECs to the membrane, via β_1-integrins. The inserts were then placed into a 24-well tissue culture plate, into which had been pipetted 500 µl of DMEM with F-12 HAM nutrient mixture per well. HUVECs were allowed to grow to confluence (2-3 days) at 37°C in an atmosphere of 95% air/5% CO_2 and 100% humidity. The medium was replaced with fresh medium after 36-48 hours. Prior to the assay, the HUVEC monolayer was washed twice in HBSS.

For the eosinophil transmigration assay, cell culture inserts with a pore size of 5 µm were used, since eosinophils can not pass through smaller pores. Therefore, the transwell system (Corning Costar, High Wycombe, UK) was used. Since these cell inserts are not available ready-coated with fibronectin, they were prepared as follows: Human plasma fibronectin was diluted to 6 µg/ml in serum-free DMEM with F-12 HAM and 250 µl of this solution was added to each transwell insert. The inserts were incubated at room temperature for 1 hour, following which the solution was removed and the inserts washed in PBS. 5×10^4 HUVECs in 100 µl of HUVEC medium were then seeded into the inserts (diameter 6.5 mm) and grown to confluence as before. The HUVEC monolayer was washed twice in HBSS prior to the assay.

2.5.2 Chemotaxis assay

For the chemotaxis assay, transwell cell culture inserts (Corning Costar, High Wycombe, UK) of pore size 3 µm and diameter 6.5 mm were used. Neutrophils were prepared as above and suspended in HBSS at a cell density of 15×10^6 cells/ml and prewarmed to 37°C. 100 µl of this suspension (approximately 1½ million cells) was added to each cell insert. 600 µl of HBSS (prewarmed to 37°C) containing the appropriate stimulus was added to the bottom wells, and the inserts lowered into the wells and incubated for 2 hours in an atmosphere of 95% air/5% CO_2. After the incubation, migration was halted by incubating the plate at 4°C for 10 minutes. The cell culture inserts were removed and 100 µl of ice-cold cell dissociation fluid (Sigma, Poole, UK) was added to each well, to prevent cell adherence to the bottom of the wells. The cells in the bottom of each well were resuspended and counted using trypan blue stain as described.

In those experiments which investigated the role of pharmacological antagonists on neutrophil chemotaxis, the inhibitor was added to both the bottom well and the cell suspension in the cell culture inserts, at the appropriate concentration. In addition, the neutrophils were incubated with the antagonist for 10 minutes at 37°C prior to the commencement of the assay.

2.5.3 Neutrophil transmigration assay

Neutrophils were prepared as above and suspended in HBSS at a cell density of 15×10^6 cells per ml and prewarmed to 37°C. 100 µl of this suspension (approximately 1½ million cells) was added to each cell insert. 500 µl of HBSS (prewarmed to 37°C) containing the appropriate stimulus was added to the bottom wells, and the inserts lowered into the wells and incubated for 2 hours, at 37°C in an atmosphere of 95% air/5% CO_2 and 100% humidity. After the incubation, migration was halted as for the chemotaxis assay, and the number of cells in the lower well counted. In experiments where the effect of antagonists on neutrophil transmigration was investigated, the HUVEC monolayer on the cell culture inserts were first incubated in DMEM with F-12 HAM containing the antagonist (or culture medium alone as a control) at the

appropriate concentration, for 1 hour at 37°C in an atmosphere of 95% air/5% CO_2 and 100% humidity. The inserts were then washed once in HBSS, and the assay set up as before, with the antagonist added to both the bottom well and the cell suspension in the cell culture inserts, at the relevant concentration. Neutrophils were incubated with the antagonist for 10 minutes at 37°C prior to the commencement of the assay.

In some experiments, the effect of bradykinin generation on cell transmigration was also investigated. The HUVEC monolayers, on the cell culture inserts, were grown to confluence as before, and washed twice in serum-free medium. The monolayers were then incubated with 200 µl of medium, containing 1 µg/ml HMWK, at 37°C in an atmosphere of 95% air/5% CO_2 and 100% humidity. The HUVEC monolayers in the control inserts were incubated with medium without HMWK. After 2 hours, the inserts were removed and washed three times in HBSS to remove any excess HMWK. The cell transmigration then proceeded as above.

2.5.4 Eosinophil transmigration assay

The eosinophil transmigration assay was set up as for neutrophils, except the assay incubation time was extended to 3 hours (identified as the optimum incubation period from pilot experiments). In order to investigate the role of neutrophils on eosinophil transmigration, in some experiments, a mixture of 50% eosinophils and 50% neutrophils was added to the cell culture inserts, at an overall cell density of 1-1.5 $\times$ 10^6 cells per insert. At the termination of the experiment, the absolute number of eosinophils only was counted (using carbol chromotrope stain as described). In order to control for cell number, in these experiments, eosinophils only were added to some inserts at a density of 0.5-0.75 $\times$ 10^6 as well as at 1-1.5 $\times$ 10^6 cells per insert.

2.5.5 Assessment of "cell drop off"

Any neutrophils or eosinophils remaining trapped in the membrane on the underside of the cell culture inserts would influence the results of the migration assay. Ideally, "cell drop off" would be maximal, so that all cells passing through pores in the

membrane would migrate to the lower chamber. In order to assess this, in some experiments, the cell culture inserts were washed in PBS and then placed in 4% v/v formaldehyde in PBS for 10 minutes, to fix any cells left in the membrane. The membranes were then washed in distilled water, stained with haematoxylin for 10 minutes, washed again and dried, and finally cut away from the inserts and mounted. The number of cells retained in the membrane was then counted using a light microscope, to give an indication of "cell drop off".

2.5.6 Data and statistical analysis

For both chemotaxis and transmigration studies, the migration of cells from the upper chamber (cell culture insert) to the lower chamber (the lower well of the cell culture plate) was expressed as the percentage net stimulated migration (%NSM):

$$\%NSM = \frac{\text{No. cells in lower chamber of experimental well} \quad \textbf{MINUS} \quad \text{No. cells in lower chamber of control well (HBSS only)}}{\text{No. cells added to the top chamber (cell culture insert)}}$$

Statistical analysis was carried out using the Student's paired t-test. A probability value, p, of less than 0.05 was considered statistically significant.

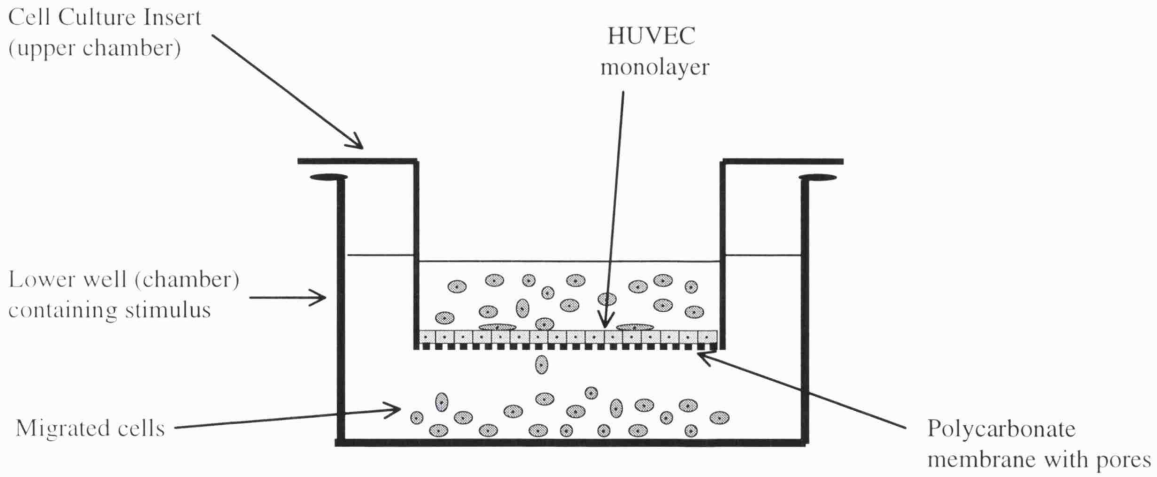

Figure 2.2: The transmigration assay system.

2.6 Biochemical assays

2.6.1 Albumin measurement

The albumin content of nasal lavage fluid was determined using a radial immunodiffusion assay (Mancini *et al.*, 1965). 20 µl volumes of nasal lavage samples, human serum albumin standards and control samples were loaded into wells on the immunodiffusion plates (Behring AG, Germany). The plates were then left open for 30 minutes at room temperature to allow the sample to diffuse into the agar-gel layer, following which the plates were sealed and incubated for 48 hours at room temperature. The diameters of the precipitation rings were measured using a special tool and a standard curve constructed from the albumin standards. A standard curve was constructed for each immunodiffusion plate used, and control serum samples were used to check the function of each plate. The albumin concentrations of the samples were determined from the standard curves. The assay was sensitive to albumin concentrations in the range 2.5 to 44 mg/dl.

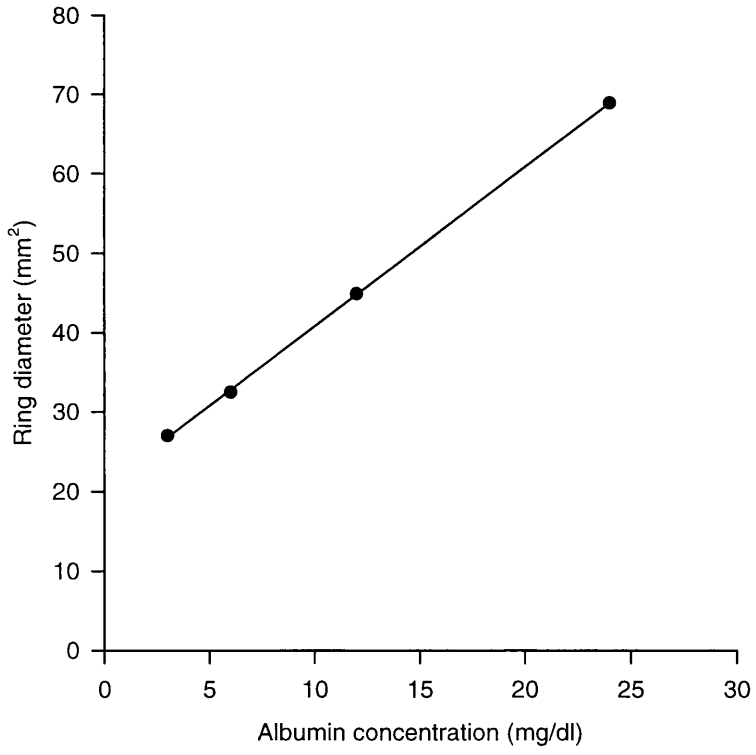

Figure 2.3: A typical standard curve for albumin measured by radial immunodiffusion.

2.6.2 Bradykinin assay

In order to determine the bradykinin content of nasal lavage fluid, a commercially available bradykinin radioimmunoassay was used (Peninsula Laboratories, USA). The assay is based upon the competition of radiolabelled ^{125}I-[Tyr^{0}]-bradykinin with unlabelled bradykinin or kallidin (in the standards or samples) binding to a limited quantity of specific antibody. The more unlabelled peptide present in the sample, the lower the quantity of radiolabelled peptide which will bind to the antibody. Therefore, by measuring the amount of radiolabelled bradykinin in the presence of different concentrations of bradykinin standards, a standard curve can be constructed from which the concentration of kinins in the unknown samples can be determined (Figure 2.4). The assay was equally sensitive for bradykinin and kallidin, and so results are expressed as kinin levels in nasal lavage samples.

2.6.2.1 Sample preparation

Nasal lavage samples were prepared as follow: After collection, a 1 ml aliquot was removed and EDTA added to give a final concentration of 40 mM, so as to inhibit any kininase activity present (Proud *et al.*, 1983). The sample was then centrifuged at 4°C for 10 min at 1000g, and the supernatant assayed immediately or stored at -70°C. Samples from the cell culture experiments were prepared as described in those experiments.

2.6.2.2 Assay protocol

1) Bradykinin standard or nasal lavage fluid was added to an antiserum specific for kinin raised in the rabbit. A new standard curve was used for every assay, and each standard or sample was assayed in duplicate. Tubes were then vortexed and incubated at 4°C overnight.

2) ^{125}I-[Tyr^{0}]-bradykinin was added and the tubes vortex-mixed and again stored at 4°C overnight.

3) Goat anti-rabbit IgG serum and normal rabbit serum were added to each tube and the tubes mixed and left at room temperature for 90 minutes. The tubes were then centrifuged at 1700g for 20 min at 4°C, the supernatant aspirated and the radioactivity present in the residual pellets counted in a gamma counter (Wallac 1261, Wallac-Pharmacia, Finland).

The assay was sensitive over the range of 1-128 pg kinin per tube.

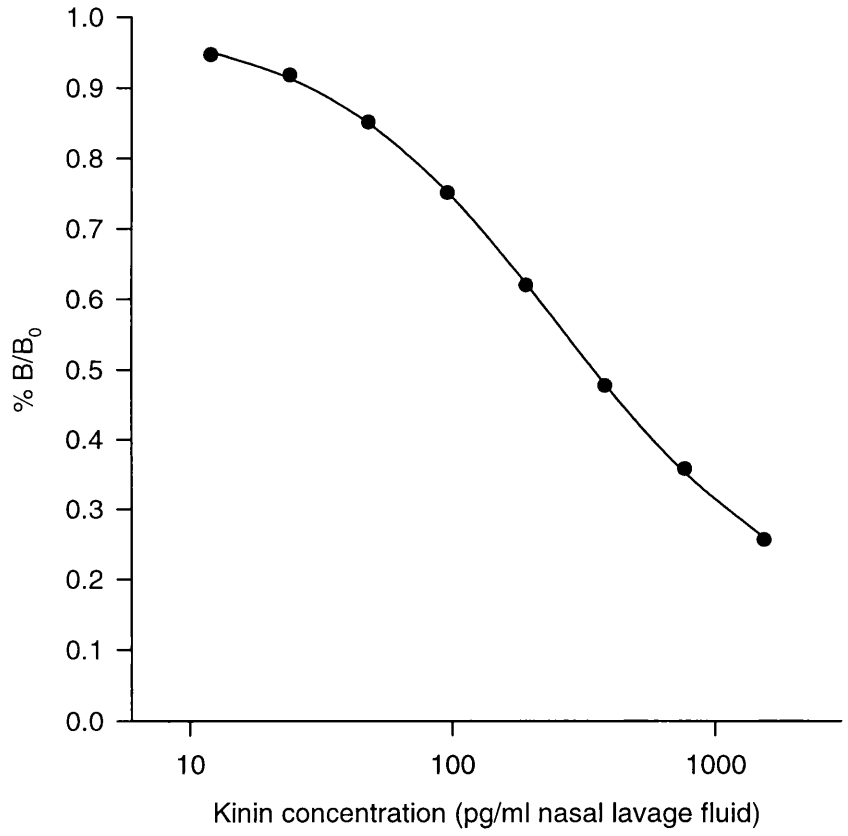

Figure 2.4: A typical standard curve for bradykinin as measured by radioimmunoassay. B and B_0 represent the specific binding of ^{125}I-[Tyr^0]-bradykinin to kinin-specific antibody in the presence and absence (respectively) of unlabelled bradykinin.

2.6.3 Eosinophil cationic protein (ECP) assay

The ECP content of nasal lavage was determined by a double antibody radioimmunoassay (Pharmacia, Sweden), using a method originally described by Venge *et al.* in 1977, and subsequent modified in 1991 (Peterson *et al.*, 1991). The principle of the assay and protocol are similar to that used to assay bradykinin, except the human ECP standards, radiolabelled [^{125}I]-ECP and antibody to ECP (raised in the rabbit) were used. Samples and standards were assayed in duplicate, and a new standard curve was constructed for each assay (Figure 2.5).

The detection limit of the assay was <2 µg/l. The assay was almost totally specific for ECP only, with a minimal cross-reactivity with Eosinophil Peroxidase (less than 0.06%, data from Pharmacia Diagnostics, Sweden).

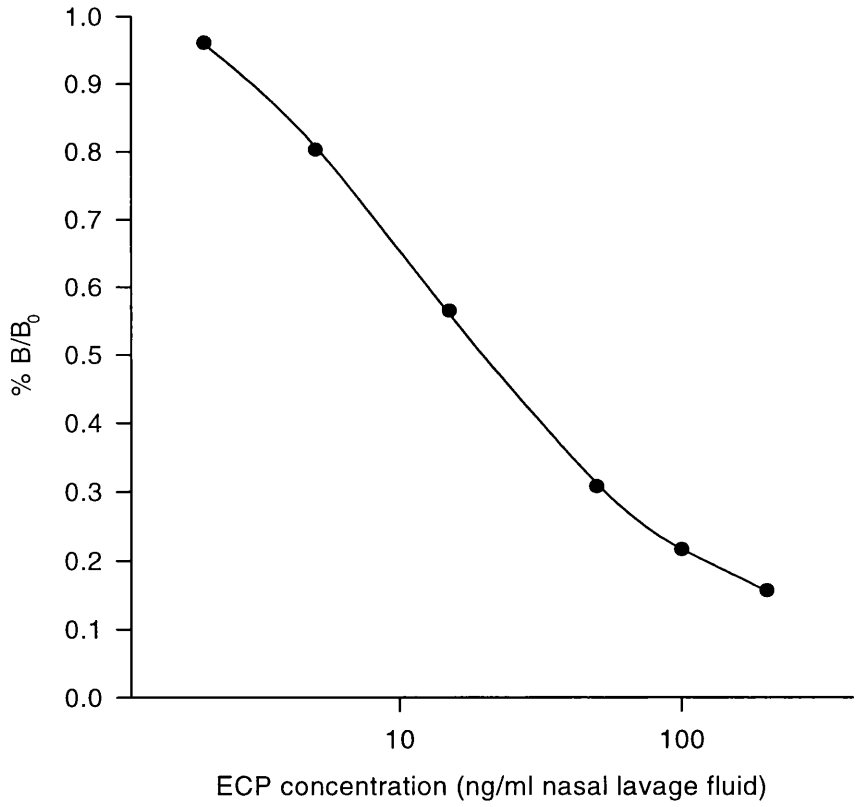

Figure 2.5: A typical standard curve for ECP measured by radioimmunoassay. B and B_0 represent the specific binding of [^{125}I]-ECP to ECP-specific antibody in the presence and absence (respectively) of unlabelled ECP.

2.6.4 Substance P assay

Substance P in nasal lavage samples was measured by a commercially available radioimmunoassay (Peninsula Laboratories, USA). The assay functioned in essentially the same way as the bradykinin assay used, except the radiolabel was ^{125}I-[Tyr^{8}]-substance P and the antibody was specific to substance P and substance P fragments 2/3/4/5-11. In order to prevent the metabolism of any substance P present in the samples, 100 µl of a peptidase inhibitor cocktail was added to 0.9 ml of lavage fluid immediately after collection (Schultz *et al.*, 1996). The inhibitor mixture consisted of aprotinin (400 kIU), EDTA (400 mM) and phosphoramidon (100 µM). Furthermore, after centrifugation, tubes were filled with nitrogen (to prevent autooxidation of substance P to Met-suphoxide-substance P) and stored at -70°C until analysis. The sensitivity of the assay was 1-64 pg per tube.

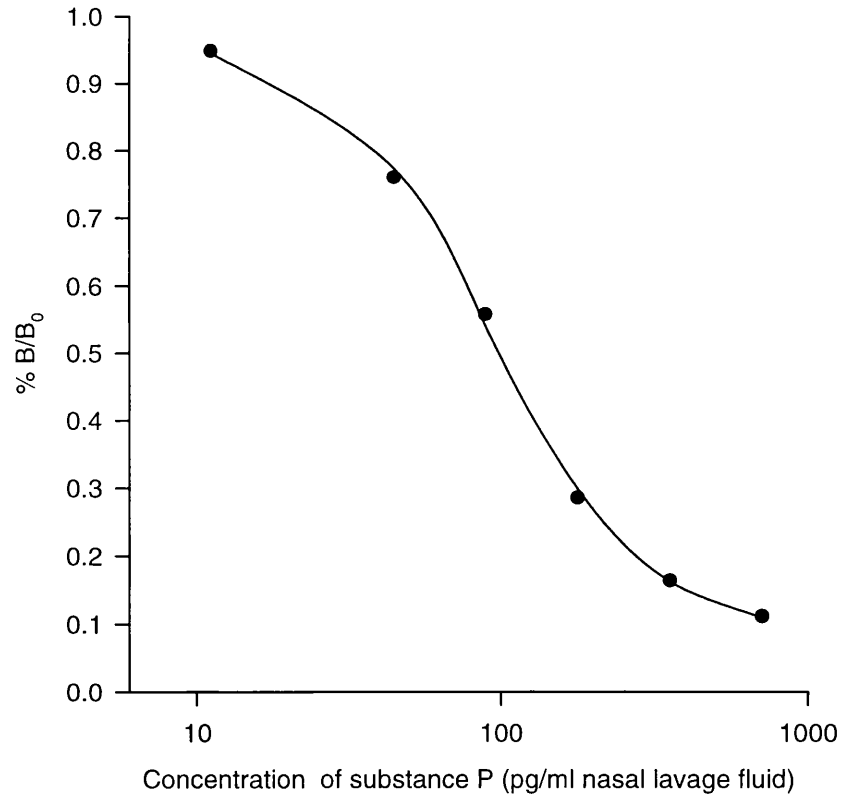

Figure 2.6: A typical standard curve for substance P measured by radioimmunoassay. B and B_0 represent the specific binding of ^{125}I-[Tyr^{8}]-substance P to a specific antibody in the presence and absence (respectively) of unlabelled substance P.

2.6.5 Interleukin-5 and Interleukin-8 assay

Interleukin-5 (IL-5) and IL-8 were determined using commercially available, solid phase enzyme-linked immunosorbent assays (ELISA) based on the sandwich immunoassay principle. In both cases, samples or standards were incubated in microtitre wells, coated with a murine monoclonal antibody to the cytokine being measured. A second 'tracer' antibody was then added after washing, which bound to the IL-5 or IL-8 captured in the microtitre wells.

In the IL-5 assay (Cytimmune Sciences, USA), a further solution consisting of an alkaline phosphatase conjugate was added, and the reaction allowed to proceed for 20 minutes. The absorbance of the solution in the microtitre wells was measured at 492 nm, using a spectrophotometric plate reader (Titertek Multiskan MCC340, Flow Laboratories, Switzerland). The sensitivity of the assay was 0.92 pg/ml, with a range of detection of 8 pg/ml to 500 pg/ml, and a cross-reactivity with other cytokine standards of <0.5%. A typical standard curve is shown in Figure 2.7.

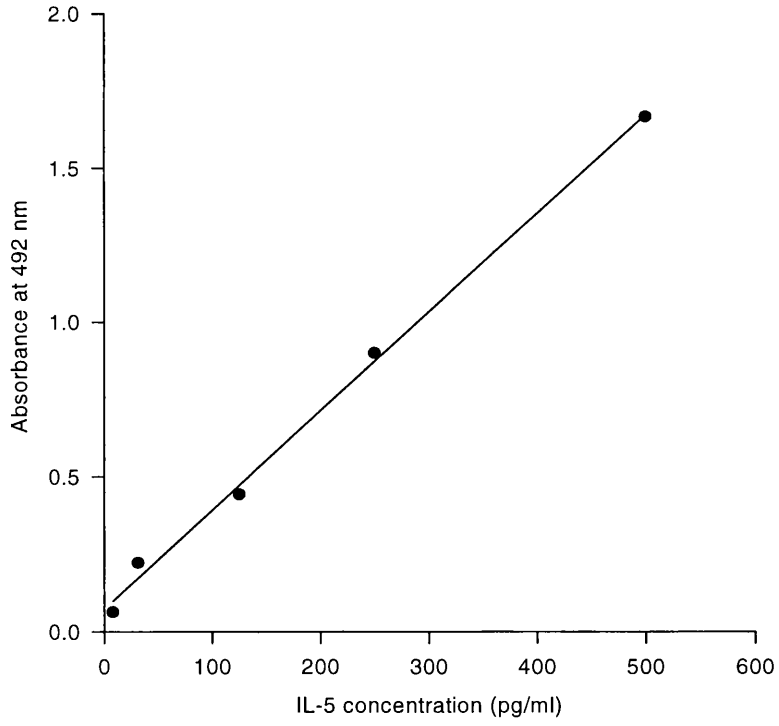

Figure 2.7: A typical standard curve for human IL-5 measured by ELISA

For the IL-8 assay (Hycult biotechnology, Netherlands), a biotinylated the tracer antibody was added after the first incubation. Addition of a streptavidin-peroxidase conjugate, followed by tetramethylbenzidine, resulted in a colour change proportional to the amount of IL-8 present. The reaction was stopped by the addition of 0.5 M citric acid, and then the absorbance of the solutions measured at 450 nm. A standard curve was then constructed, from which the levels of IL-8 in the samples could be determined (Figure 2.8).

The sensitivity of the assay was <5 pg/ml, with a detection range of 4 pg/ml to 1000 pg/ml. There was no significant cross-reactivity with other cytokines (manufacturer's specifications).

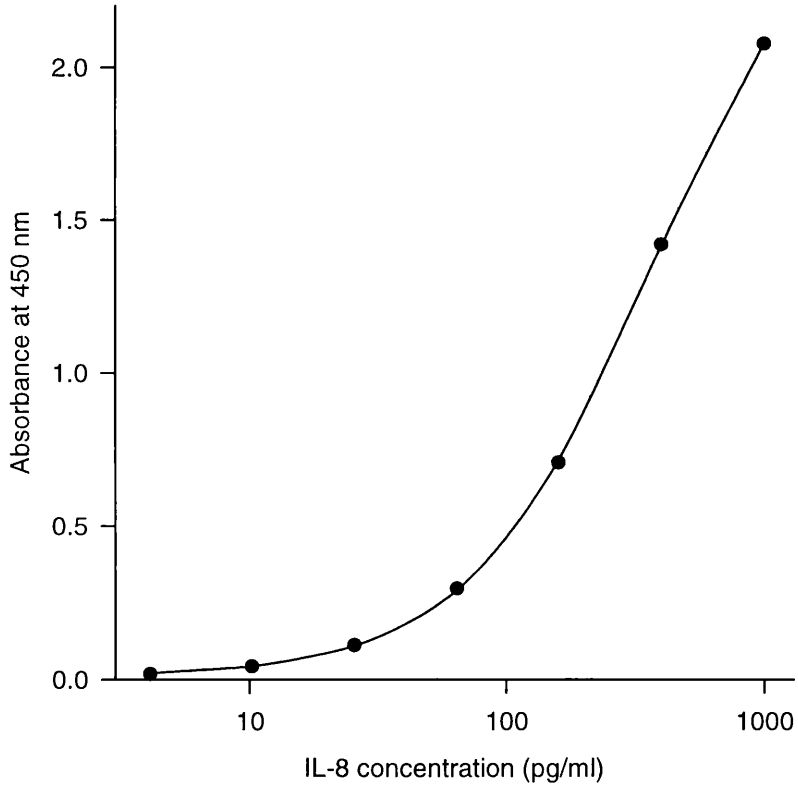

Figure 2.8: A typical standard curve for human IL-8 measured by ELISA.

2.6.6 Nitric oxide

Nitric oxide activity is commonly measured in two ways, either by direct detection using a chemiluminescene detector, or by measuring the levels of nitrate and nitrite, both metabolites of nitric oxide, in biological fluids.

2.6.6.1 Direct Chemiluminescence

Chemiluminescence analysers function by measuring the light emitted following the photochemical reaction between nitric oxide (NO) and ozone (O_3):

$$NO + O_3 \longrightarrow NO_2 + O_2 + \text{photon of light}$$

The amount of light produced is directly proportional to the amount of NO present, provided that excess ozone is available.

Using a chemiluminescene detector designed to measure NO in expired air (LR2000 detector, Logan Research Ltd., Rochester, UK), the NO present in the nasal airways was measured by inserting a probe into each nares in turn while the subject was holding his/her breath following full inspiration. When measuring nasal NO, it is important to prevent the dilution of the nasal air with air (containing much lower levels of NO) from the lower airways. Using the technique of breath-holding, dilution of nasal NO was minimised. In order to ensure this, readings were only accepted if CO_2 levels in the sampled air were below 0.5% (expired air from the lower airways contains higher levels of CO_2; therefore, any dilution of nasal air would result in an increase in the amount of CO_2 detected). The measurement of nasal NO followed the guidelines recommended by the European Respiratory Society (Kharitonov *et al.*, 1997a).

2.6.6.2 Griess reaction

To determine the level of nitrate and nitrite in nasal lavage samples, the method used by Schmidt & Kelm (1996) was modified. This method involves the diazotisation of sulphanilic acid by nitrite and its subsequent coupling with N-(L-naphthyl)-ethylene

diamine (NED), which can be detected using a spectrophotometer. This reaction is known as the Griess reaction. However, since this method only detects nitrite, it is was first necessary to convert the nitrate in the samples to nitrite enzymatically. The conversion of nitrate to nitrite was monitored by using both nitrate and nitrite as standard solutions in the assay.

2.6.6.2.1 Conversion of nitrate to nitrite

An enzyme cocktail of nitrate reductase (640 mU/ml) and flavin adenine dinucleotide (FAD) (40 µM) was made up in PBS (pH 7.4). 25 µl of this mixture was added to 150 µl of the standard solution or lavage sample in a 96 well plate. 25 µl of 1.6 mM nicotinamide adenine dinucleotide phosphate (NADPH) in PBS was added, and the plate incubated in the absence of light at 37°C for 45 minutes. The conversion of nitrate to nitrite requires the oxidation of NADPH to $NADP^+$. Unfortunately, NADPH interferes with the Griess reaction, so any excess NADPH was oxidised by the addition of lactate dehydrogenase and sodium pyruvate (to a final concentrations of 50 U/ml and 1 mM respectively). The reaction was allowed to proceed for a further 10 minutes at 37°C in the absence of light, following which the reaction solutions were placed on ice.

2.6.6.2.2 Deproteination of samples

Any protein in the samples might be precipitated by the reagents used in the Griess reaction, and therefore interfere in the spectrophotometric detection of the diazonium salt. To prevent this, reaction solutions were then deproteinated by the addition of 30 µl of 1.3M zinc sulphate and 20 µl of 1M sodium hydroxide, in that order (Garrrelds *et al.*, 1995). Solutions were left for 15 minutes on ice and then centrifuged at 3000g for 5 minutes at 4°C (pre-cooling samples increases the stability of the diazonium salt produced by the Griess reaction). The supernatants were then assayed for nitrite content.

2.6.6.2.3 Griess reaction

For the final stage of the assay, commercially prepared and standardised Griess reagents were used. 100 µl of sample or standard were transferred to a 96-well flat-bottomed assay plate, and 50 µl of sulphanilamide solution was added and the plate incubated at room temperature (20°C) and in the absence of light. After 5 minutes, 50 µl of NED was added and the plate incubated for a further 20 minutes under the same conditions. The absorbance of the solution in the wells was then determined using a spectrophotometric plate reader (Titertek Multiskan MCC340) with a filter of 540 nm. The limit of detection for the assay was 1 µM.

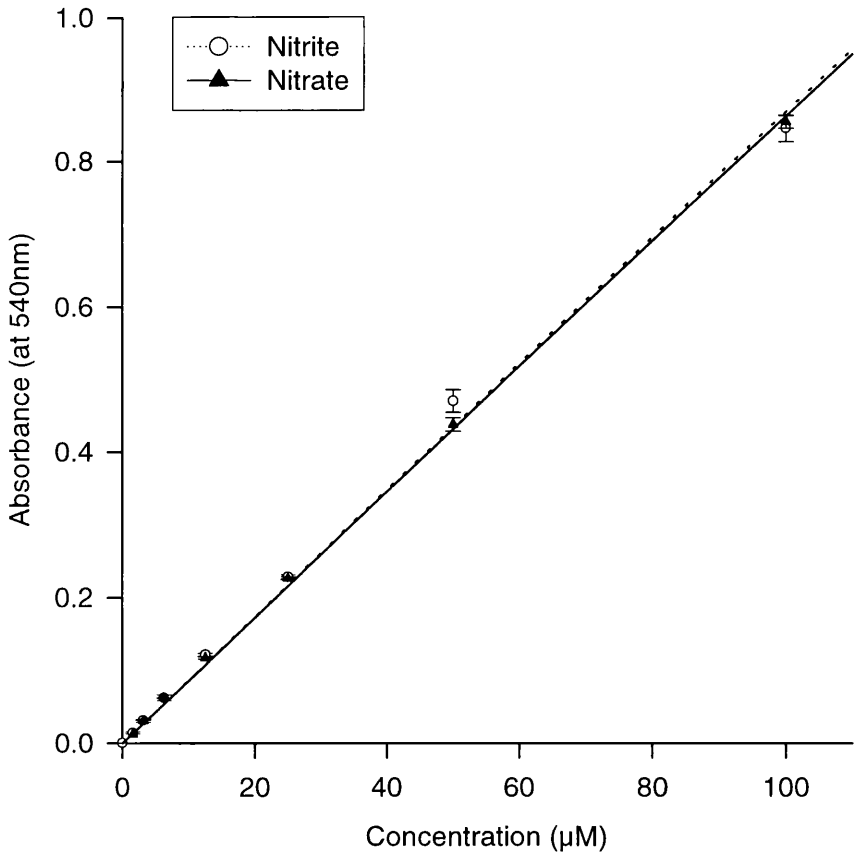

Figure 2.9: Typical standard curves for nitrate and nitrate, determined by the Griess reaction. Data are mean±s.e.mean from three measurements. The correlation coefficient between the two standard curves shown is 0.9992.

CHAPTER 3

THE ROLE OF KININS IN PLATELET ACTIVATING FACTOR-INDUCED HYPERRESPONSIVENESS IN THE HUMAN NASAL AIRWAY

3.1 Introduction

One of the characteristic signs of allergic rhinitis is an increased response of the nasal airway to challenge with a range of stimuli, including histamine and bradykinin. In non-atopic subjects, platelet activating factor (PAF), but not lyso-PAF, can be used to induce a nasal airway hyperresponsiveness (AHR) similar to that caused by antigen in allergic rhinitis (Andersson & Pipkorn, 1988; Austin & Foreman, 1993). It also causes the recruitment of eosinophils into the nasal airway, and the subsequent activation of eosinophils, resulting in the increased production of eosinophil cationic protein (ECP) (Tedeschi *et al.,* 1994a). Furthermore, the release of ECP, by PAF, corresponds to the presence of AHR in the human nose; implying that eosinophil activation is an important step in the development of PAF-induced AHR (Austin & Foreman, 1993). One possible mechanism by which PAF may cause AHR is via the activation of eosinophils, resulting in the release of eosinophil proteins such as ECP and major basic protein (MBP).

In the lower airways of rats, MBP caused an AHR which was dependent upon the generation of kinins (Coyle *et al.,* 1995). Furthermore, pretreatment with NPC 17713, a bradykinin B_2 receptor antagonist, prevented the MBP-induced AHR. It has also been reported that bradykinin alone produced AHR to acetylcholine in guinea pig airways (Omini *et al.,* 1989). This chapter investigates the involvement of kinins in the development of AHR, induced by PAF, in the human nasal airway.

3.2 Experimental Protocol

In the following experiments, healthy non-atopic human volunteers were used as subjects.

3.2.1 The effect of bradykinin B_2 receptor antagonists on platelet activating factor-induced hyperresponsiveness

In order to assess the presence or absence of AHR, the change in nasal patency induced by histamine challenge was measured, following pretreatment of the nasal airways with PAF. The effect of bradykinin B_2 receptor antagonists on PAF-induced AHR was studied. The antagonists used were icatibant, 200 µg, and [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin ([Ad]-BK), also at 200 µg.

Subjects received, by intranasal spray, either icatibant or saline (as a control). Two minutes later, subjects were given a further spray containing either PAF, 60 µg, or a saline control. Since the duration of action of icatibant is approximately two hours (Dear, 1996), a further dose of icatibant or a saline control was administered two and four hours later. Six hours after the start, subjects were challenged with histamine, 200 µg. The time course was chosen on the basis of previous studies which identified a significant increase in the response of the human nasal airways to histamine 6 hours after PAF administration (Austin & Foreman, 1993). Immediately prior to, and 2, 5 and 10 minutes after histamine challenge, the minimal cross-sectional area (Amin.) of the nasal airways was measured by acoustic rhinometry. The protocol is summarised in Figure 3.1.

Each subject received all four possible combinations of treatment, in a cross-over manner, i.e.:

- Icatibant, 200 µg (three administrations) and PAF 60 µg (once)
- Icatibant, 200 µg (three administrations) and saline (once)
- Saline (three administrations) and PAF 60 µg (once)
- Saline (three administrations) and saline (once)

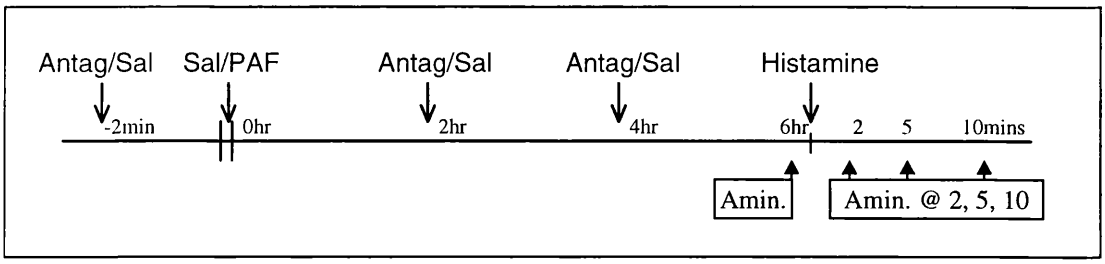

Figure 3.1: Protocol for investigating the effect of bradykinin B_2 antagonists on PAF-induced hyperresponsiveness, by measuring the change in nasal patency following intranasal histamine challenge. Antag = antagonist, Sal = saline.

The action of PAF persists for up to 48 hours, therefore each combination was administered on separate occasions at least 2 days apart (Austin, 1994). The order of treatments was determined using a randomised, balanced-block design. Subjects were randomly assigned to the treatment protocols.

The experiment was repeated on a separate occasion, using [Ad]-BK, 200 µg, instead of icatibant.

3.2.2 The effect of PAF and lyso-PAF on mediator release in the nasal cavity

Subjects received either saline, PAF, 60 µg, or lyso-PAF, 60µg by intranasal spray. Immediately prior to treatment, three nasal lavages were performed, the third being retained as a baseline. At 2, 4 and 6 hours later, a further nasal lavage was carried out. All subjects received all three treatments on separate occasions, in a random order, at least 48 hours apart. The protocol is summarised in Figure 3.2.

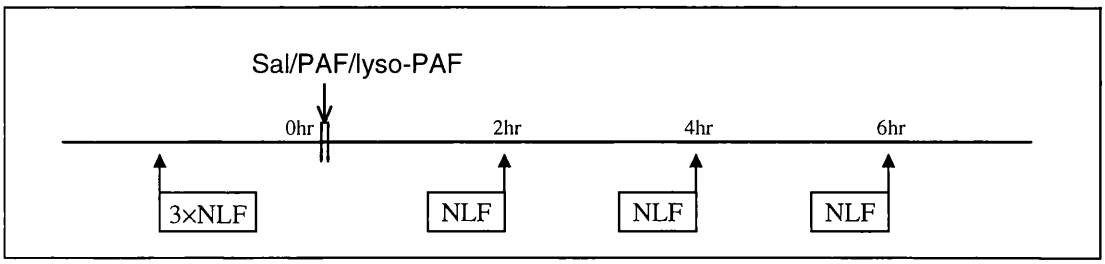

Figure 3.2: Protocol for investigating the effect of PAF and lyso-PAF on mediator release in the nasal cavity. NLF = nasal lavage; Sal = saline.

Nasal lavage samples were processed, and stored at -70°C, until assay for their kinin, ECP and albumin content, as described in chapter 2. For the measurement of ECP, the three nasal lavage samples collected over the six hours were combined, and the mean ECP determined using the assay.

3.2.3 The effect of bradykinin B_2 receptor antagonists on PAF-induced changes in mediator release in the nasal cavity

In order to determine whether PAF-induced mediator release could be modulated by bradykinin B_2 receptor antagonists, a separate experiment was carried out, utilising a protocol summarised in Figure 3.3. Initially, a nasal lavage was carried out three times per nostril, the third being retained and used as a baseline. The nasal cavity was then allowed to dry, following which subjects received a spray of either icatibant or a saline control. Two minutes later, PAF, 60 µg, or saline (as a control) was instilled into each nostril. Nasal lavage was again carried out 2, 4 and 6 hours later. In addition, following the lavage at 2 and 4 hours, subjects were again administered icatibant or a saline control as before. All subjects received all 4 combinations of treatment in a random order on separate occasions. The experiment was repeated on a separate occasion, using [Ad]-BK, 200 µg, instead of icatibant.

The nasal lavage samples were processed and stored at -70°C, until assayed for their kinin and ECP content, as described in chapter 2.

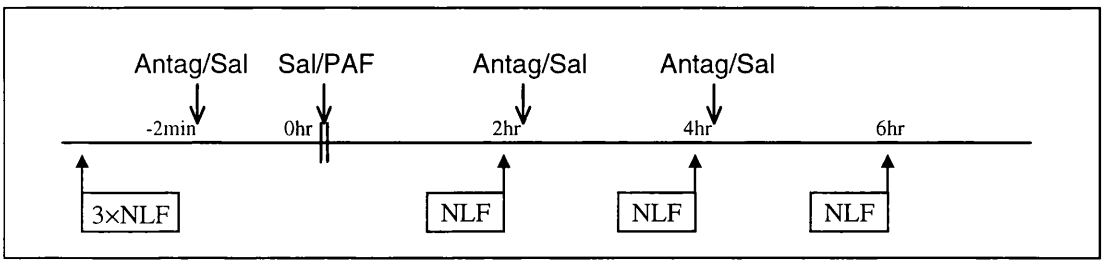

Figure 3.3: Protocol for investigating the effect of bradykinin B_2 antagonists on PAF-induced changes in mediator release in the nasal cavity. NLF = nasal lavage; Antag = antagonist, Sal = saline.

3.2.4 The effect of bradykinin on the responsiveness of the human nasal airway to histamine

In this study, an initial value of Amin. was obtained. This value was used to determine any change in the resting level of Amin. resulting from pretreatment of the nose with bradykinin or the saline control. Subjects then received 5 intranasal administrations of either bradykinin, 500 µg, or a saline control, at 30 min intervals. At 3, 4 and 6 hours later, a baseline measurement of Amin. was taken, followed by nasal provocation with histamine, 200 µg. Three further values of Amin. were measured 2, 5 and 10 min after each histamine challenge. Subjects received both bradykinin and saline pretreatments on separate occasions, at least 72 hours apart. The protocol is summarised in Figure 3.4.

In a second study following a similar protocol, subjects received 5 intranasal doses of bradykinin, either 0, 200 or 500 µg, as before. At 3 and 4 hours later, a baseline measurement of Amin. was determined, which was followed by a histamine challenge of 30, 100 or 300 µg. The nasal response was then monitored, at 2, 5 and 10 min after each histamine challenge. Subjects received all possible combinations of bradykinin pretreatment and histamine on separate occasions, at least 72 hours apart.

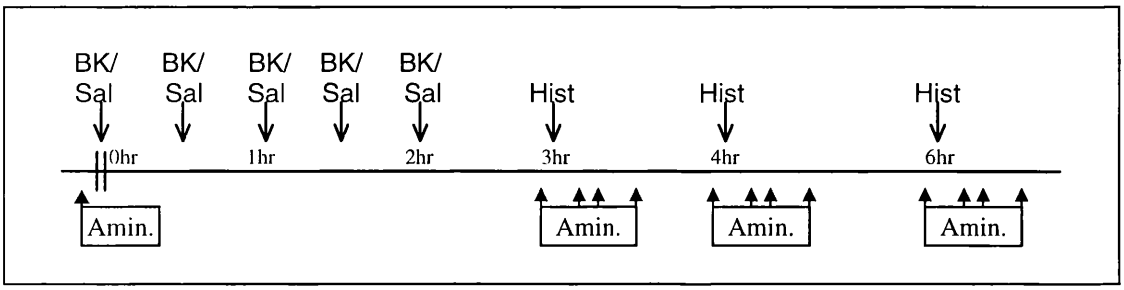

Figure 3.4: Protocol for investigating the ability of bradykinin to induce a hyperresponsiveness to histamine, by measuring the change in nasal patency following intranasal histamine challenge. BK = bradykinin, Sal = saline, Hist = histamine.

3.2.5 Data analysis

Measurements of nasal patency using Amin. following nasal challenge were normalised, as described in chapter 2. For each challenge, a response-time curve was plotted using the percentage decrease in Amin. from baseline against time, and the area under the curve (AUC) determined. The data are presented as mean values, together with s.e.mean. Data from nasal lavage are expressed as medians, together with the interquartile range and 80% central range. The appropriate non-parametric statistical test is given with each data set. A value of $p<0.05$ is taken as significant.

3.3 Results

3.3.1 Effect of bradykinin B_2 receptor antagonists PAF-induced hyperresponsiveness

Histamine, 200 µg, caused a significant increase in AUC, corresponding to an increase in obstruction of the nasal airway ($p<0.05$, Wilcoxon sign-rank test) (Figure 3.5). Treatment with icatibant or [Ad]-BK, 200 µg, did not affect the response to histamine ($p>0.05$, Wilcoxon sign-rank test).

Pretreatment with PAF, 60 µg, resulted in an increased nasal response to histamine, significantly greater than that induced by histamine following pretreatment with saline control ($p<0.05$, Wilcoxon sign-rank test). This hyperresponsiveness to histamine was abolished if the nasal cavity was pretreated with icatibant, 200 µg ($p<0.05$, Wilcoxon sign-rank test) (Figure 3.5). Similarly, [Ad]-BK also prevented the increase in nasal response to histamine caused by PAF ($p<0.05$, Wilcoxon sign-rank test) (Figure 3.6). There were no differences in the initial baseline values of Amin. between treatment groups in either experiment (0.64 ± 0.03 and 0.61 ± 0.03 cm^2 respectively; $p>0.05$, Friedman's test).

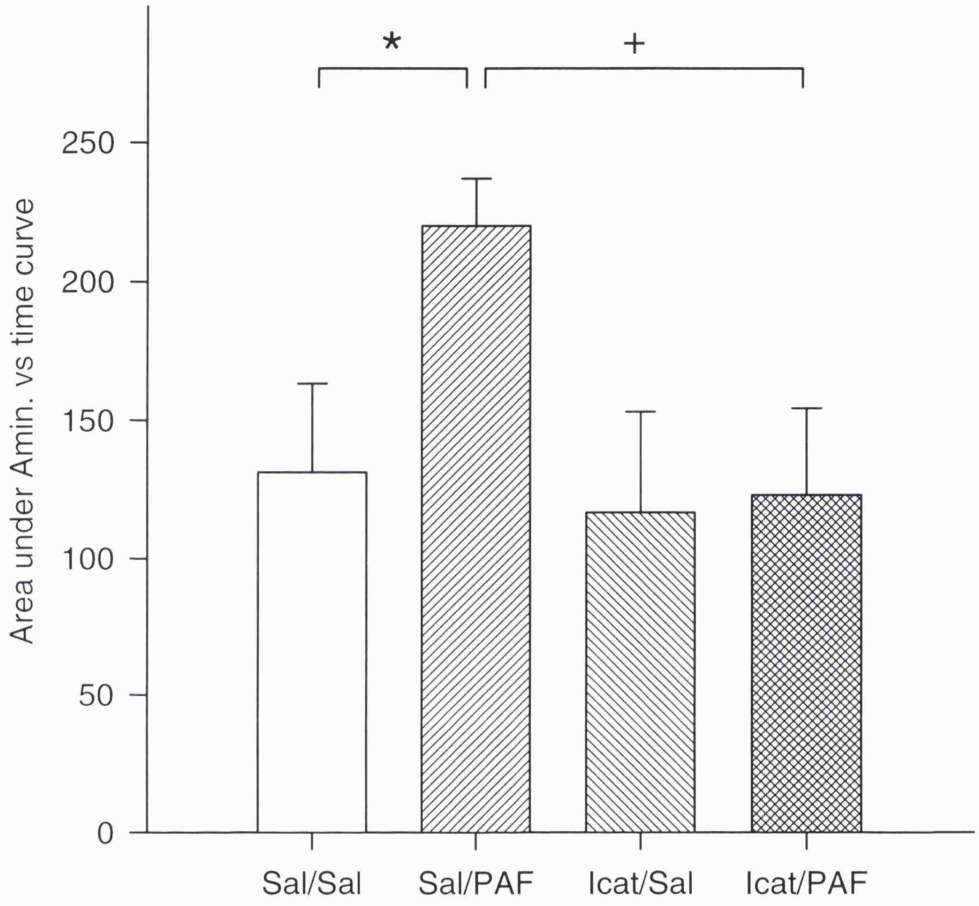

Figure 3.5: Effect of icatibant on the hyperresponsiveness to histamine, 200 µg, induced by PAF after 6 hours. The nasal airway was first pretreated with saline (Sal) or icatibant (Icat), 200 µg, following which subjects received a nasal spray of PAF, 60 µg, or a saline control. Amin. was measured immediately before, and 2, 5 and 10 minutes after histamine challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve was determined. Data are means from 8 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value of Amin. was 0.64±0.03 cm^2. *Significant difference in AUC following pretreatment with saline/PAF compared to saline control ($p<0.05$, Wilcoxon sign-rank test). +Significant difference in AUC when pretreatment with saline/PAF is compared to icatibant/PAF ($p<0.05$, Wilcoxon sign-rank test).

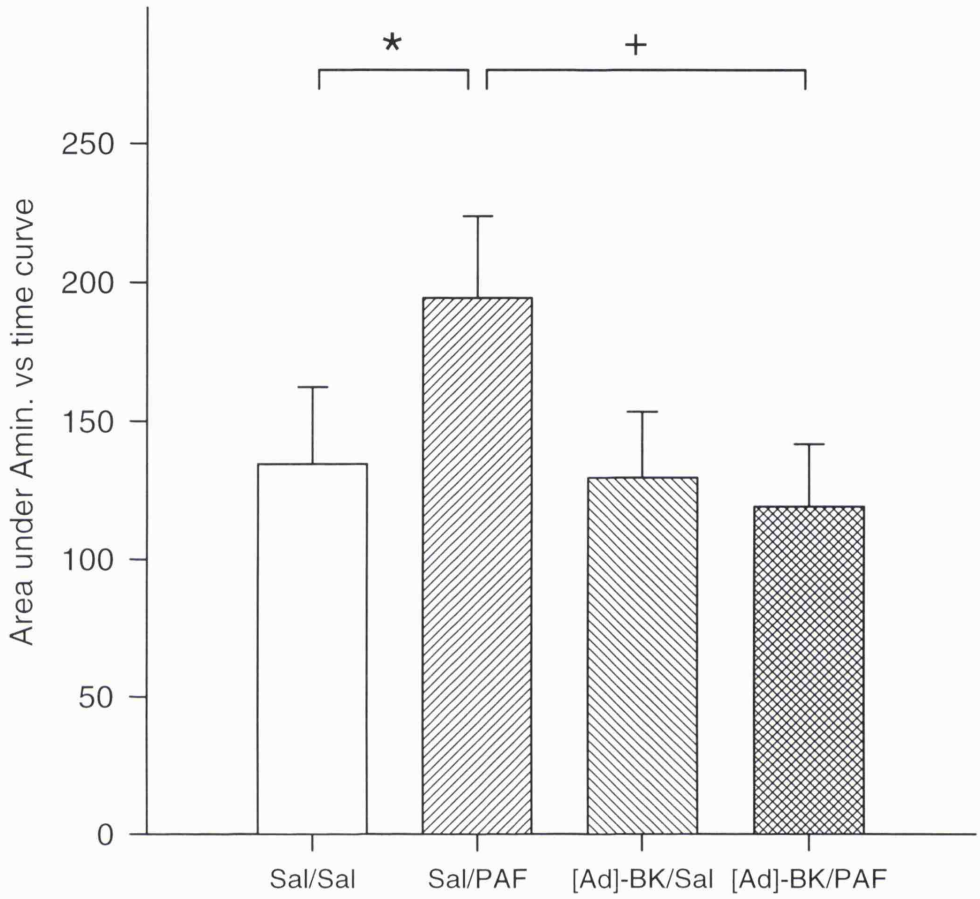

Figure 3.6: Effect of [1-adamantane acetyl-D-Arg^{0}, Hyp^{3}, Thi^{5,8}, D-Phe^{7}]-bradykinin ([Ad]-BK) on the hyperresponsiveness to histamine, 200 µg, induced by PAF after 6 hours. The nasal airway was first pretreated with saline (Sal) or [Ad]-BK, 200 µg, following which subjects received a nasal spray of PAF, 60 µg, or a saline control. Amin. was measured immediately before, and 2, 5 and 10 minutes after histamine challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve was determined. Data are means from 9 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value of Amin. was 0.61±0.03 cm^{2}. *Significant difference in AUC following pretreatment with saline/PAF compared to saline control ($p<0.05$, Wilcoxon sign-rank test). +Significant difference in AUC when pretreatment with saline/PAF is compared to [Ad]-BK/PAF ($p<0.05$, Wilcoxon sign-rank test).

3.3.2 Effect of PAF and lyso-PAF on mediator release in the nasal cavity

Administration of PAF, 60 µg, into the nasal cavity resulted in a significant increase in the level of ECP detected in nasal lavage fluid in the ensuing six hours, compared to saline control ($p=0.043$, Wilcoxon sign-rank test) (Figure 3.7). PAF also caused a significant increase in the kinin content of nasal lavage fluid at 2 and 6 hours after administration, compared to saline control ($p=0.014$ and 0.042 respectively, Wilcoxon sign-rank test) (Figure 3.8).

To investigate whether these increases were due to the action of PAF as a surfactant, subjects were also challenged with lyso-PAF, 60 µg. Lyso-PAF did not increase the levels of either ECP or kinins compared to treatment with saline control ($p>0.05$, Wilcoxon sign-rank test). PAF also induced an increase in the albumin content of nasal lavage fluid in some subjects (Figure 3.9), though the overall increase at any particular timepoint was not statistically significant ($p>0.05$, Wilcoxon sign-rank test). Furthermore, no significant correlations were identified between the albumin content of the lavage samples and kinin or ECP levels.

There were no significant differences in baseline levels of ECP, kinin or albumin between treatments ($p>0.05$, Friedman's test).

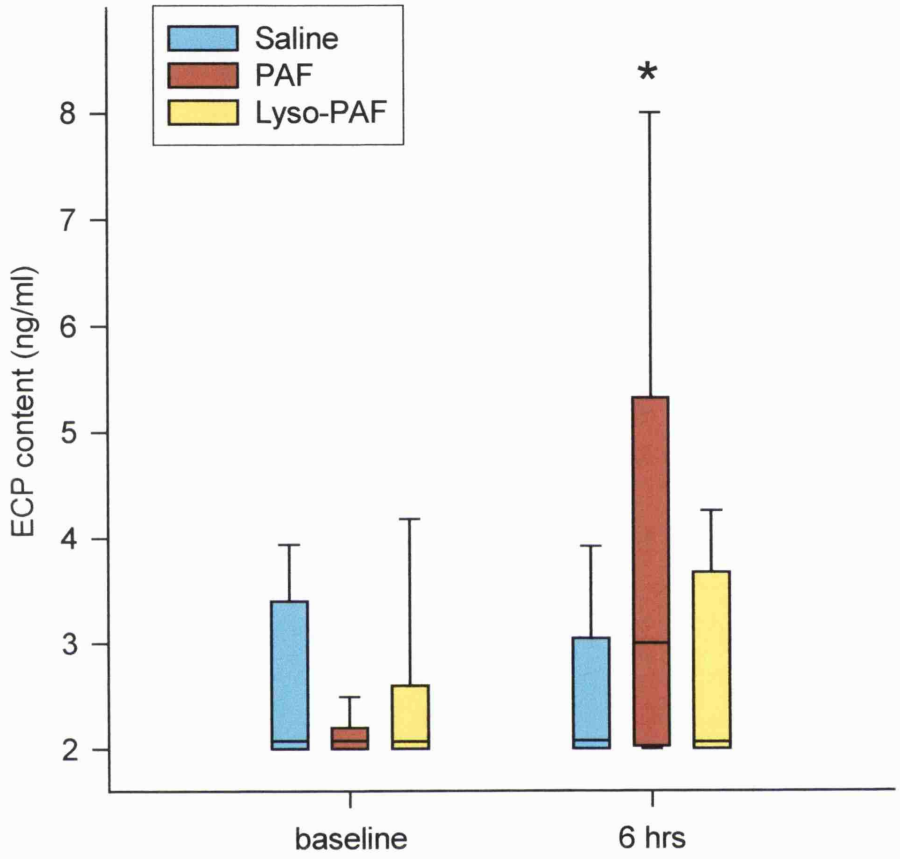

Figure 3.7: The effect of PAF, 60 µg, and lyso-PAF, 60 µg, on the ECP content of nasal lavage fluid. Data are medians from 8 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values. *Significant increase in ECP levels for the pretreatment shown, compared to pretreatment with saline only ($p<0.05$, Wilcoxon sign-rank test).

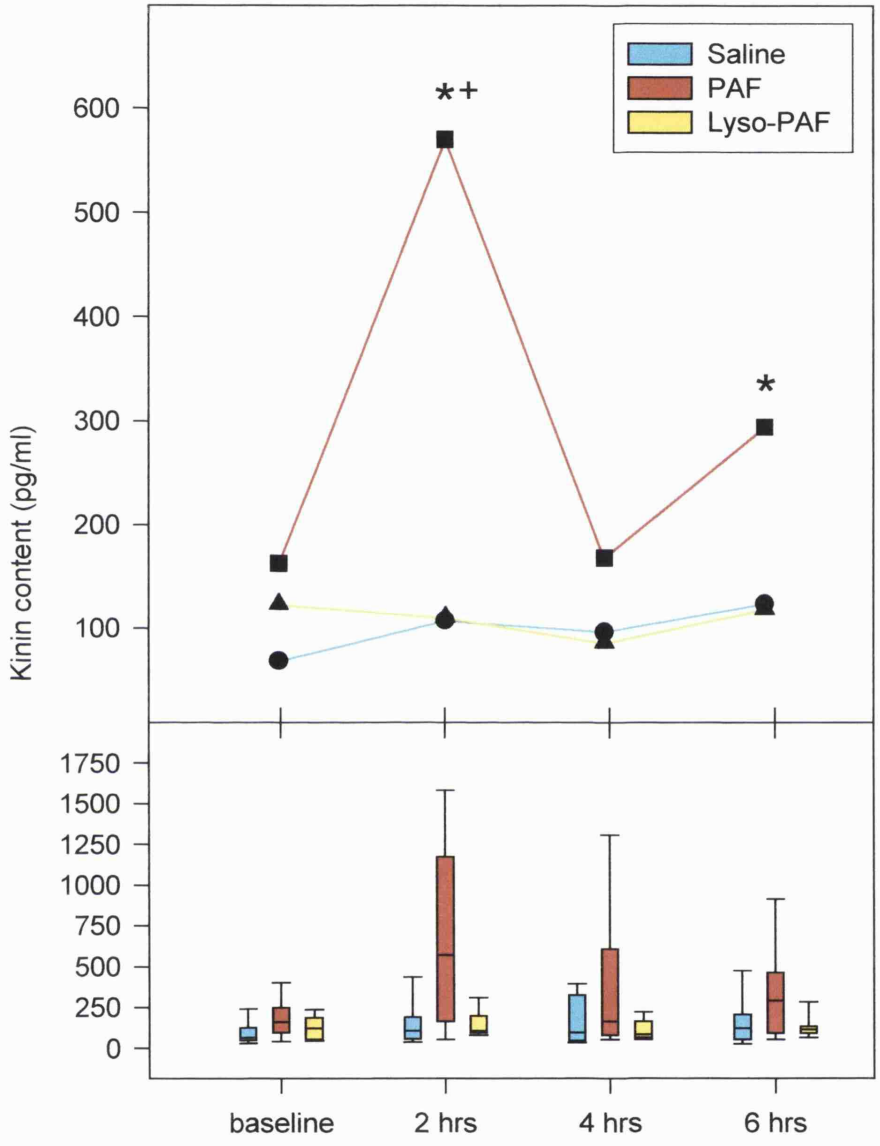

Figure 3.8: The effect of PAF, 60 µg, and lyso-PAF, 60 µg, on the kinin content of lavage fluid. Data are medians from 8 subjects. The bottom window indicates the interquartile range of values for each median, with the horizontal line indicative of the median value. Vertical bars represent the 80% central range of values. *Significant increase in kinin content for the pretreatment shown, compared to saline control ($p<0.05$, Wilcoxon sign-rank test). +Significant difference in kinin content following treatment with PAF compared to lyso-PAF ($p<0.05$, Wilcoxon sign-rank test).

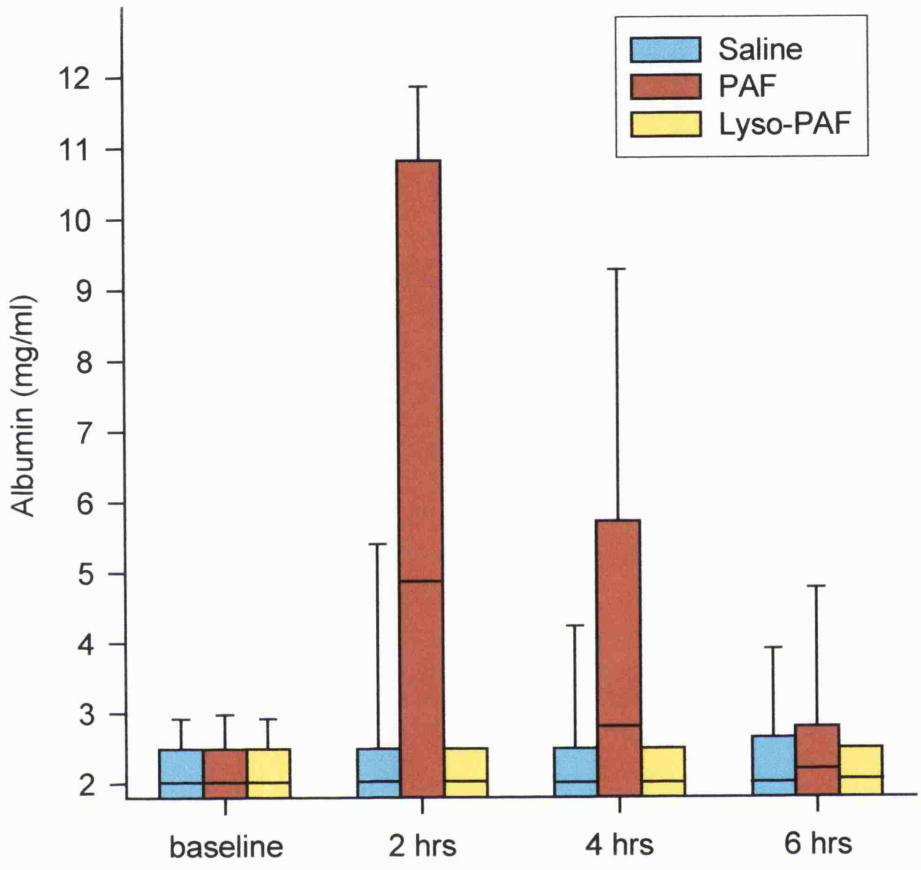

Figure 3.9: The effect of PAF, 60 µg, and lyso-PAF, 60 µg, on albumin levels in nasal lavage fluid. Data are medians from 8 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values.

3.3.3 Effect of bradykinin B_2 receptor antagonists on PAF-induced changes in mediator release in the nasal cavity

Figures 3.10 and 3.11 show the levels of ECP detected in the lavage samples immediately prior to (baseline) and 6 hours after pretreatment with PAF, 60 µg, or saline, with or without pretreatment using a bradykinin B_2 receptor antagonist. No significant differences in baseline values were observed between treatments ($p>0.05$, Friedman's test). Administration of PAF caused the release of ECP into the nasal cavity; this was detected as a significant increase in the ECP content of the nasal lavage samples compared to the saline control ($p=0.009$, Wilcoxon sign-rank test). Analysis of baseline levels of kinin content did not yield any significant differences between treatments ($p>0.05$, Friedman's test). Icatibant alone did not alter ECP levels ($p>0.05$, Wilcoxon sign-rank test), but did significantly reduce the increase in ECP induced by PAF ($p=0.009$, Wilcoxon sign-rank test) (Figure 3.10). It should be noted that in the presence of icatibant, PAF did cause an increase in ECP content, but this was not significant ($p=0.076$, Wilcoxon sign rank test).

Interestingly, pretreatment with [Ad]-BK did not significantly reduce the increase in ECP levels in nasal lavage fluid, 6 hours after PAF administration, as shown in Figure 3.11 ($p>0.05$, Wilcoxon sign-rank test).

In the six hours following challenge with PAF, there was an overall increase in the concentration of kinins detected in nasal lavage fluid compared to saline controls ($p<0.03$, Wilcoxon sign-rank test) (Figure 3.12). The maximal increase occurred 2 hours after PAF administration ($p=0.003$, Wilcoxon sign-rank test). Icatibant alone did not alter the kinin content of the nasal lavage samples ($p>0.05$, Wilcoxon sign-rank test). However, treatment with icatibant, 200 µg, every two hours attenuated the increase in kinin caused by PAF; this effect was statistically significant six hours after PAF administration ($p<0.05$, Wilcoxon sign-rank test).

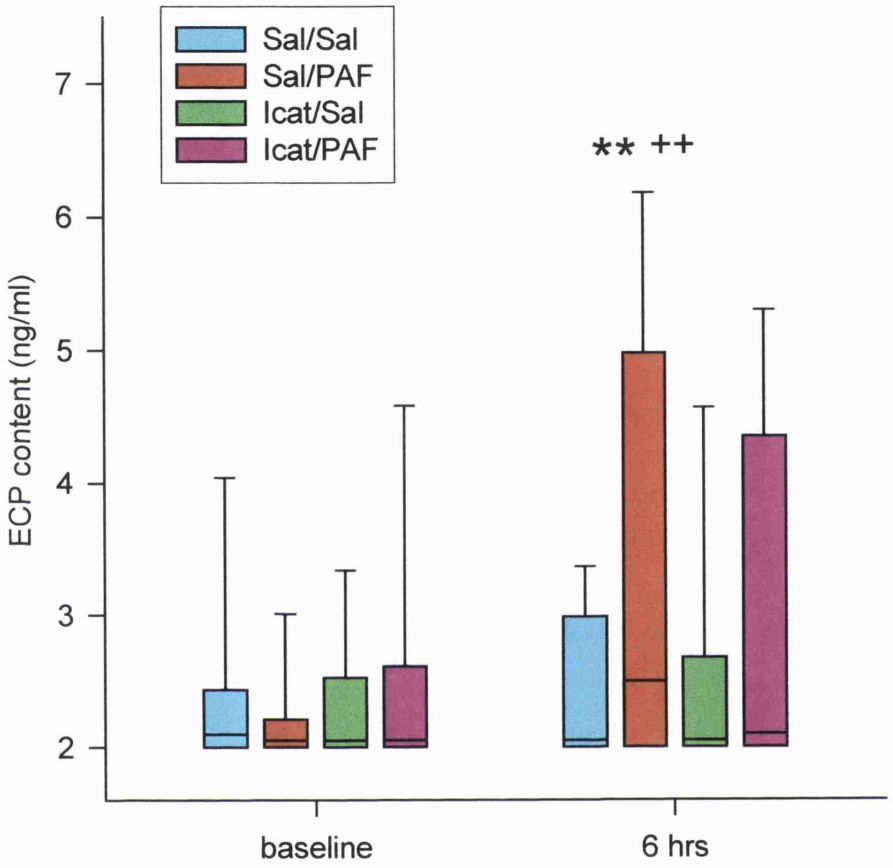

Figure 3.10: The change in the ECP content of nasal lavage fluid following pretreatment of the nasal cavity with saline (Sal), icatibant (Icat), 200 µg, and/or PAF, 60 µg, as described in the experimental protocol. Data are medians from 13 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values **Significant increase in ECP levels for the pretreatment shown, compared to pretreatment with saline only ($p<0.01$, Wilcoxon sign-rank test). ++Significant difference in ECP levels following pretreatment with saline/PAF compared to icatibant/PAF ($p<0.01$, Wilcoxon sign-rank test).

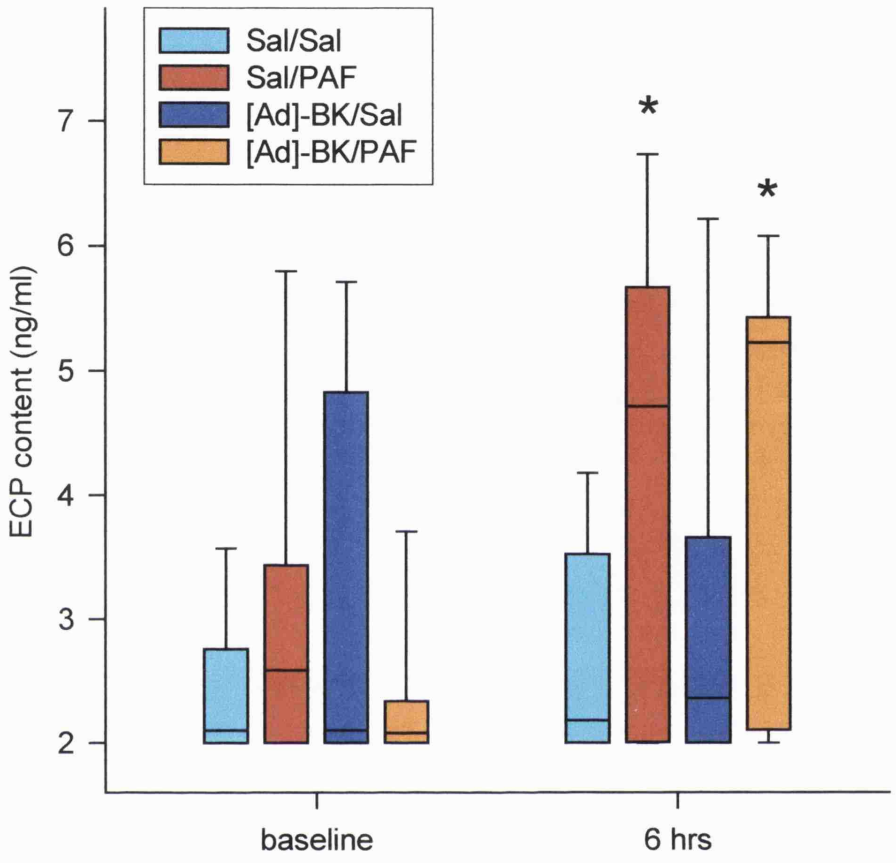

Figure 3.11: The change in the ECP content of nasal lavage fluid following pretreatment of the nasal cavity with saline (Sal), [1-adamantane acetyl-D-Arg^{0}, Hyp^{3}, Thi^{5,8}, D-Phe^{7}]-bradykinin ([Ad]-BK), 200 µg, and/or PAF, 60 µg, as described in the experimental protocol. Data are medians from 10 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values. *Significant increase in ECP levels for the pretreatment shown, compared to pretreatment with saline only ($p<0.05$, Wilcoxon sign-rank test).

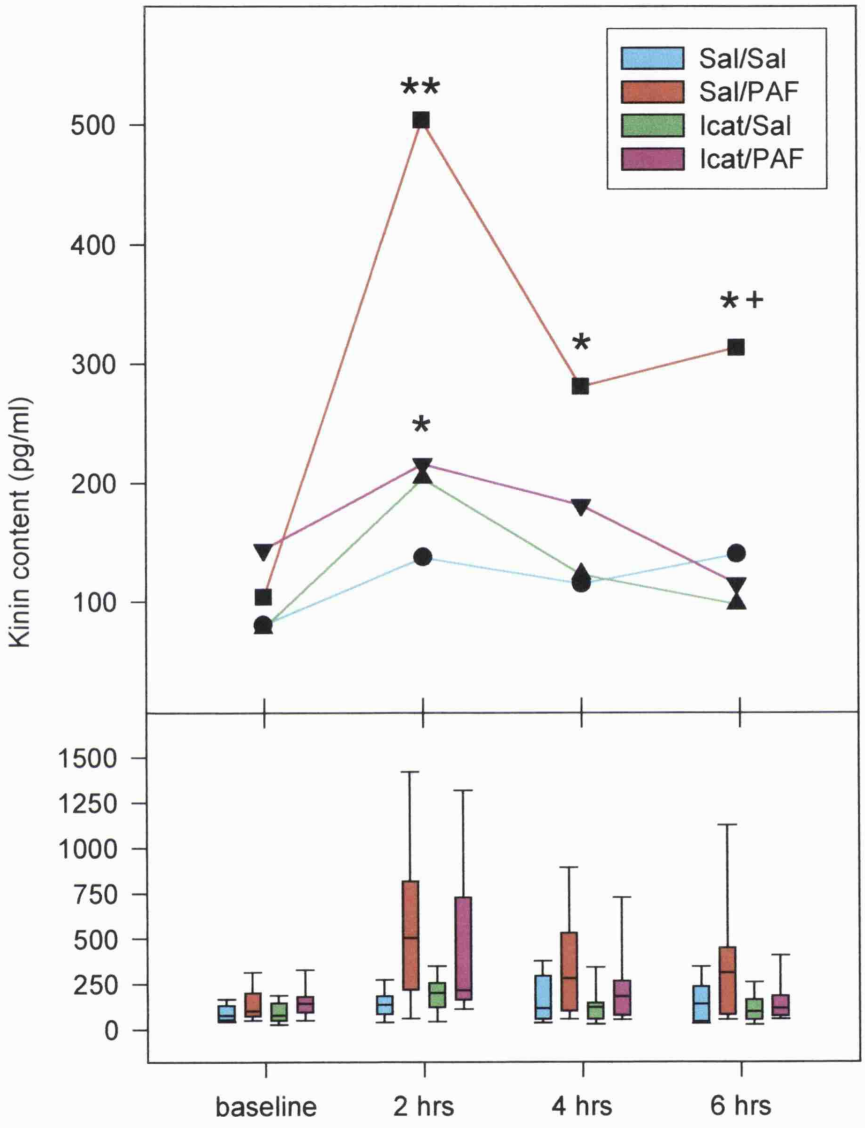

Figure 3.12: The change in the kinin content of lavage fluid following pretreatment of the nasal cavity with saline (Sal), icatibant (Icat), 200 µg, and/or PAF, 60 µg, as described in the experimental protocol. Data are medians from 13 subjects. The bottom window indicates the interquartile range of values for each median, with the horizontal line indicative of the median value. Vertical bars represent the 80% central range of values. */**Significant increase in kinin content for the pretreatment shown, compared to saline control (*$p<0.05$, **$p<0.01$, Wilcoxon sign-rank test). +Significant difference in kinin content following pretreatment with saline/PAF compared to icatibant/PAF ($p<0.05$, Wilcoxon sign-rank test).

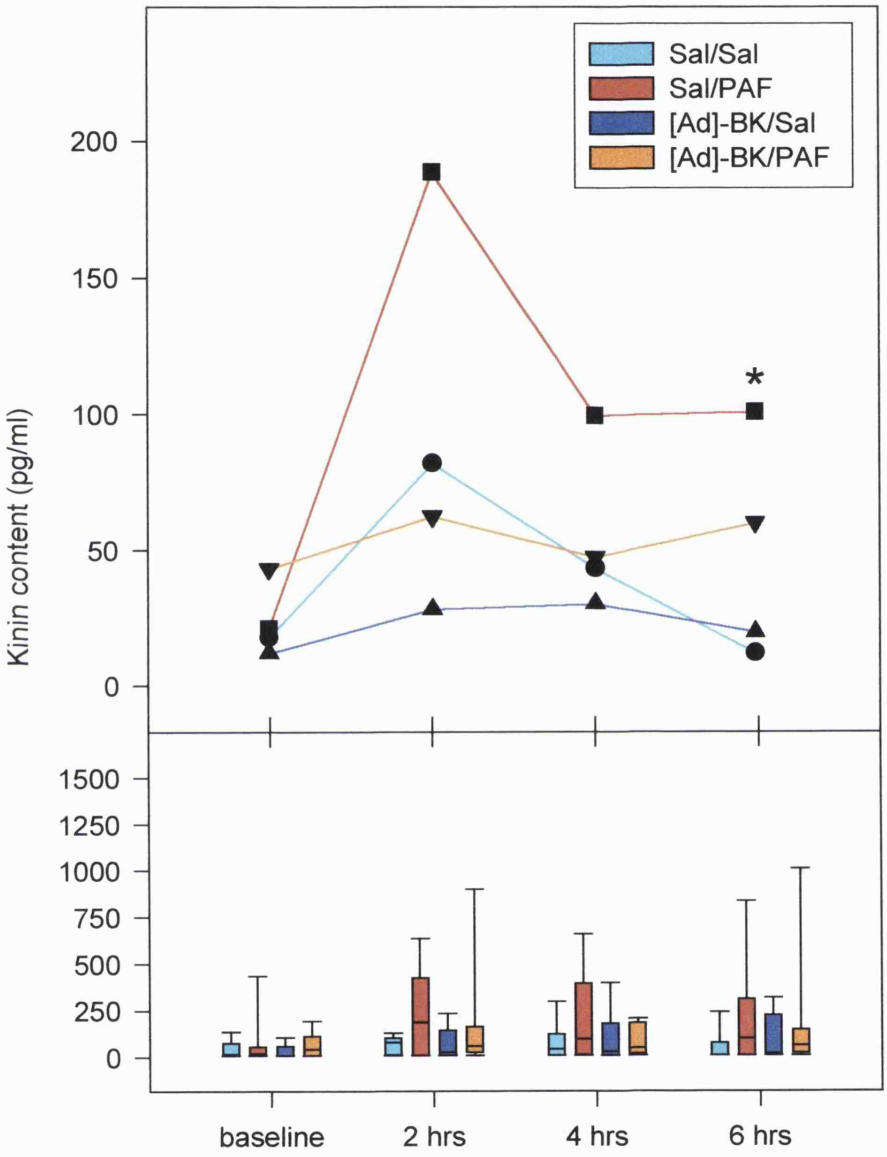

Figure 3.13: The change in the kinin content of lavage fluid following pretreatment of the nasal cavity with saline (Sal), [1-adamantane acetyl-D-Arg^{0}, Hyp^{3}, Thi^{5,8}, D-Phe^{7}]-bradykinin ([Ad]-BK), 200 µg, and/or PAF, 60 µg, as described in the experimental protocol. Data are medians from 10 subjects. The bottom window indicates the interquartile range of values for each median, with the horizontal line indicative of the median value. Vertical bars represent the 80% central range of values. *Significant increase in kinin content for the pretreatment shown, compared to pretreatment with saline only ($p<0.05$, Wilcoxon sign-rank test).

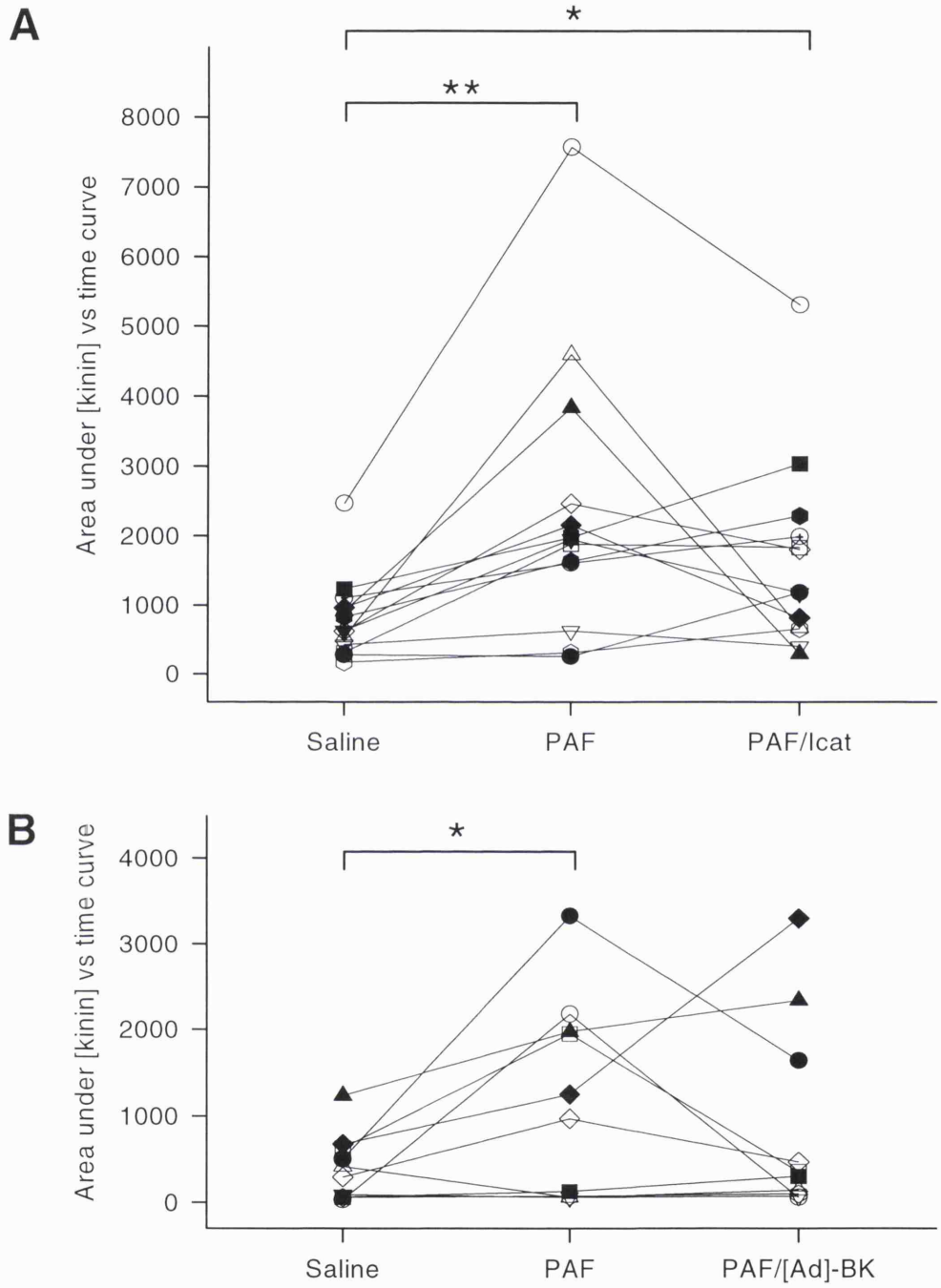

Figure 3.14: Area under [kinin] against time curves (AUC) following pretreatment of the nasal cavity with saline (Sal), PAF, 60 µg, and PAF with icatibant (graph A) or [1-adamantane acetyl-D-Arg^{0}, Hyp^{3}, Thi^{5,8}, D-Phe^{7}]-bradykinin ([Ad]-BK) (graph B), both at 200 µg. Each symbol represents a separate subject. */**Significant increase in kinin content for the pretreatment shown, compared to saline control (*$p<0.05$, **$p<0.01$, Wilcoxon sign-rank test).

Similarly, following treatment with [Ad]-BK, PAF failed to cause an increase in the kinin content of nasal lavage fluid, compared to saline control ($p>0.05$, Wilcoxon sign-rank test) (Figure 3.13). In order to analyse the overall changes in kinin detected over the six hours, the area under the kinin content against time curve was determined for each subject, and plotted in Figure 3.14. In both experiments, PAF caused a significant increase in the levels of kinin detected in the lavage samples ($p<0.05$, Wilcoxon sign-rank test) which was reduced by treatment with the antagonist.

3.3.4 Effect of bradykinin on the responsiveness of the nasal airway

In this experiment, determinations of nasal patency were normalised to the Amin. value determined immediately prior to each histamine challenge. This was necessary because pretreatment with bradykinin, 500 μg, caused a significant decrease (13.7±4.0%) in the baseline Amin. measured before the histamine challenge at 3 and 4 hours after the start ($p=0.03$, Friedman's test).

Pretreatment of the nasal airway with bradykinin did not significantly alter the response to histamine challenge at either dose of bradykinin, compared to saline control, at any timepoint ($p>0.05$, Wilcoxon sign-rank test) (Figure 3.15). No significant shift was observed in any of the histamine dose-response curves following bradykinin pretreatment, as shown in Figure 3.16 ($p>0.05$, Friedman's test). This confirms the observation the pretreatment with bradykinin did not potentiate the nasal response to histamine.

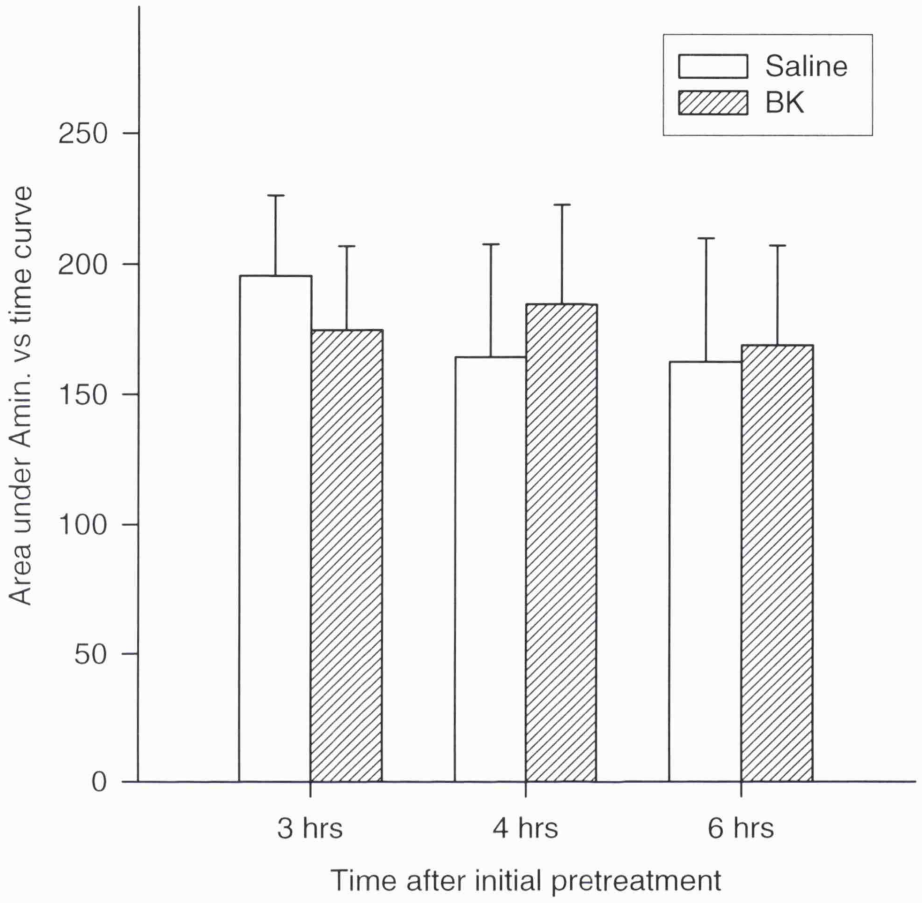

Figure 3.15: Effect of bradykinin on the response to nasal challenge with histamine, 200 µg. The nasal cavity was pretreated with saline or bradykinin (BK), 500 µg, every 30 minutes for two hours, as described in the experimental protocol. Amin. was measured immediately before, and 2, 5 and 10 minutes after each histamine challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve (AUC) was determined. Data are means from 10 subjects. Vertical bars represent s.e.mean. The baseline Amin. values (mean ± s.e.mean) were 0.64±0.02 cm^2 for saline and 0.65±0.03 cm^2 for bradykinin.

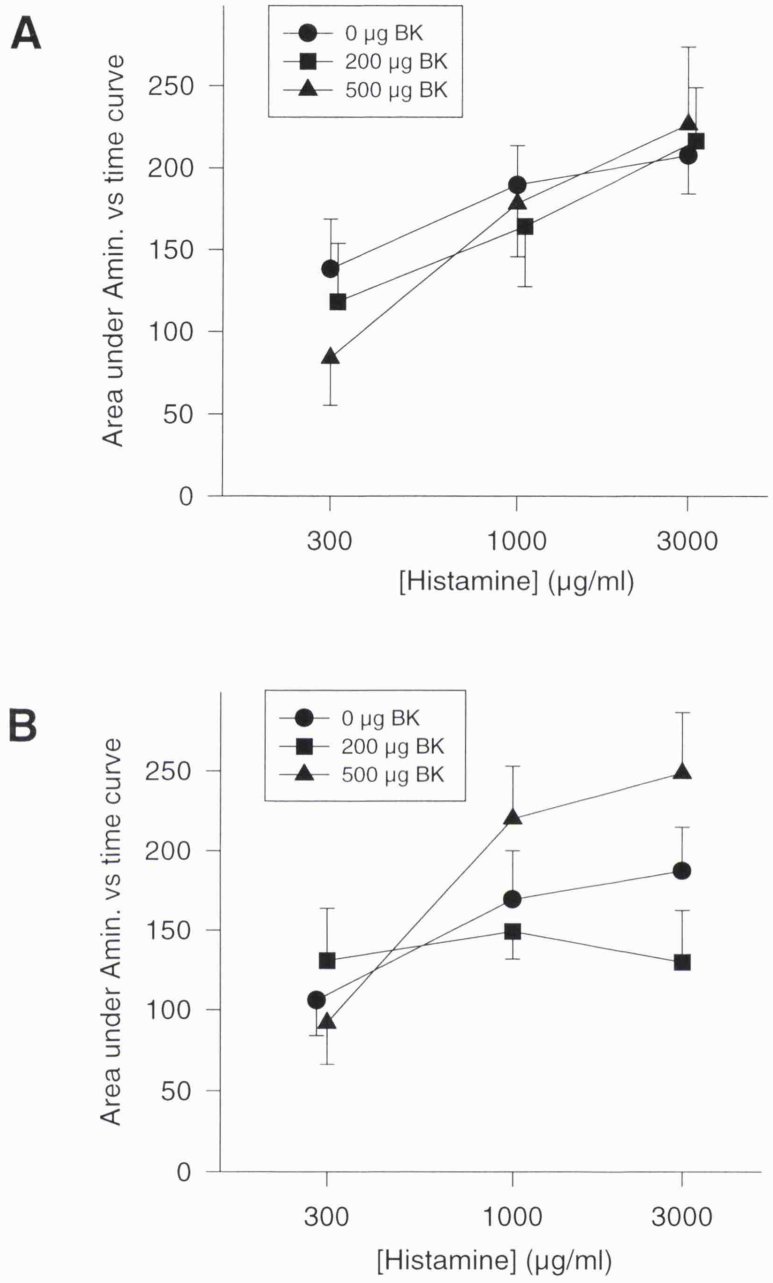

Figure 3.16: Dose-response curves for histamine, following pretreatment of the nasal cavity with one of three doses of bradykinin (BK) (0 µg, 200 µg and 500 µg). A histamine challenge was carried out one hour (graph A) and two hours (graph B) after the final pretreatment with bradykinin, as described in the experimental protocol. Amin. was measured immediately before, and 2, 5 and 10 minutes after each histamine challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curves (AUC) was determined. Data are means from 10 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value for Amin. was 0.64 ± 0.03 cm^2.

3.4 Discussion

In this study, histamine, 200 µg, produced a reduction in the patency of the human nasal airway. Pretreatment of the nasal airway with the bradykinin B_2 receptor antagonists, icatibant or [Ad]-BK, had no effect on the response to histamine challenge, demonstrating that the action of histamine is not mediated through the release of kinins, nor mediated through the bradykinin B_2 receptor. Pretreatment of the nasal airway with PAF, 60 µg, enhanced the nasal action of histamine, as previously reported (Austin & Foreman, 1993).

The PAF-induced nasal AHR to histamine was abolished following pretreatment with either icatibant or [Ad]-BK, suggesting that kinin generation, and, in turn, activation of bradykinin B_2 receptors, are required for PAF-induced AHR. This is further supported by the detection of increased levels of kinins in nasal lavage fluid, 2, 4 and 6 hours after the administration of PAF. The development of AHR by PAF, at the dose used in this study, is only apparent at six hours and not at two hours after administration (Austin & Foreman, 1993), yet the increase in kinin production induced by PAF occurs within two hours of administration. Therefore, kinin production by PAF appears to be an early event in the pathway which results in AHR. The assay used was equally sensitive to bradykinin and kallidin, so it was not possible to distinguish between the production of these two peptides by PAF, and their relative roles in promoting AHR.

Interestingly, when the nasal airway was pretreated with icatibant or [Ad]-BK, the degree of kinin production stimulated by PAF was reduced. There is some evidence that icatibant inhibits kallikrein in an animal model, in addition to its action as an antagonist at the bradykinin B_2 receptor (Dr K. Wirth, unpublished), and some bradykinin analogue antagonists inhibit human urinary kallikrein, measured using a bioassay *in vitro* (Spragg *et al.*, 1988). Furthermore, previous studies on allergic rhinitis have found that icatibant reduces the production of kinins *in vivo* following challenge with house dust-mite (Dear *et al.,* 1996). PAF causes plasma extravasation in both the skin and lower airways (Barnes *et al.,* 1988) and, at higher doses, in the human nasal airway (Leggieri *et al.,* 1991). At the dose used in this study, PAF did not increase albumin in nasal lavage fluid, so the increase in kinin levels, and the

effect of pretreatment with icatibant or [Ad]-BK, are likely to be due to a change in kinin production (and/or metabolism), rather than a consequence of altered vascular permeability.

The levels of kinin detected in this study are greater than those found in the nasal lavage of subjects with perennial allergic rhinitis, following challenge with house dust mite (Dear *et al.,* 1996). This is probably because in this study, the nasal cavity was lavaged after two hours, and not after just ten minutes as in the study by Dear *et al.* Nonetheless, it is somewhat of an anomaly that the nasal obstruction, induced by antigen challenge in house-dust mite sensitive subjects, is abolished by icatibant (Dear *et al.*, 1996) (implying that the kinin released is responsible for the reduction in nasal patency), yet the administration of PAF, 60 µg, which caused more kinin generation, was not associated with any nasal blockage (Austin & Foreman, 1993). This may be because the nasal cavity in atopics is more sensitive to bradykinin compared to normal, non-atopic subjects. Alternatively, the release of kinins, following allergen challenge, occurs together with the release of enzymes, such as carboxypeptidases, which will metabolise both bradykinin and kallidin (Proud *et al.*, 1987). These metabolites (e.g. [des-Arg^9]-bradykinin) are not detected by the kinin assay used, so this might explain the lower kinin levels detected in the study by Dear *et al.* Although higher levels of kinin were detected in the experiment using icatibant, compared to when [Ad]-BK was used, this was probably due to variation between individuals (the two studies used different groups of subjects).

Lyso-PAF causes an eosinophilia in the human nasal airway, but does not increase ECP in nasal lavage (Tedeschi *et al.,* 1994a), nor induce nasal AHR (Austin & Foreman, 1993). In this experiment, administration of lyso-PAF did not cause ECP release nor stimulate kinin production, indicating that kinin generation by PAF is not due to its action as a surfactant. This data is compatible with the finding that kinin generation is required for the development of PAF-induced nasal AHR.

PAF is a potent chemotactic and chemokinetic factor for eosinophils, *in vitro* (Wardlaw *et al.,* 1986) and *in vivo* in both the lower airways (Barnes *et al.,* 1988) and nasal airways in man (Klementsson & Andersson, 1992; Tedeschi *et al.,* 1994a). ECP

is released from activated eosinophils, and it is therefore not surprising that in this experiment, PAF caused an increase in the amount of ECP detected in nasal lavage fluid. It has been demonstrated that the increase in ECP follows a similar time course to the presence of AHR following PAF administration into the nasal cavity (Austin & Foreman, 1993; Tedeschi *et al.,* 1994a). It is, therefore, possible that PAF causes AHR by stimulating ECP production. ECP is a cytotoxic protein, which may cause AHR by damaging the epithelium lining the airways (Gleich *et al.*, 1988). Alternatively, the increase in ECP may signify eosinophil activation, which would also cause the release of MBP and EPO. MBP is also a cytotoxic mediator, causing AHR in a number of animal models of airway allergy, possibly as a result of epithelial damage (Flavahan *et al.*, 1988; Gundel *et al.*, 1991; Coyle *et al.*, 1993). However, it has also been shown that cationic proteins may induce AHR without causing epithelial damage (Coyle *et al.*, 1993).

Coyle *et al.* (1995) reported that MBP, released from activated eosinophils, produced AHR of the rat lower airways by a kinin-dependent mechanism which involved the bradykinin B_2 receptor. ECP can activate plasma kallikrein (Venge *et al.,* 1979). Therefore, PAF may induce a AHR by recruiting eosinophils and releasing cationic proteins such as ECP and MBP which, in turn, activate kallikrein-like enzymes and generate kinins. However, at the dose used in this study, PAF did not cause an increase in albumin levels, which can be used as a marker of plasma extravasation (Naclerio *et al.,* 1983). Therefore, it is unlikely that the kinins were generated by the action of plasma kallikrein on kininogen from plasma. Although the levels of tissue kallikrein in nasal secretions are increased in allergic rhinitis (Baumgarten *et al.,* 1986b), this enzyme is not activated by cationic proteins (Coyle *et al.,* 1995). Neutrophils, however, contain tissue kallikrein and bind plasma kallikrein, together with high and low molecular weight kininogen, on the cell surface (Gustafson *et al.,* 1989; Henderson *et al.,* 1994). Nasal challenge with antigen or PAF causes a neutrophilia, in addition to eosinophil recruitment (Tedeschi *et al.*, 1994b), and it is therefore possible that neutrophils provide the components needed for the generation of kinins by ECP and MBP. However, there is no evidence to suggest that neutrophils are associated with PAF-induced AHR in the lower airways of human subjects (Wardlaw *et al.*, 1990).

This hypothesis does not explain the reduction in PAF-induced ECP production following pretreatment with icatibant. Whether this effect was due to a decrease in eosinophil activation or a consequence of the decrease in kinin production is unknown. Farmer *et al.* (1992) found that bradykinin antagonists reduced the eosinophilia caused by antigen challenge in the lower airway of the guinea pig. Therefore, icatibant may have inhibited the eosinophilia caused by PAF and, consequently, the release of ECP. A number of antagonists at the histamine H_1 receptor possess additional anti-eosinophil properties which are independent of their action at histamine receptors. The reduction in ECP by icatibant may have been due to a similar anti-eosinophil activity of icatibant, which is not shared by [Ad]-BK. Alternatively, the results could be due to the different degrees to which the antagonists are also able to inhibit kinin production.

There are, of course, alternative models to explain the kinin dependency of PAF-induced nasal AHR. The production of kinins by PAF and the kinin-dependent development of PAF-induced AHR could result from an action of PAF which is independent of eosinophils. Antigen-induced AHR can occur without an obvious eosinophilia in both animals (Spina *et al.*, 1991) and man (Klementsson *et al.*, 1991). However, in the current experiment, the administration of bradykinin alone failed to induce an AHR to histamine. This is in agreement with studies in animal models (Abraham *et al.*, 1991; Coyle *et al.*, 1995), though another study found that bradykinin can potentiate the response of the airway to acetylcholine in the guinea pig (Omini *et al.*, 1989). It is, therefore, likely that PAF also caused the release of other mediators, particularly those derived from eosinophils and neutrophils, which are needed to generate kinins and induce AHR.

Icatibant and [Ad]-BK may have prevented the PAF-induced AHR by their ability to inhibit kinin production, and, therefore, the induction of AHR by PAF could be independent of bradykinin B_2 receptor activation. However, while the bradykinin B_1 receptor may be upregulated in the nasal airways of subjects with active allergic rhinitis (Woessner and Zuraw, 1998), there is no evidence to suggest the involvement of the bradykinin B_1 receptor in the physiology of the nasal airways of normal, non-atopic subjects (Rajakulasingam *et al.*, 1991; Austin & Foreman, 1994). In addition,

icatibant and [Ad]-BK abolished the AHR even though kinin levels in nasal lavage samples remained high, thus implying receptor involvement in the development of AHR.

There are also no reports of PAF mediating its actions through bradykinin generation. In a study by Sakamoto *et al.* (1992), icatibant did not inhibit PAF-induced bronchoconstriction or plasma extravasation into the lower airway in guinea pigs. However, it is possible that these actions of PAF are not mediated via kinins, while the development of AHR is. Unfortunately, the authors did not investigate the action of icatibant on PAF-induced AHR. Interestingly, an interaction between PAF and the kinins has been implicated in acute pancreatitis. PAF has an important role as an initiator of acute pancreatitis, both in experimental models (Emanuelli *et al.*, 1989) and man (Kingsnorth *et al.*, 1995), and it has been suggested that the underlying inflammation, in which kinins have a major role, may be triggered by PAF (Griesbacher & Lembeck, 1997).

The mechanism by which kinins may induce AHR remains unknown. Neuropeptide release may be important in the AHR induced by PAF in the lower airway of the guinea pig (Spina *et al.*, 1991), and cationic proteins in the rat lower airway (Coyle *et al.,* 1994). Furthermore, neuropeptide depletion prevents antigen-induced AHR in animals (Ladenius & Biggs, 1989; Matsuse *et al.*, 1991). Bradykinin can stimulate sensory nerve endings, causing the release of substance P and other neuropeptides (Saria *et al.,* 1988; Bertrand & Geppetti, 1996). Therefore, the development of AHR may depend on the kinin-mediated release of neuropeptides. In fact, the release of both substance P and neurokinin A from guinea pig bronchi after antigen challenge *in vitro* are significantly reduced by pretreatment with icatibant (Lindstrom & Andersson, 1997).

Fox *et al.* (1996) reported that bradykinin can cause sensitisation of C-fibres in the guinea-pig trachea, and bradykinin increases airway neural responses *in vitro*, possibly through the activation of a prejunctional B_2 receptor on airway nerves (Miura *et al.*, 1992). A similar process may happen in the human nasal airway, though the data presented in this chapter indicates that this does not occur in the absence of

PAF. Alternatively, the kinins may stimulate the production of cytokines IL-1, IL-6 and IL-8 by inflammatory cells and sensitised neurones (Ferreira *et al.,* 1993), which may be involved in the development of AHR (Howarth, 1995). In addition, PAF and eosinophil-derived cationic proteins are cytotoxic to the epithelial lining of the airway (Ganbo and Hisamatsu, 1990; Ohashi *et al.,* 1997), and this could further increase the responsiveness of the nasal airway. This might explain why bradykinin does not cause AHR in the absence of PAF.

In conclusion, previous studies have demonstrated a strong association between eosinophil activation and the development of PAF-induced AHR in the human nasal airway (Austin & Foreman, 1993; Tedeschi *et al.,* 1994a), and this study has shown that these processes are modulated by kinins. It is, therefore, likely that endogenous kinins have a role in the induction of AHR by PAF.

Summary

- Histamine, 200 µg, induced a significant decrease in the patency of the human nasal airway, observed as an increase in nasal obstruction.

- Administration of PAF, 60 µg, six hours prior to histamine challenge, potentiated the nasal response to histamine.

- Pretreatment with either of the bradykinin B_2 receptor antagonists, icatibant or [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin ([Ad]-BK), both at 200 µg, prevented the PAF-induced airway hyperresponsiveness to histamine.

- PAF, 60 µg, also caused a significant increase in the kinin content of nasal lavage fluid. This increase was abrogated by both icatibant or [Ad]-BK, 200 µg.

- Icatibant, 200 µg, but not [Ad]-BK, also significantly reduced the increase in ECP in nasal lavage fluid following administration of PAF, 60 µg.

- Administration of lyso-PAF, 60 µg, did not cause a rise in kinin or ECP levels detected in nasal lavage fluid.

- Bradykinin, at either 200 µg or 500 µg, given repeatedly over 2 hours, did not cause a nasal hyperresponsiveness to histamine.

- The data indicates that bradykinin itself does not cause AHR, but is involved in the AHR induced by PAF in the normal, non-atopic nasal airway, possibly via an interaction between kinins and other inflammatory mediators.

CHAPTER 4

THE ROLE OF KININS IN THE RECRUITMENT OF NEUTROPHILS AND EOSINOPHILS, INDUCED BY PLATELET ACTIVATING FACTOR, *IN VITRO* AND *IN VIVO* IN THE HUMAN NASAL AIRWAY

4.1 Introduction

Intranasal administration of platelet activating factor (PAF) causes the recruitment of eosinophils into the human nasal airway, and their subsequent activation, resulting in the increased production of eosinophil cationic protein (ECP) (Austin & Foreman, 1993; Tedeschi *et al.,* 1994a). In the previous chapter, it was found that the PAF-induced increase in ECP levels detected in nasal lavage fluid was inhibited by pretreatment with icatibant, a bradykinin B_2 receptor antagonist.

It has been reported that bradykinin antagonists reduce antigen-induced eosinophilia in the lower airways of the guinea pig (Farmer *et al.*, 1992). Therefore, the reduction in ECP, observed in chapter 3, could have been a consequence of a fall in eosinophil recruitment following pretreatment of the nasal airway with icatibant. While this may be due to a specific action of icatibant against eosinophil recruitment, Evans *et al.* (1996) demonstrated that the antigen-induced eosinophilia in guinea pigs could also be prevented by inhibitors of tissue kallikrein (TK). This suggests that the generation of kinins may contribute to the recruitment of eosinophils following antigen challenge.

Interestingly, neutrophils can release TK, and also bind plasma kallikrein, low molecular weight kininogen and high molecular weight kininogen (HMWK) on their cell membrane (Gustafson *et al.*, 1989; Henderson *et al.*, 1994). Human endothelial cells also bind HMWK, and can generate kinin via a kallikrein dependent pathway

(Nishikawa *et al.*, 1992). Marcus *et al.* (1997) reported that the loss of human endothelial cell barrier function requires the adhesion of neutrophils to the endothelium. This may be dependent upon the release of elastase from neutrophils, a process which can be modulated by kinins (Carl *et al.*, 1996). Therefore, the generation of kinins, by neutrophils or endothelial cells, may be involved in the migration of inflammatory cells across the vascular endothelium (Naidoo & Bhoola, 1997).

Therefore, the experiments in this chapter examine the effect of bradykinin B_2 receptor antagonists on PAF-induced eosinophilia in the human nasal airway. In addition, an *in vitro* model of neutrophil and eosinophil transmigration was developed, to study further the role of kinins in the recruitment of inflammatory cells.

4.2 Experimental Protocol

4.2.1 The effect of bradykinin B_2 receptor antagonists on platelet activating factor-induced inflammatory cell recruitment, in the human nasal airway

In this experiment, the nasal lavage samples obtained from the study carried out in chapter 3 (section 3.2.3) were processed, cytological specimens prepared and examined for neutrophils and eosinophils, as described in chapter 2.

Briefly, healthy non-atopic human volunteers were used as subjects. Three nasal lavages were carried out, the third was retained as a baseline. The nasal cavity was then allowed to dry, following which subjects received a spray of either icatibant, 200 µg, or a saline control. Two minutes later, PAF, 60 µg, or saline (as a control) was instilled into each nostril. Nasal lavage was again carried out 2, 4 and 6 hours later. The duration of action of icatibant, in the human nasal airway, is approximately two hours (Dear, 1996). Therefore, subjects were again administered icatibant or a saline control following the lavage at 2 and 4 hours. Cytological specimens were then prepared from the nasal lavage samples. The protocol is summarised in Figure 4.1.

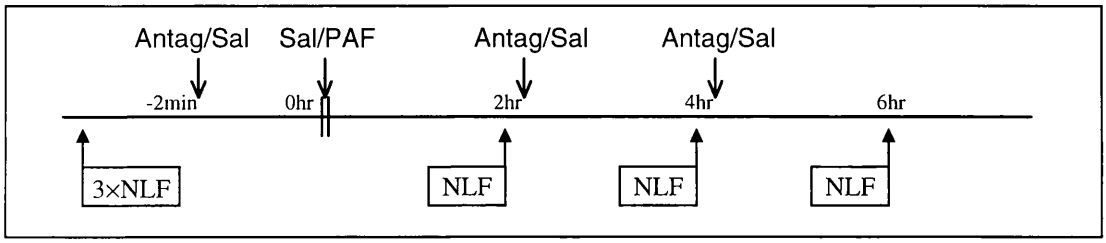

Figure 4.1: Protocol for investigating the effect of bradykinin B_2 antagonists on PAF-induced changes in nasal cytology. NLF = nasal lavage; Antag = antagonist; Sal = saline.

Each subject received all four possible combinations of treatment, in a cross-over manner, on separate occasions at least 2 days apart i.e.:

- Icatibant, 200 µg (three administrations) and PAF 60 µg (once)
- Icatibant, 200 µg (three administrations) and saline (once)
- Saline (three administrations) and PAF 60 µg (once)
- Saline (three administrations) and saline (once)

The order of treatments was determined using a randomised, balanced-block design. Subjects were randomly assigned to the treatment protocol.

The experiment was repeated on a separate occasion, using [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin ([Ad]-BK), 200 µg, instead of icatibant.

4.2.2 Effect of bradykinin on inflammatory cell recruitment in the nasal airway

To investigate the effect of bradykinin on nasal cytology, the nasal cavity was lavaged three times, and the third sample retained for analysis. The nasal cavity was then allowed to dry, following which subjects received a spray of either bradykinin, 200 µg, or saline as a control. This administration was repeated every 30 minutes for two hours, following which a nasal lavage was carried again. Cytological specimens were then prepared from the lavage samples, and examined for neutrophils and eosinophils, as described in chapter 2. Subjects received both bradykinin and saline on separate occasions, at least 72 hours apart, in a randomised, cross-over fashion. The protocol is summarised in Figure 4.2.

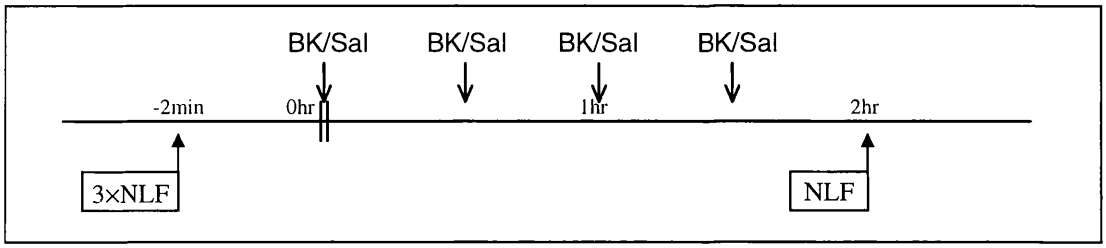

Figure 4.2: Protocol for investigating the effect of bradykinin on nasal cytology. NLF = nasal lavage; BK = bradykinin; Sal = saline.

4.2.3 Initial experiments on the effect of platelet activating factor and N-formyl-met-leu-phe on neutrophil migration

In order to ascertain the optimum concentration of chemotactant to be used in the following experiments, neutrophil migration was assessed in response to different concentrations of PAF and N-formyl-met-leu-phe (FMLP). Neutrophil migration was measured both across a porous membrane (chemotaxis), and through a confluent monolayer of EA.hy926 endothelial cells (transmigration), using the method described in chapter 2.

In some experiments, an assessment of "cell drop off" was made, as described in chapter 2. Also, the relative contributions of chemokinesis and chemotaxis to neutrophil migration across a porous membrane was investigated by "checkerboard analysis", as follows: the migration of neutrophils was measured in response to the presence of FMLP in either the lower, upper or both the lower and upper compartments of the chemotaxis system. This allowed the migratory response of neutrophils to be characterised (Wilkinson *et al.*, 1982).

4.2.4 The effect of icatibant and bradykinin on neutrophil migration

The effect of icatibant, 1 μM and 10 nM, and bradykinin, 1 μM, on both PAF- and FMLP-induced chemotaxis across a porous membrane was investigated, using the system described in chapter 2. The experiment was repeated to study the same conditions on neutrophil transmigration across an endothelial cell monolayer, as described in chapter 2.

4.2.5 The effect of icatibant and bradykinin on eosinophil transmigration

The effect of icatibant and bradykinin, both at a concentration of 1 μM, on PAF-induced eosinophil transmigration across an endothelial cell monolayer, was investigated, as described in chapter 2. In some experiments, the effect of neutrophils on PAF-induced transmigration of eosinophils was also investigated.

4.2.6 Characterisation of the effect of bradykinin on neutrophil transmigration

In order to investigate the role of bradykinin B_1 and B_2 receptors on PAF-induced neutrophil transmigration in the presence of bradykinin, the transmigration system was set up as described in chapter 2, with the addition of a bradykinin receptor antagonist, at a concentration of 1 μM, to both compartments. The antagonists used were Lys-[des-Arg^9, Leu^8]-bradykinin and icatibant, which are antagonists at the bradykinin B_1 and B_2 receptors, respectively. Both the endothelial cell monolayer and the neutrophils were pre-incubated with the antagonist or control (HBSS) prior to the commencement of the assay, as described in chapter 2. The effect of the bradykinin B_1 receptor agonist [des-Arg^9]-bradykinin, on PAF-induced neutrophil transmigration, was also investigated.

Furthermore, in some experiments, the role of neutrophil elastase was assessed, by allowing the neutrophil transmigration to proceed in the presence of 50 μM N-methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone (AAPVK). AAPVK is an inhibitor of neutrophil elastase, and has been shown to inhibit the permeability of endothelial cells in the presence of activated neutrophils, at the dose used in this study (Carl *et al.*, 1996).

4.2.7 The generation of kinin from kinin moieties bound to neutrophils and endothelial cells, and their effect on PAF-induced neutrophil transmigration

In order to examine the role of kinins in the migration of neutrophils across an endothelium, the ability of neutrophils and EA.hy926 endothelial cells to generate kinins was first investigated, as described in chapter 2.

Neutrophils were suspended in 450 µl of HBSS, at a density of 2×10^7 cells/ml, and incubated for 30 minutes at 37°C with one of the following stimuli: PAF, 1 µM; FMLP, 1 µM; calcium ionophore A23187, 1 µM; eosinophil cationic protein (ECP), 10 ng/ml; human plasma kallikrein, 0.1-100 µg/ml. A quench agent was then added and the cells were centrifuged as described in chapter 2. The supernatants were aspirated and assayed for kinins, as described.

EA.hy926 endothelial cells were grown to confluence and then incubated with 1 µg/ml high molecular weight kininogen (HMWK). At various time intervals thereafter, human plasma kallikrein, 100 ng/ml, was added and the supernatants aspirated at various timepoints and assayed for kinins, as described in chapter 2. The experiment was also conducted in cell culture plates without any endothelial cells, to control for the binding of HMWK to the plastic surface.

The effect of kinin generation, by endothelial cells previously incubated with HMWK, on PAF-induced neutrophil transmigration was then studied, as described in chapter 2. The cell culture inserts were incubated with 1 µg/ml HMWK or culture medium alone for 2 hours, following which they were washed in HBSS. Kinin generation was stimulated by the addition of plasma kallikrein, at a concentration of 10 µg/ml, using HBSS without plasma kallikrein as a control. Neutrophil transmigration to PAF was then assessed as before. In addition, the effect of adding icatibant to the transmigration system was investigated.

4.2.8 Data analysis

Data from nasal lavage do not fit a normal distribution, and are, therefore, expressed as medians, together with the interquartile range and 80% central range. The appropriate non-parametric statistical test is given with each data set. The results from the *in vitro* experiments are presented as mean values, together with s.e.mean, and were analysed using parametric statistical tests. A value of $p<0.05$ is taken as significant.

4.3 Results

4.3.1 Effect of bradykinin B_2 receptor antagonists PAF-induced inflammatory cell recruitment in the human nasal airway

Figure 4.3 shows the percentage neutrophils in nasal lavage fluid following administration of PAF, with or without icatibant pretreatment. PAF, 60 µg, induced a significant increase in the percentage neutrophils in nasal lavage fluid collected 2 hours later, compared to the saline control ($p=0.031$, Wilcoxon sign-rank test). Icatibant alone did not affect the differential neutrophil count ($p>0.05$, Wilcoxon sign-rank test). However, after pretreatment with icatibant, 200 µg, PAF failed to induce a significant neutrophilia 2 hours later ($p>0.05$, Wilcoxon sign-rank test). No significant increases in the differential neutrophil count were observed with any of the treatments used in the experiment with [Ad]-BK ($p>0.05$, Friedman's test).

PAF also caused a significant increase in the percentage eosinophils isolated in nasal lavage fluid obtained 2 and 6 hours after administration, compared to the saline control ($p=0.007$ and $p=0.034$ respectively, Wilcoxon sign-rank test). A similar increase was seen in the absolute number of eosinophils in nasal lavage fluid ($p=0.21$, Wilcoxon sign-rank test). Pretreatment of the nasal airway with icatibant, 200 µg, significantly reduced the PAF-induced nasal eosinophilia 6 hours later ($p=0.045$, Wilcoxon sign-rank test) (Figure 4.4a). Interestingly, if the nasal cavity was treated with [Ad]-BK (instead of icatibant), the eosinophilia was not reduced ($p>0.05$, Wilcoxon sign-rank test) (Figure 4.4b). The different actions of these bradykinin B_2 receptor antagonists is particularly apparent in Figure 4.5, which shows the absolute number of eosinophils isolated in the lavage samples. While PAF failed to induce an eosinophilia if the nasal airway was pretreated with icatibant ($p>0.05$, Wilcoxon sign-rank test), an eosinophilia was still present following treatment with [Ad]-BK ($p=0.006$, Wilcoxon sign-rank test).

There were no differences between baseline levels of neutrophils and eosinophils, for the treatments used in either experiment ($p>0.05$, Friedman's test).

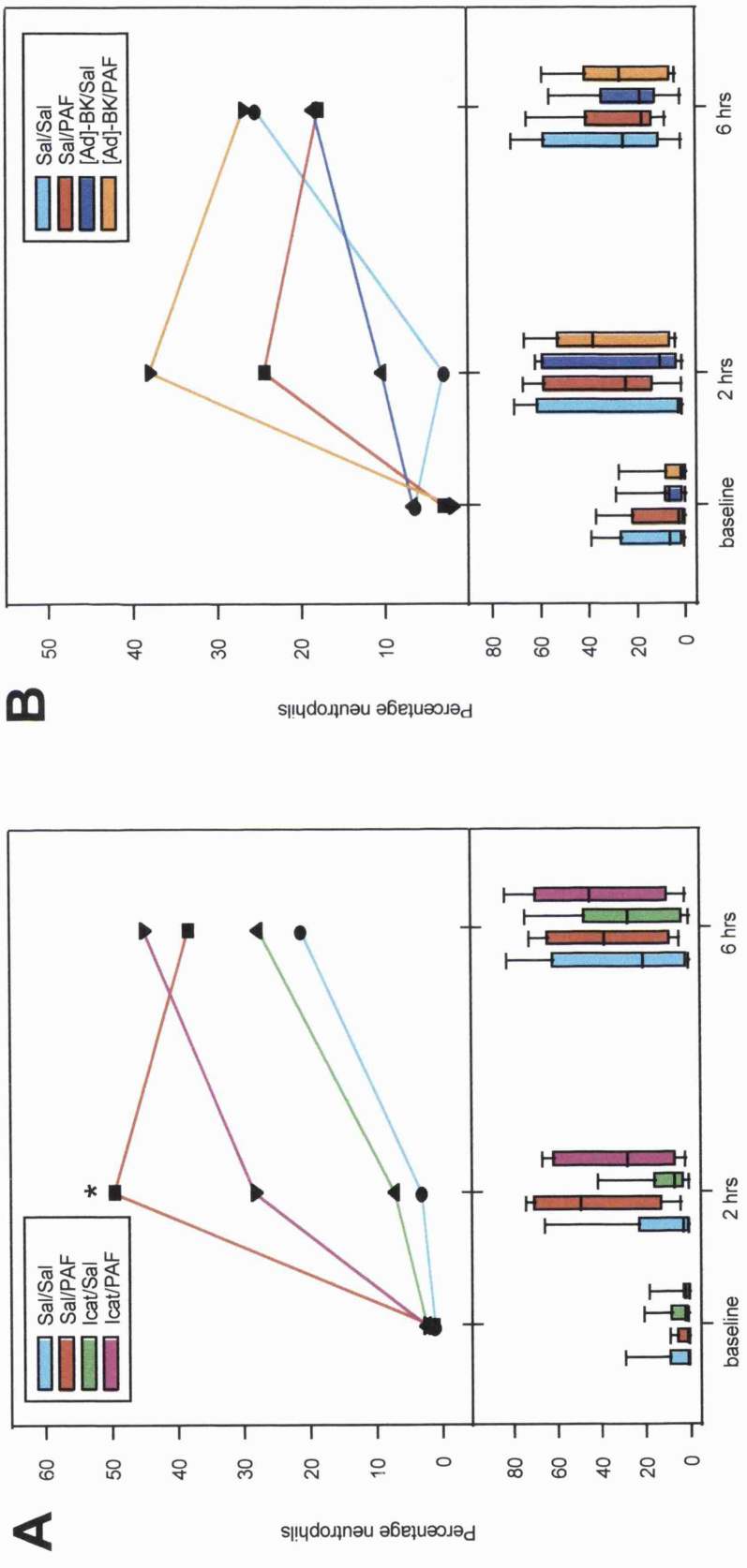

Figure 4.3: The change in the percentage neutrophils isolated from nasal lavage fluid following pretreatment of the nasal cavity with saline (Sal), a bradykinin B2 receptor antagonist and/or PAF, 60 µg, as described in the experimental protocol. The bradykinin B2 receptor antagonists used were icatibant, 200 µg (graph A) or [1-adamantane acetyl-D-Arg0, Hyp3, Thi5,8, D-Phe7]-bradykinin ([Ad]-BK), 200 µg (graph B). Data are medians from 13 and 10 subjects respectively. The bottom window indicates the interquartile range for each median, with the horizontal line indicative of the median value. Vertical bars represent the 80% central range of values. *Significant increase in the percentage neutrophils isolated for the treatment shown, compared to treatment with saline control (*p<0.05, Wilcoxon sign-rank test).

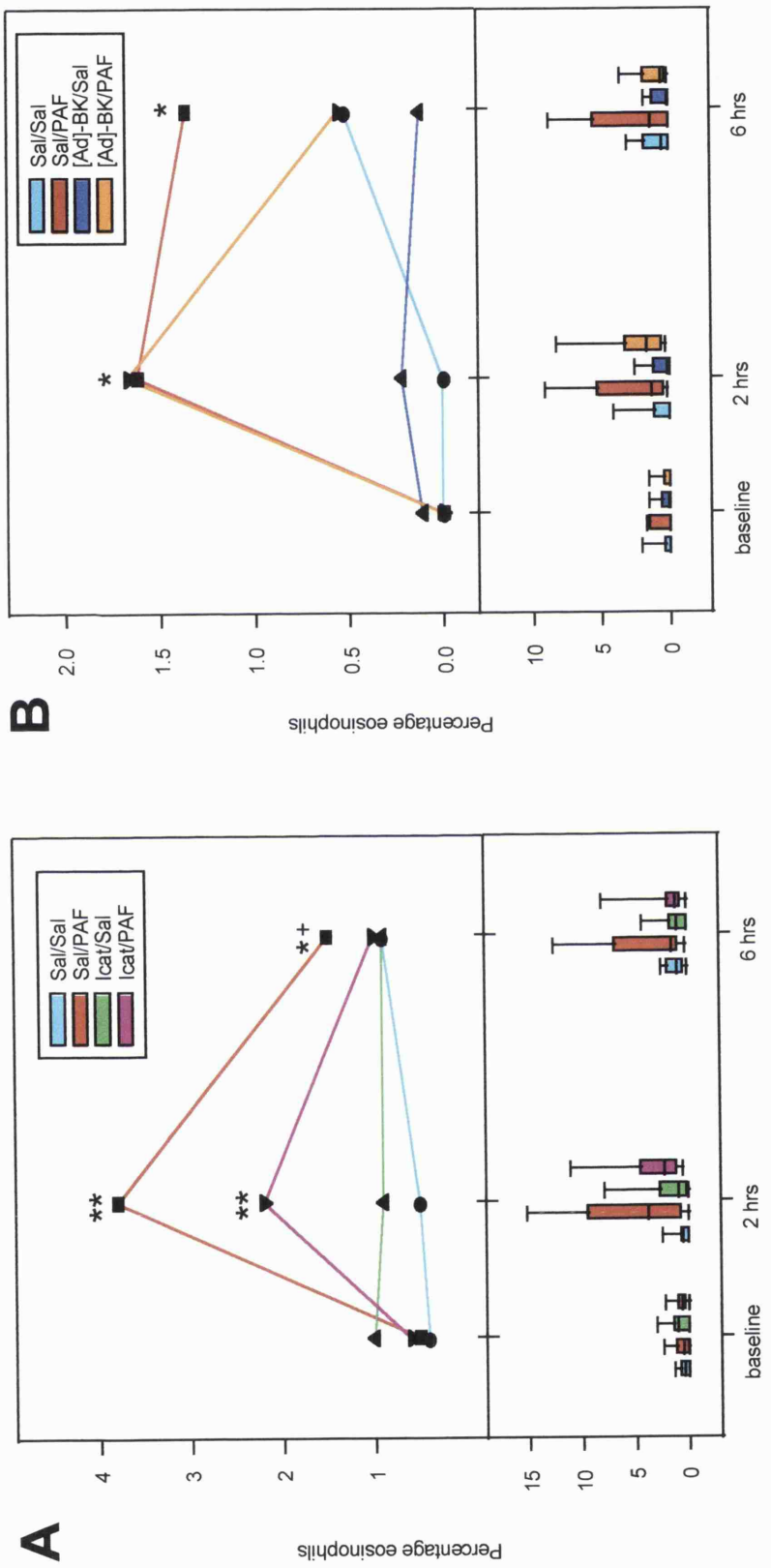

Figure 4.4: The change in the percentage eosinophils isolated from nasal lavage fluid following pretreatment of the nasal cavity with saline (Sal), a bradykinin B2 receptor antagonist and/or PAF, 60 µg, as described in the experimental protocol. The bradykinin B2 receptor antagonists used were icatibant, 200 µg (graph A) or [1-adamantane acetyl-D-Arg0, Hyp3, Thi5,8, D-Phe7]-bradykinin ([Ad]-BK), 200 µg (graph B). Data are medians from 13 and 10 subjects respectively. The bottom window indicates the interquartile range for each median, with the horizontal line indicative of the median value. Vertical bars represent the 80% central range of values. */**Significant increase in the percentage eosinophils isolated for the treatment shown, compared to treatment with saline control (*p<0.05, **p<0.01, Wilcoxon sign-rank test). +Significant difference in the PAF-induced increase in the percentage eosinophils isolated, following pretreatment with icatibant (p<0.05, Wilcoxon sign-rank test).

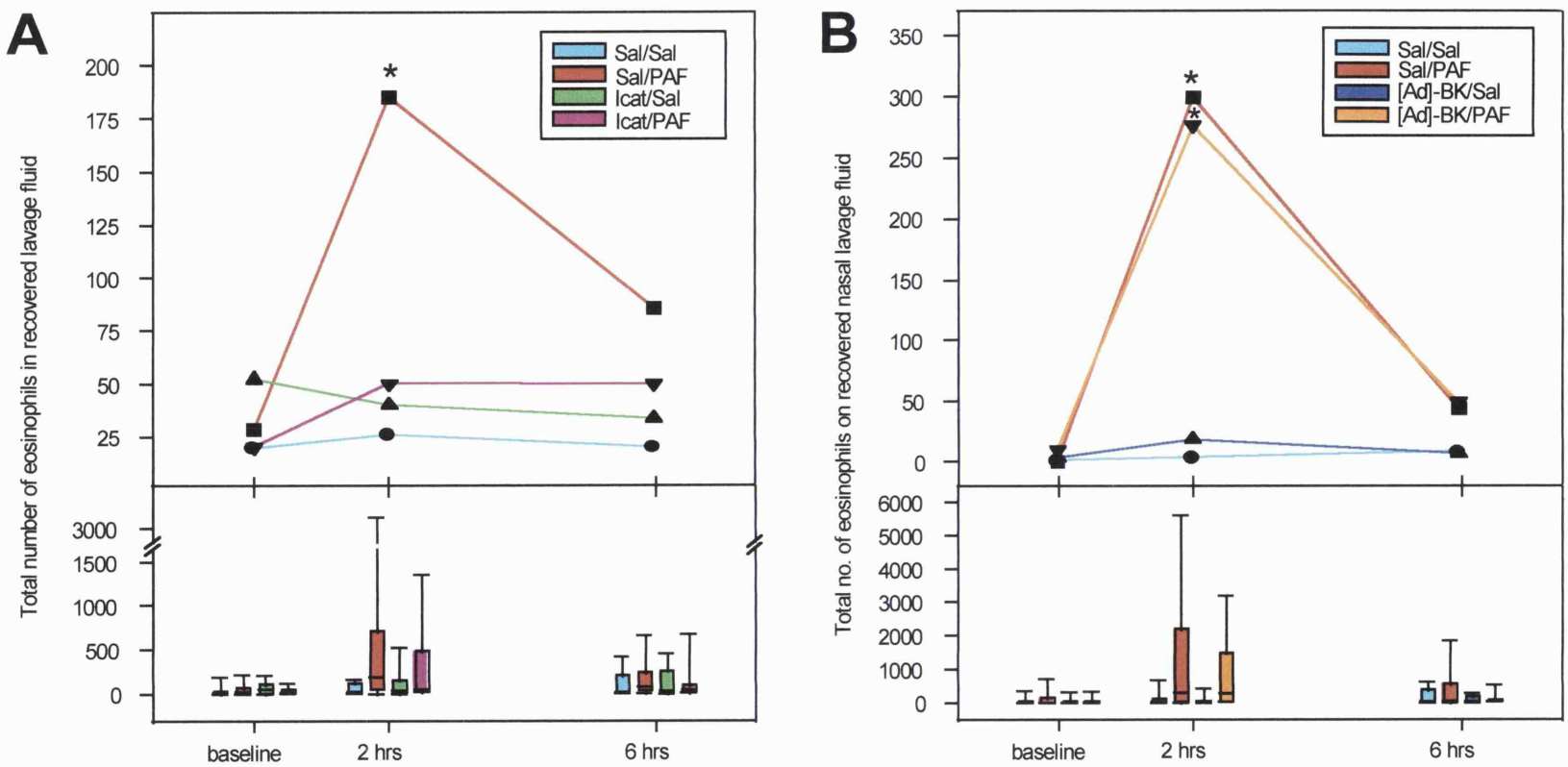

Figure 4.5: The change in the total number of eosinophils isolated from nasal lavage fluid following treatment of the nasal cavity with saline (Sal), a bradykinin B2 receptor antagonist and/or PAF, 60 µg, as described in the experimental protocol. The bradykinin B2 receptor antagonists used were icatibant, 200 µg (graph A) or [1-adamantane acetyl-D-Arg0, Hyp3, Thi5,8, D-Phe7]-bradykinin ([Ad]-BK), 200 µg (graph B). Data are medians from 13 and 10 subjects respectively. The bottom window indicates the interquartile range for each median, with the horizontal line indicative of the median value. Vertical bars represent the 80% central range of values. *Significant increase in the total number of eosinophils isolated for the treatment shown, compared to treatment with saline control (*$p<0.05$, Wilcoxon sign-rank test).

4.3.2 Effect of bradykinin on inflammatory cell recruitment in the nasal airway

Figure 4.6 shows the cytological profile of nasal lavage fluid, following repeated treatment of the nasal airway with bradykinin, 500 µg, or a saline control. There were no differences in the neutrophil or eosinophil counts following repeated administration of bradykinin, compared to the saline control ($p>0.05$, Wilcoxon sign-rank test). There were no differences between baseline levels of neutrophils and eosinophils, for the treatments used ($p>0.05$, Friedman's test).

4.3.3 Preliminary experiments into neutrophil migration

Figures 4.7 and 4.8 display dose-response curves for neutrophil migration in response to the chemotactants FMLP and PAF. Maximum migration across a porous membrane occurred in response to 1 µM FMLP. This was also the concentration of PAF which resulted in peak neutrophil migration. It was decided to use PAF at 1 µM and FMLP at 0.1 µM in subsequent experiments, since these concentrations caused approximately equal responses.

The chemotactants generally caused a greater of degree of neutrophil transmigration across an endothelial monolayer than through a porous membrane, at the same concentrations. Peak migration was observed with 1 µM PAF and 10 nM FMLP. In subsequent experiments investigating neutrophil transmigration, the stimuli were used at the following concentrations: FMLP, 0.1 µM and 1 nM; PAF, 1 µM and 0.1 µM.

In both the chemotaxis and transmigration studies, the number of neutrophils adhering to the underside of the porous membrane was negligible, with "cell drop-off" greater than 98%. The results of the "checkerboard" analysis for chemotaxis are shown in Table 4.1. Neutrophil migration occurred in response to a positive chemotactic gradient . However, a significant degree of migration also occurred when FMLP was present in both the upper and lower chambers of the migration system ($p<0.05$, Student's t-test), implying that the migratory response to FMLP, across the porous membrane, was partly chemokinetic in nature i.e. due to an increase in cell locomotion, irrespective of the presence of a chemotactic gradient.

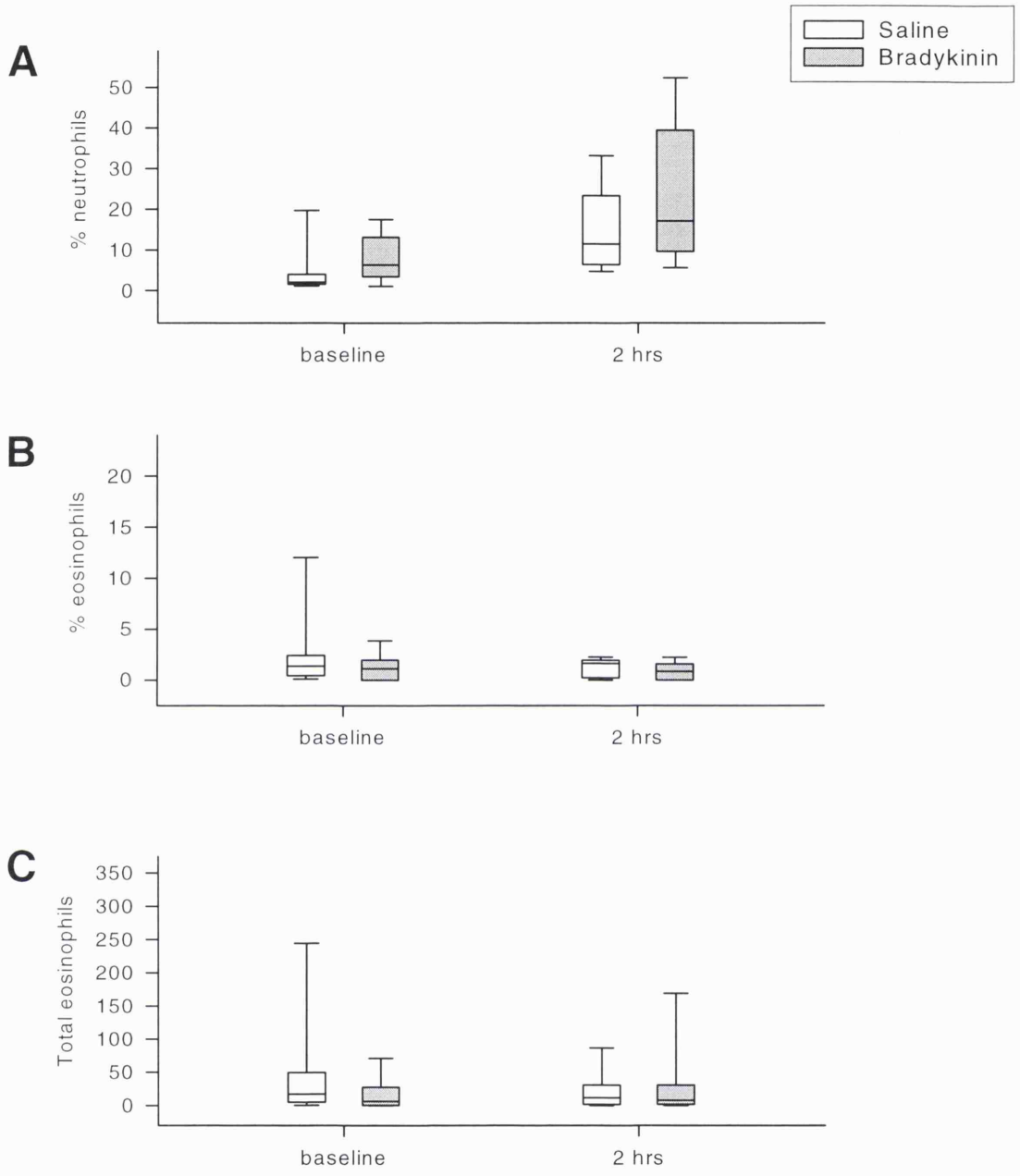

Figure 4.6: Changes in nasal cytology after treatment of the nasal cavity with saline or bradykinin, 500 µg, as described in the experimental protocol. The changes shown are percentage neutrophils (graph A), percentage eosinophils (graph B) and total number of eosinophils in lavage sample (graph C). Data are medians from 8 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values.

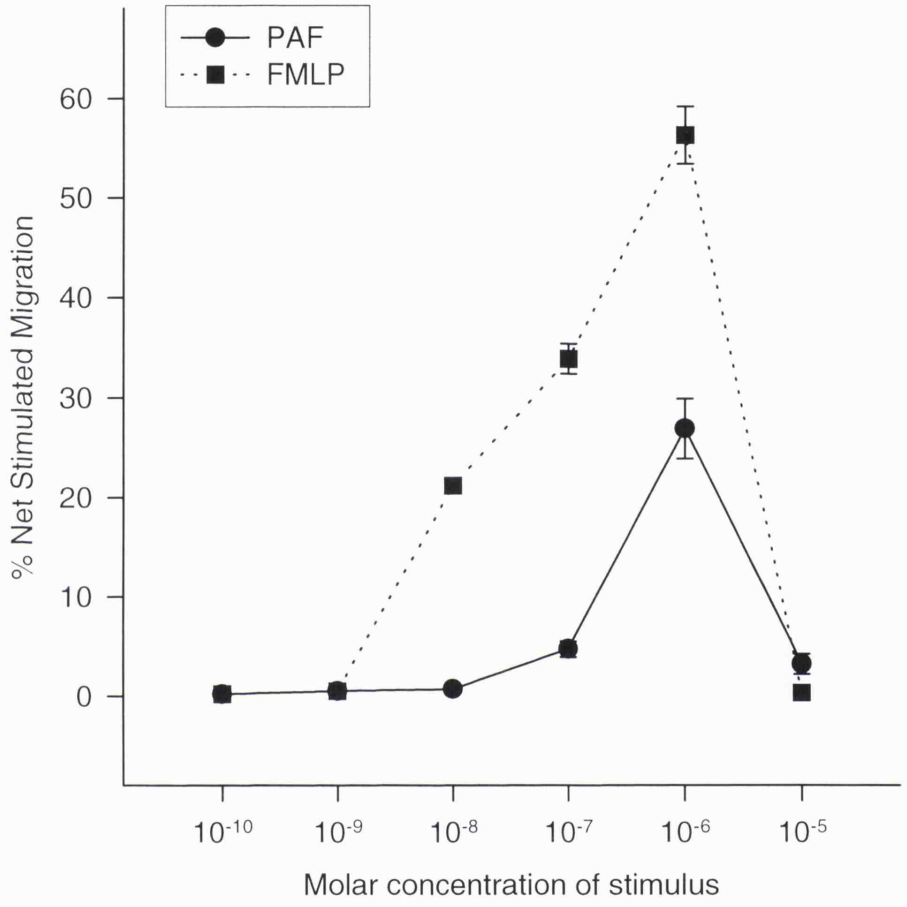

Figure 4.7: Dose-response curves for the degree of neutrophil chemotaxis across a porous membrane, in response to a range of concentrations of PAF and FMLP. Neutrophil migration is expressed as the percentage net stimulated migration above control. Data are means ± s.e.mean from 4 experiments. Control migration was <0.1%.

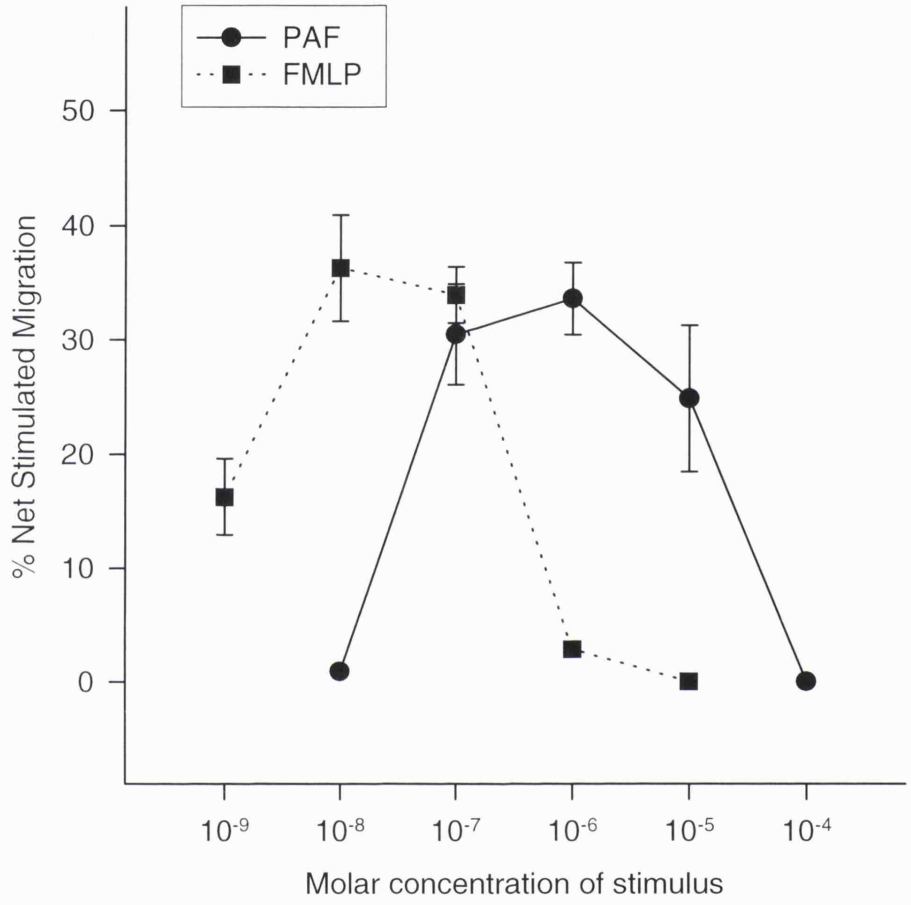

Figure 4.8: Dose-response curves for the degree of neutrophil transmigration across an endothelial cell monolayer, in response to a range of concentrations of PAF and FMLP. Neutrophil migration is expressed as the percentage net stimulated migration above control. Data are means ± s.e.mean from 5 experiments. Control migration was 12.6±1.3%.

		Concentration of FMLP below filter	
		0 μM	0.1 μM
Concentration of FMLP above filter	0 μM	0.3 ± 0.1 %	31.2 ± 1.9 %
	0.1 μM	0.9 ± 0.2 %	22.2 ± 2.2 %

Table 4.1: Checkerboard analysis of neutrophil migration across a porous membrane, in response to FMLP, 0.1 μM. Neutrophil migration is expressed as the percentage net stimulated migration (%NSM) above control. Data are means ± s.e.mean from 5 experiments. Control migration was <0.1%.

4.3.4 Effect of icatibant and bradykinin on neutrophil migration

Neither icatibant, 10 nM or 1 μM, or bradykinin, 1μM, affected neutrophil chemotaxis across a porous membrane ($p>0.05$, Student's t-test), as shown in Figure 4.9. Bradykinin, 10 nM to 100 μM, did not induce significant neutrophil migration ($p>0.05$, Student's t-test). Icatibant, 1 μM, did not alter neutrophil transmigration across an endothelial cell monolayer ($p>0.05$, Student's t-test) (Figure 4.10a). However, addition of bradykinin, 1 μM, potentiated the transmigration induced by PAF, 1 μM, by 22.1% ($p=0.02$, Student's t-test), but not in response to FMLP ($p>0.05$, Student's t-test), as shown in Figure 4.10b. Bradykinin alone, at a concentration of 1 μM, did not cause a significant degree of neutrophil transmigration, compared to control ($p>0.05$, Student's t-test).

4.3.5 Effect of icatibant and bradykinin on eosinophil transmigration

Addition of bradykinin or icatibant, both at 1 μM, did not significantly alter eosinophil migration in response to PAF, 1 μM ($p>0.05$, Student's t-test) (Figure 4.11). However, in the presence of neutrophils in a 50/50 mixture, bradykinin potentiated PAF-induced eosinophil transmigration across an endothelial cell monolayer by 30.4% ($p=0.033$, Student's t-test) (Figure 4.12). This increase was not the result a change in the number of cells added to the migration system ($p>0.05$, Student's t-test).

4.3.6 Characterisation of the effect of bradykinin on neutrophil transmigration

Addition of bradykinin, or the bradykinin B_1 receptor agonist, [des-Arg^9]-bradykinin, 1 μM, significantly potentiated neutrophil transmigration induced by PAF, 1 μM ($p=0.047$ and $p=0.031$ respectively, Student's t-test), as shown in Figure 4.13. Neither Lys-[des-Arg^9, Leu^8]-bradykinin nor icatibant, bradykinin B_1 and B_2 receptor antagonists respectively, altered the ability of bradykinin, 1 μM, to increase PAF-induced neutrophil transmigration across an endothelial cell monolayer.

In the presence of the neutrophil elastase inhibitor N-methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone (AAPVK), at a concentration of 50 μM, the level of neutrophil transmigration in response to both FMLP and PAF increased. Unfortunately, AAPVK caused a significant degree of neutrophil migration, in the absence of any other stimulus ($p=0.004$, Student's t-test), as shown in Figure 4.14.

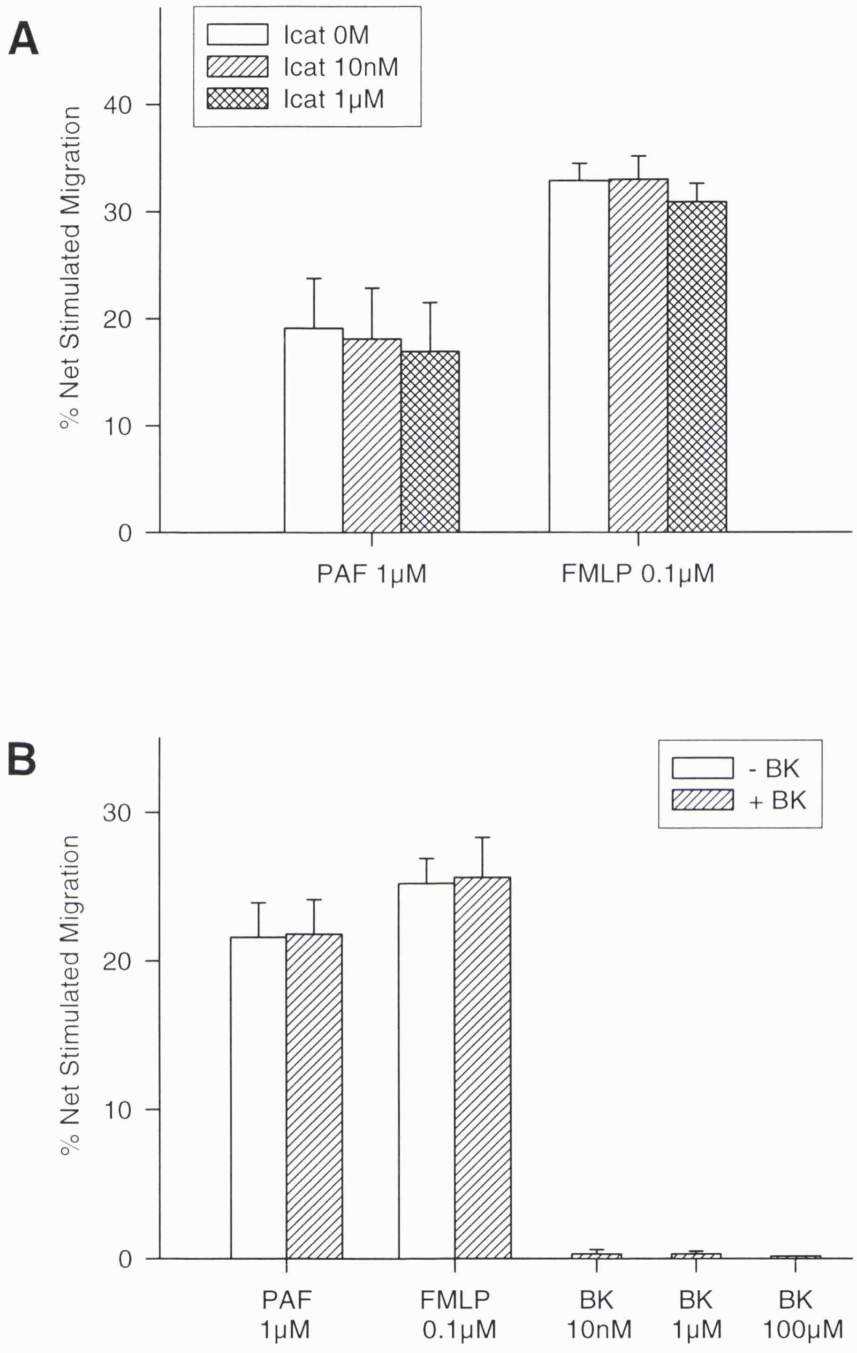

Figure 4.9: Effect of icatibant (Icat), 10 nM to 1 μM (graph A), and bradykinin (BK), 1 μM (graph B), on neutrophil chemotaxis across a porous membrane, in response to PAF and FMLP. Neutrophil migration is expressed as the percentage net stimulated migration above control. Data are means ± s.e.mean from 4-5 experiments. Control migration was <0.1%.

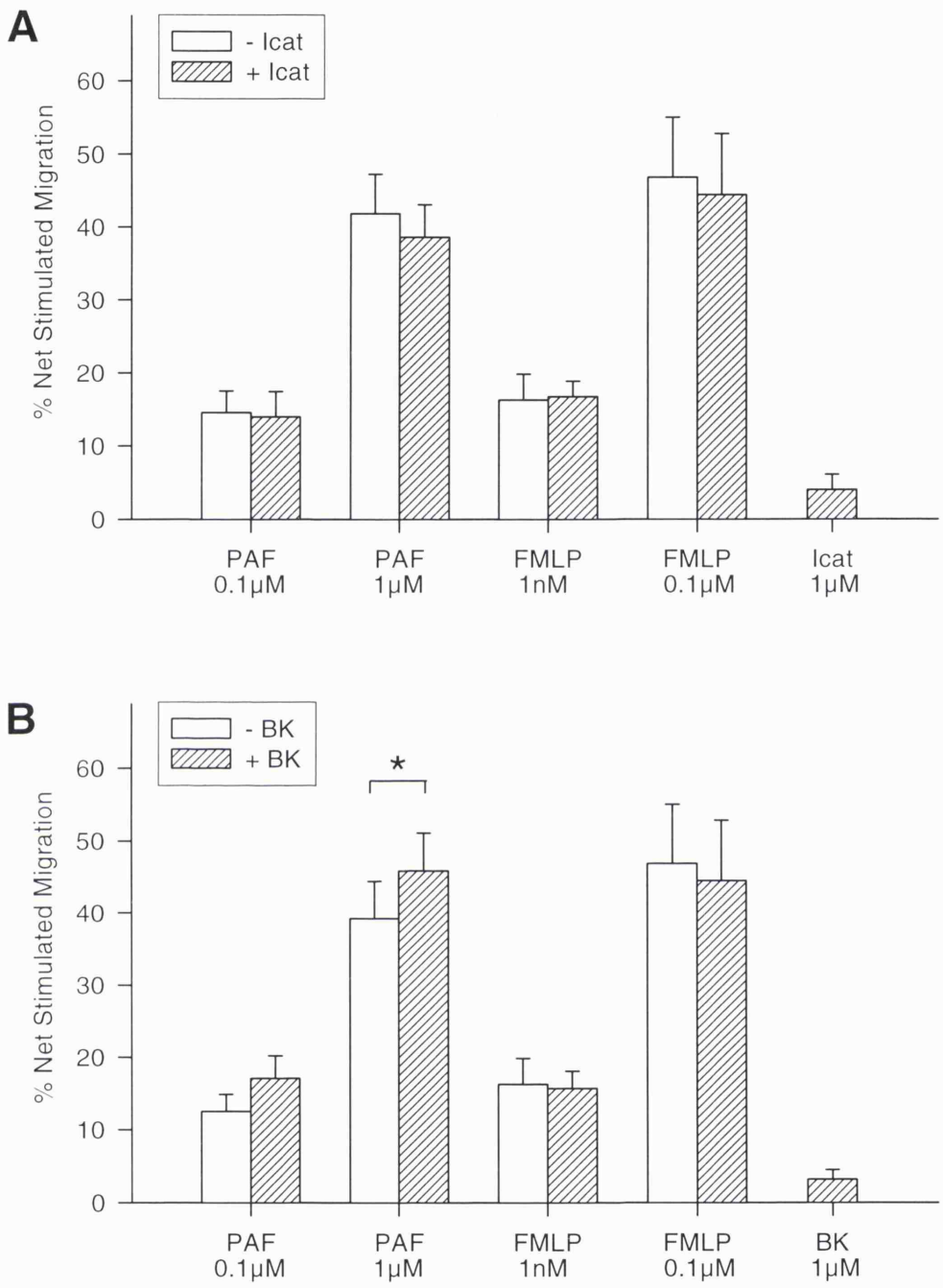

Figure 4.10: Effect of icatibant (Icat) (graph A) and bradykinin (BK) (graph B), both at 1 μM, on neutrophil transmigration across an endothelial cell monolayer, in response to PAF and FMLP. Neutrophil migration is expressed as the percentage net stimulated migration (%NSM) above control. Data are means ± s.e.mean from 5-9 experiments. Control migration was 9.6±1.2%. *Significant increase in %NSM induced by PAF, 1 μM, in the presence of BK, compared to PAF without BK ($p<0.05$, Student's t-test).

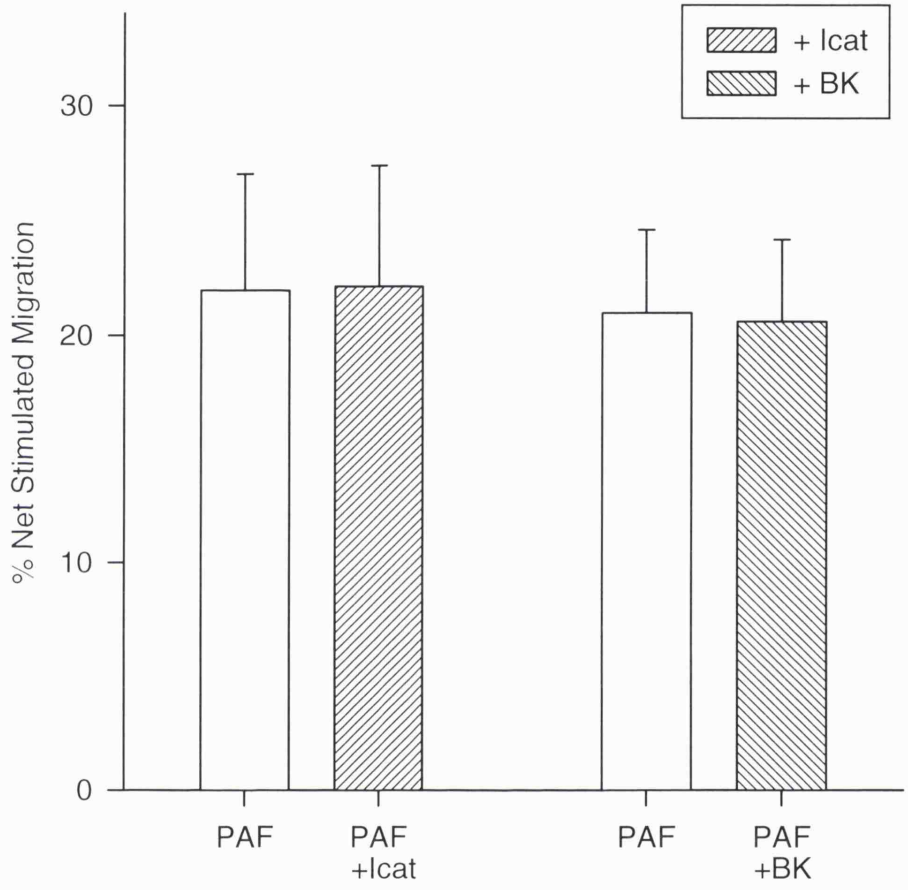

Figure 4.11: Effect of icatibant (Icat) and bradykinin (BK), both at 1 μM, on eosinophil transmigration across an endothelial cell monolayer, in response to PAF, 1 μM. Eosinophil migration is expressed as the percentage net stimulated migration above control. Data are means ± s.e.mean from 5-7 experiments. Control migration was 1.6±0.6%.

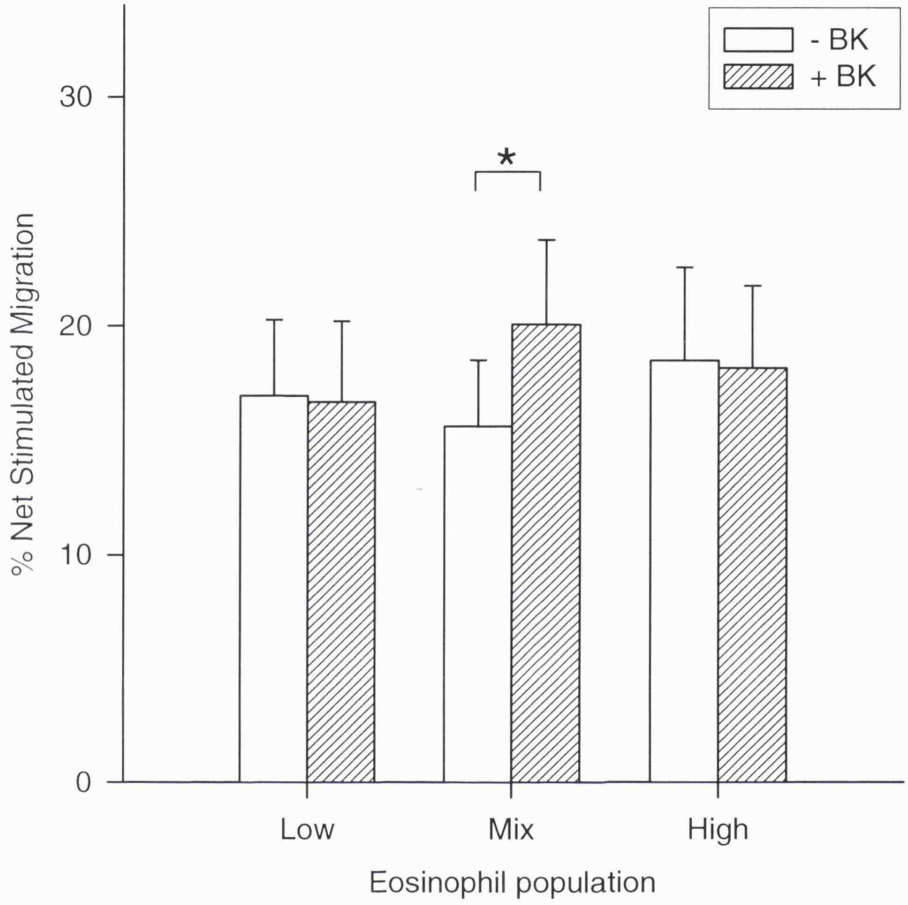

Figure 4.12: Effect of neutrophils on PAF-induced eosinophil transmigration across an endothelial cell monolayer, in the presence of bradykinin (BK), 1 μM. The cell populations used were composed of 0.5×10^6 eosinophils (low), 0.5×10^6 eosinophils and 0.5×10^6 neutrophils (mix) and 1×10^6 eosinophils (high). Eosinophil migration is expressed as the percentage net stimulated migration (%NSM) above control. Data are means ± s.e.mean from 5 experiments. Control migration was 1.0±0.6%. *Significant increase in %NSM induced by PAF, 1 μM, in the presence of BK and neutrophils, compared to PAF without BK and/or the presence of neutrophils ($p<0.05$, Student's t-test).

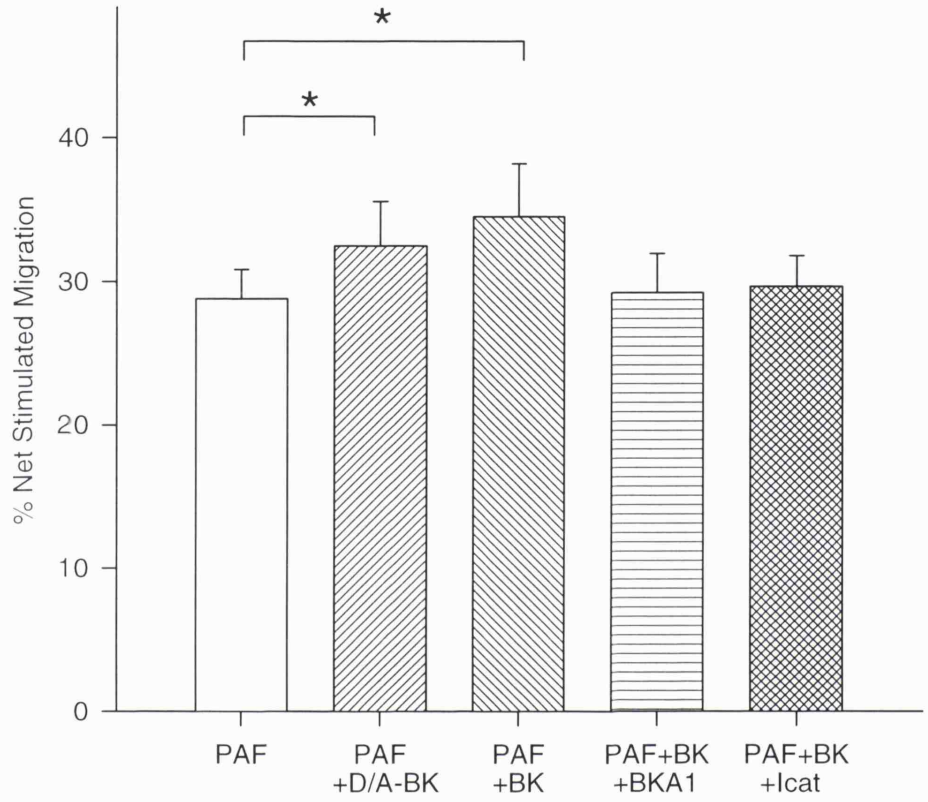

Figure 4.13: Modulation of neutrophil transmigration across an endothelial cell monolayer, stimulated by PAF, 1 μM, in the presence of various agonists and antagonists at bradykinin receptors. The agonists used were bradykinin (BK), and the BK B_1 receptor agonist, [des-Arg^9]-BK (D/A-BK), both at 1 μM. The antagonists used were Lys-[des-Arg^9, Leu^8]-BK (BKA1) and icatibant, antagonists at the BK B_1 and B_2 receptor respectively, both at 1 μM. Neutrophil migration is expressed as the percentage net stimulated migration (%NSM) above control. Data are means ± s.e.mean from 8-9 experiments. Control migration was 9.6±1.2%. *Significant increase in %NSM induced by PAF, 1 μM, in the presence of BK or D/A-BK, compared to PAF without BK agonist ($p<0.05$, Student's t-test).

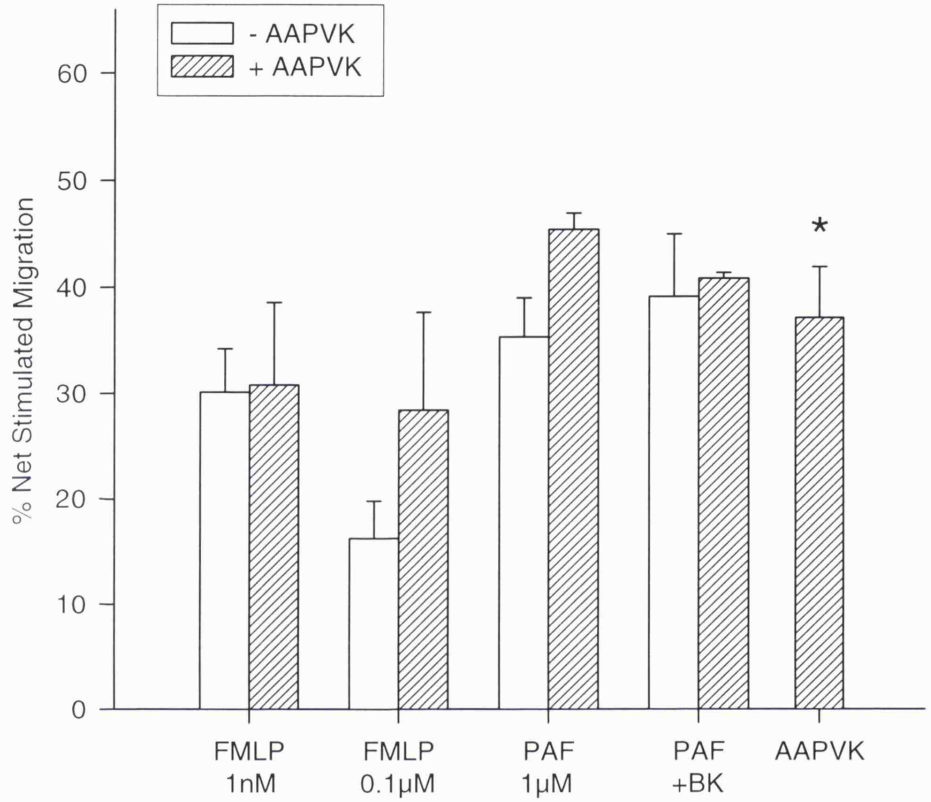

Figure 4.14: Modulation of neutrophil transmigration across an endothelial cell monolayer in response to FMLP and PAF, in the presence or absence of 50 μM N-methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone (AAPVK), an inhibitor of neutrophil elastase. The effect of AAPVK on PAF-induced neutrophil migration in the presence of bradykinin (BK) was also investigated. Neutrophil migration is expressed as the percentage net stimulated migration (%NSM) above control. Data are means ± s.e.mean from 3-4 experiments. Control migration was 13.5±3.4%. *Significant increase in %NSM induced by AAPVK, 50 μM ($p<0.05$, Student's t-test).

4.3.7 Generation of kinin from kinin moieties bound to neutrophils and endothelial cells, and their effect on PAF-induced neutrophil transmigration

Incubation of neutrophils with plasma kallikrein, 10-100 µg/ml, caused an increase in the amount of kinin generated in the cell suspension, as shown in Figure 4.15. However, this increase failed to reach statistical significance ($p>0.05$, one-way ANOVA). Neither PAF, FMLP or calcium ionophore A23187 stimulated the production of kinin ($p>0.05$, Student's t-test). ECP, at a concentration of 10 µg/ml, resulted in the significant generation of kinin, compared to control ($p=0.03$, Student's t-test). Unfortunately, the diluent for ECP also caused significant kinin release ($p=0.05$, Student's t-test), so the effect of ECP on kinin production (if any) could not be ascertained.

Incubation of EA.hy926 endothelial cells with 1 µg/ml HMWK, followed by 100 ng/ml human plasma kallikrein, for various incubation periods, resulted in a significant increase in kinin generation (Figure 4.16). This increase was significant both with respect to the incubation time with HMWK ($p<0.001$, two-way ANOVA) and plasma kallikrein ($p<0.001$, two-way ANOVA). In the absence of the endothelial monolayer, no kinin production was observed, indicating that the HMWK was binding to the endothelial cell monolayer, rather than the tissue culture flask.

Using the transmigration model, the effect of bradykinin generation, from HMWK bound to endothelial cells, on PAF-induced migration was investigated. As expected, addition of bradykinin, 1 µM, significantly increased the migration of neutrophils to 1 µM PAF ($p=0.024$, Student's t-test). Without the pre-incubation of the endothelial cell monolayer with HMWK, plasma kallikrein did not alter the degree of neutrophil migration ($p>0.05$, Student's t-test). Similarly, pre-incubation with HMWK, but without plasma kallikrein, failed to alter PAF-induced migration. However, pre-incubation with HMWK, followed by addition of plasma kallikrein, significantly increased neutrophil migration to PAF ($p=0.009$, Student's t-test). Addition of icatibant, 1 µM, prevented this effect, as shown in Figure 4.17 ($p=0.024$, Student's t-test). HMWK, with or without subsequent addition of plasma kallikrein, did not cause significant neutrophil migration ($p>0.05$, Student's t-test).

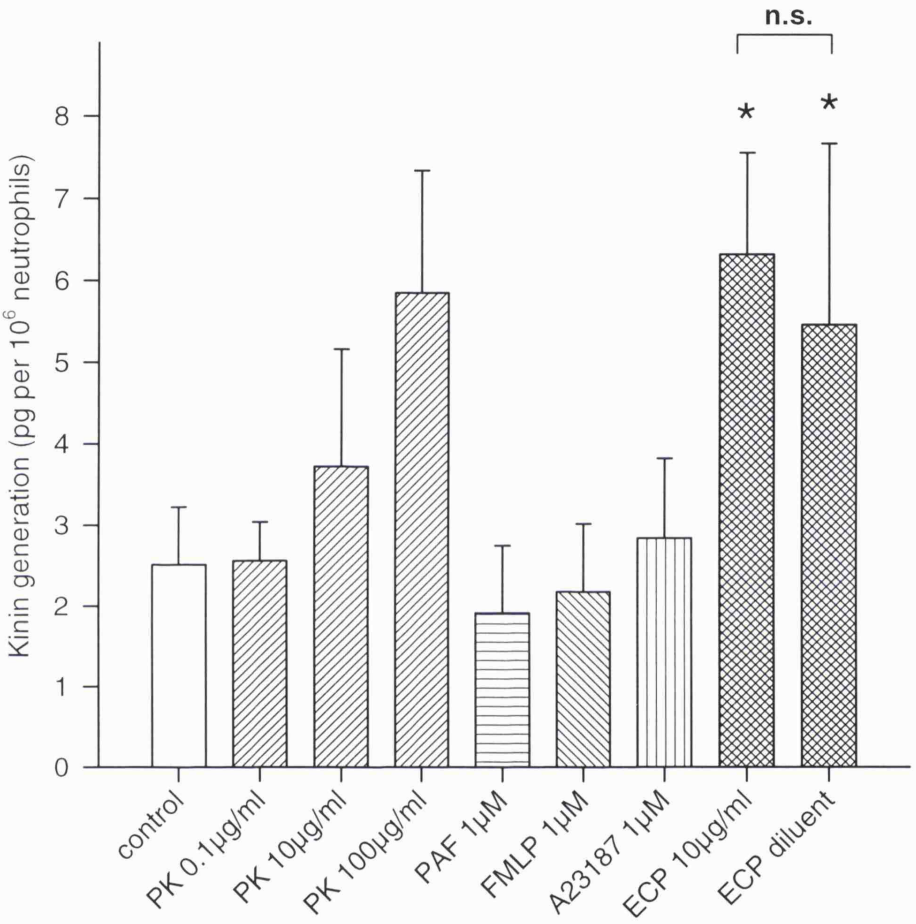

Figure 4.15: The levels of kinin generated in a suspension of neutrophils, after the addition of various stimuli. PK = plasma kallikrein, ECP = eosinophil cationic protein. Data are means ± s.e.mean from 5-7 experiments. *Significant increase in kinin levels with stimulus shown, compared to control ($p<0.05$, Student's t-test). n.s. = not significant.

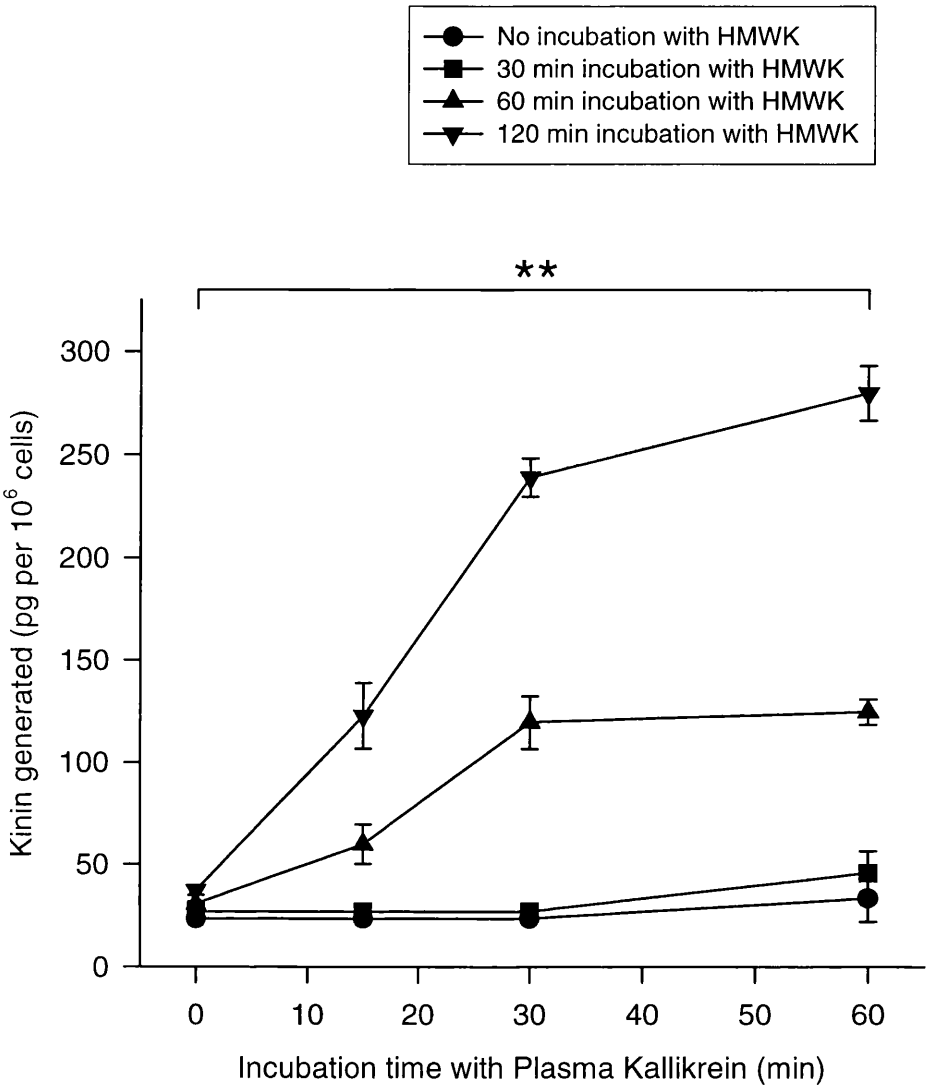

Figure 4.16: The levels of kinin generated following incubation of EA.hy926 endothelial cells with 1 µg/ml high molecular weight kininogen (HMWK), followed by 100 ng/ml human plasma kallikrein, for various incubation times. Data are means ± s.e.mean from 3 experiments. *Significant increase in kinin levels, associated with increasing incubation times for both HMWK and plasma kallikrein ($p<0.001$, two-way ANOVA).

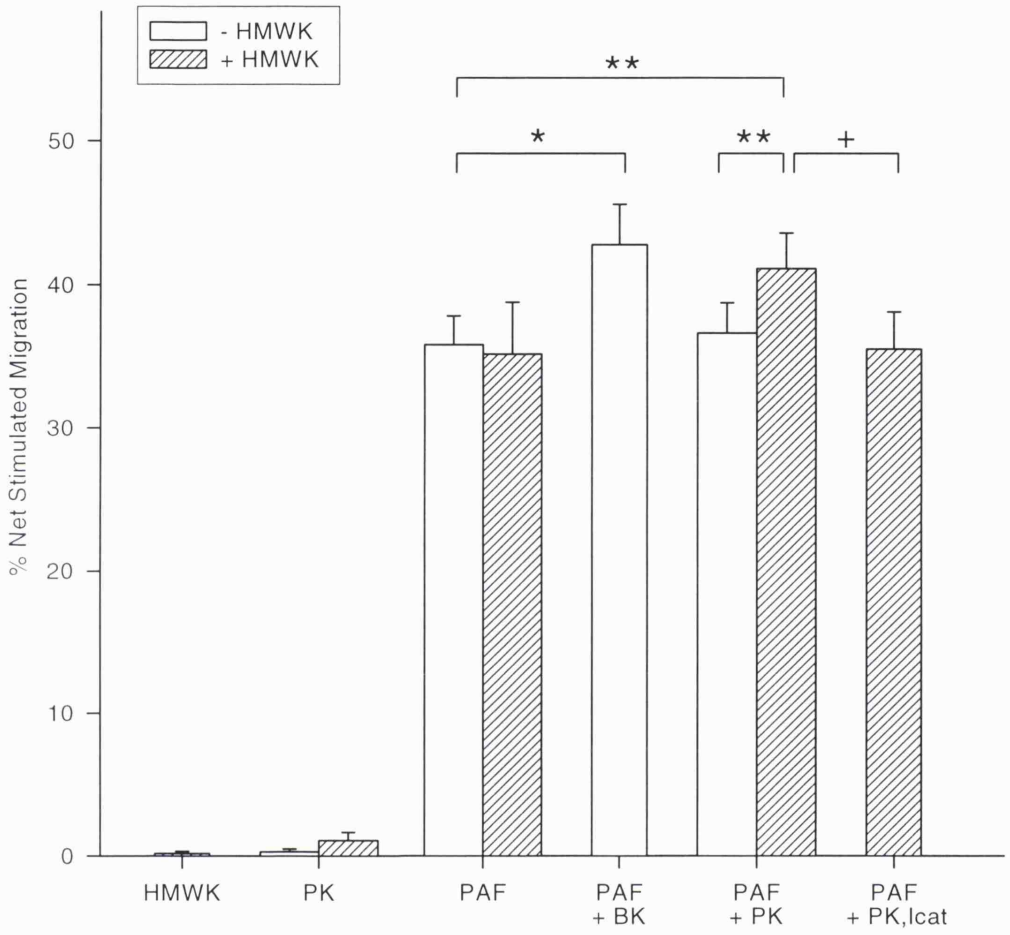

Figure 4.17: Modulation of neutrophil transmigration across an endothelial cell monolayer, stimulated by PAF, 1 μM, in the presence of bradykinin (BK), 1 μM, or plasma kallikrein (PK), 10 μg/ml. Where shown, the endothelial cell monolayer was pre-incubated with 1 μg/ml high molecular weight kininogen (HMWK) for two hours, prior to the commencement of the assay. The effect of icatibant, 1 μM, on PAF-induced neutrophil migration in the presence of PK was also investigated. Neutrophil migration is expressed as the percentage net stimulated migration (%NSM) above control. Data are means ± s.e.mean from 7 experiments. Control migration was 15.6±3.1%. */**Significant increase in %NSM induced by PAF, 1 μM, in the presence of BK or PK after pre-incubation with HMWK, compared to PAF alone (*$p<0.05$, **$p<0.01$, Student's t-test). +Significant inhibition of PAF-induced neutrophil migration by icatibant, in the presence of PK and after pre-incubation with HMWK ($p<0.05$, Student's t-test).

4.4 Discussion

The intranasal administration of PAF, 60 µg, caused an increase in the number of eosinophils, identified in nasal lavage samples collected 2 and 6 hours later. There was also a significant increase in the differential neutrophil count in samples obtained 2 hours after PAF. This data is consistent with previous studies (Klementsson & Andersson, 1992; Tedeschi *et al.*, 1994a & 1994b). The PAF-induced eosinophilia was significantly reduced by pretreatment with the bradykinin B_2 receptor antagonist, icatibant. However, [Ad]-BK, another B_2 receptor antagonist, failed to reduce the eosinophilia. This observation may, therefore, account for the reduction in PAF-induced ECP release by icatibant, but not by [Ad]-BK, found in chapter 3. However, this does not explain the ability of icatibant to reduce PAF-induced eosinophilia, while [Ad]-BK had no effect.

PAF also causes kinin release into the nasal cavity, an effect which is abrogated by pretreatment with icatibant, and to a lesser extent by [Ad]-BK (chapter 3). The generation of kinins by PAF might, in turn, cause an increase in eosinophil recruitment. Kinins could act as a chemotactant for eosinophils. Therefore, icatibant might reduce the eosinophilia, induced by PAF, by inhibiting kinin production. However, bradykinin alone failed to induce either a neutrophilia or eosinophilia into the human nasal airway. This implies that kinins only affect eosinophil recruitment in the presence of other mediators (such as PAF). Furthermore, this action is not, apparently, mediated through the bradykinin B_2 receptor, since [Ad]-BK did not reduce the eosinophilia induced by PAF. The failure of [Ad]-BK to affect the eosinophilia could not be due to the lower potency of this antagonist at the bradykinin B_2 receptor, compared to icatibant (Dear *et al.*, 1996b), since both antagonists inhibit PAF-induced AHR (chapter 3) and the increase in nasal obstruction and albumin leakage induced by bradykinin (Austin & Foreman, 1994b). Alternatively, the action of icatibant could be due to a specific, anti-eosinophil property of the antagonist (similar to that shared by some histamine H_1 receptor antagonists, such as cetirizine).

In order to investigate the role of kinins in inflammatory cell recruitment, an *in vitro* model of cell migration was developed. Both PAF and FMLP stimulated neutrophil migration across a porous membrane, and also through a cell monolayer consisting of EA.hy926 endothelial cells. This cell line is a hybridoma between human umbilical

vein endothelial cells (HUVECs) and the epithelioma A549, and is an immortalised cell line which retains the majority of the functional characteristics of HUVECs (Edgell *et al.*, 1983; Thornhill *et al.*, 1993).

The migration of neutrophils, in response to either stimulus, was not affected by icatibant. Similarly, bradykinin failed to induce significant neutrophil migration. However, the migration of neutrophils in response to PAF was increased in the presence of bradykinin, but only across an endothelial cell monolayer. Bradykinin did not affect PAF-induced neutrophil chemotaxis. This implies that bradykinin can potentiate the transmigration of neutrophils to other stimuli, via an action on endothelial cells. This may occur by increasing the permeability of the monolayer and allowing more neutrophils to migrate through. Alternatively, bradykinin could have stimulated the release of a factor from endothelial cells, which subsequently potentiated cell migration. The action of bradykinin is not, apparently, mediated by bradykinin receptors, since Lys-[des-Arg^9, Leu^8]-bradykinin and icatibant, antagonists at the bradykinin B_1 and B_2 receptors respectively, failed to prevent the ability of bradykinin to potentiate neutrophil migration. However, like bradykinin, the bradykinin B_1 receptor agonist [des-Arg^9]-bradykinin also increased PAF-induced neutrophil transmigration, implying that the effect of bradykinin may be receptor-independent.

Interestingly, bradykinin did not potentiate neutrophil transmigration in response to FMLP, at either of the concentrations used. One explanation for this could be as follows: the diapedesis of neutrophils across an endothelial barrier may be dependent on the release of proteases from neutrophils, particularly human neutrophil elastase (HNE) (Zimmerman *et al.*, 1990), though neutrophil gelatinase and collagenase may also have a role (Bhoola *et al.*, 1992). Certainly, HNE increases the permeability of endothelial cell monolayers (Suttorp *et al.*, 1993; Carl *et al.*, 1996), though this process may be distinct from the diapedesis of neutrophils through the endothelium. It has been reported that the release of HNE from neutrophils is stimulated by FMLP, but not by PAF (Rainger *et al.*, 1998). Bradykinin induces the release of HNE from neutrophils, and the elastase inhibitor AAPVK reduces bradykinin-induced increases in the permeability of endothelial cell monolayers (Carl *et al.*, 1996). A similar interaction between HNE and bradykinin has been proposed elsewhere (Leimer *et al.*, 1997). Therefore, the addition of bradykinin to the migration system may have stimulated the release of HNE from neutrophils, potentiating PAF-induced migration, but not that stimulated by FMLP (since FMLP causes HNE release *per se*).

To study this hypothesis further, the effect of AAPVK on neutrophil transmigration was investigated. Unfortunately, it was found that AAPVK itself was a strong chemotactant for neutrophils, and thus significantly interfered with the cell migration assay. In addition, in contrast to the study by Carl *et al.* (1996), Peterson *et al.* (1987) reported that AAPVK failed to inhibit HNE-induced increases in endothelial cell monolayer permeability. The authors concluded that the mechanism involved was due to a cationic action of HNE, rather than any proteolytic property, a finding which has been supported by some studies, but refuted by others (Suttorp *et al.*, 1993; Doherty & Janusz, 1994). In any event, HNE may not fully explain the action of bradykinin observed in the experiments described in this chapter. Carl *et al.* (1996) reported that the release of HNE was significantly inhibited by both bradykinin B_1 and B_2 receptor antagonists, yet the antagonists used in this study did not alter neutrophil transmigration to PAF, in the presence of bradykinin.

Alternatively, bradykinin may have stimulated the release of a chemotactic factor which potentiated neutrophil transmigration in response to PAF. Bradykinin releases a number of cytokines, including IL-8, from A549 epithelial cells (Koyama *et al.*, 1998) and cultured human airway muscle cells (Pang & Knox, 1998). There is also evidence for the kinin-mediated release of a neutrophil chemotactant, thought to be IL-8, from human endothelial cells (Farber *et al.*, 1990). IL-8 is chemotactic for both neutrophils (Peveri *et al.*, 1988) and eosinophils (Shute, 1994). Both FMLP and PAF stimulate neutrophils to generate IL-8 (Cassatella *et al.*, 1992; Siddiqui *et al.*, 1999), possibly via a mechanism dependent upon the endogenous production of PAF (Au *et al.*, 1994; Hilger *et al.*, 1996), so it is unclear if IL-8, released from endothelial cells by bradykinin, could further contribute to neutrophil transmigration. In addition, bradykinin itself did not cause significant neutrophil transmigration, a finding which is consistent with the *in vivo* data from the human nasal airway. Nonetheless, the results from the checkerboard analysis demonstrate a lack of migration in the absence of a positive chemotactic gradient, so this could explain the lack of neutrophil migration if IL-8 (or another factor) was only present in the upper chamber of the migration system. However, the release of IL-8 from A549 cells is mediated by both bradykinin B_1 and B_2 receptors (Koyama *et al.*, 1998), in contrast to bradykinin-potentiated migration. Nonetheless, preventing the bradykinin-mediated release of IL-8 (or HNE), using an antagonist of bradykinin B_1 receptors, would still allow the release of IL-8 and HNE via bradykinin B_2 receptors, and vice versa. Thus, either mechanism could

explain the observations of the current study. It is also possible that bradykinin may have caused an increase in the expression of adhesion molecules such as ICAM-1, so increasing cell migration. However, there is no evidence for such a process in the literature, and in any event such a mechanism would not explain the different results obtained in response to stimulation with PAF and FMLP.

It has been proposed that PAF and IL-8 together control the adhesion of neutrophils to endothelial cells *in vitro* (Rainger *et al.*, 1997) and their subsequent diapedesis (Kuijpers *et al.*, 1992). IL-8 also stimulates the release of HNE from adherent neutrophils (Rainger *et al.*, 1998). Furthermore, the generation of IL-8, by neutrophils, is substantially increased during neutrophil migration, compared to levels of IL-8 produced by neutrophils under static conditions (Siddiqui *et al.*, 1999). Therefore, any IL-8 generated, from endothelial cells or migrating neutrophils, could potentiate neutrophil migration by a mechanism dependent on HNE. This allows a role for HNE in the potentiation of PAF-induced neutrophil transmigration by bradykinin, via the release of IL-8 from endothelial cells, perhaps as shown in Figure 4.18. Unfortunately, further studies into the role of IL-8 were precluded by cost.

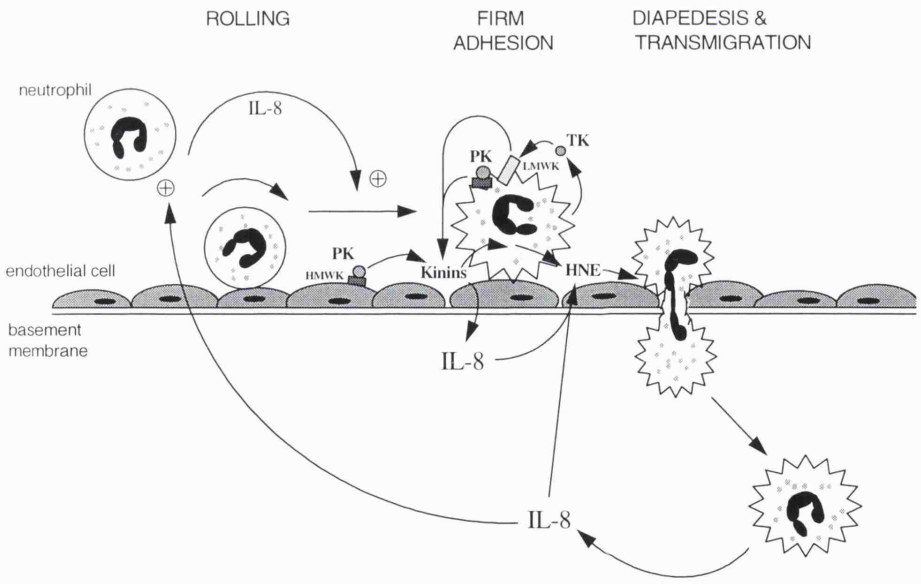

Figure 4.18: Putative mechanism for the involvement of kinins, and possibly human neutrophil elastase (HNE) and IL-8, in the migration of inflammatory cells across an endothelial barrier. See text for full details. TK = tissue kallikrein, PK = plasma kallikrein, LMWK and HMWK = low and high molecular weight kininogen (adapted from Bhoola *et al.*, 1992).

Either of these mechanisms would explain the ability of bradykinin to potentiate PAF-induced eosinophil transmigration, but only in the presence of neutrophils. Icatibant did not affect eosinophil transmigration, implying that icatibant has no direct action on eosinophil recruitment, *in vitro*, in contrast to some histamine H_1 antagonists such as cetirizine (Leprevost *et al.*, 1988). It is unlikely, therefore, that icatibant has a direct action in opposing eosinophil recruitment *in vivo*. In this study, eosinophils were isolated from donors with higher-than-average eosinophil counts, and subsequently incubated for 48 hours with GM-CSF, in order to raise eosinophil transmigration to detectable levels (Ebisawa *et al.*, 1994). In addition, eosinophils from some donors may have been primed, and therefore given different results to eosinophils from donors with a lower degree of atopy. Therefore, incubation with GM-CSF also served to prime eosinophils from less atopic donors to the same extent as cells from atopic donors (Moser *et al.*, 1992). Although only non-atopic donors would have been preferable, the large number of eosinophils required for each experiment precluded this. No attempt was made to distinguish between the relative proportions of normodense and hypodense eosinophils in the cell populations used, since the migration of eosinophils in response to PAF is not affected by their density (Little & Casale, 1991). Rozell *et al.* (1996) reported that eosinophils isolated using the MACS system do not respond to IL-8. In the current study, the PAF-induced transmigration of eosinophils, isolated using the MACS system, was potentiated by bradykinin. Therefore, it is unlikely that the mechanism responsible was dependent on IL-8 alone.

To study further the role of kinins in neutrophil transmigration, a number of experiments were conducted to investigate the production of kinin, from kinin moieties present on the cell membrane of neutrophils (Gustafson *et al.*, 1989; Henderson *et al.*, 1994) and endothelial cells (Nishikawa *et al.*, 1992). Incubation of neutrophils with plasma kallikrein resulted in an increase in kinin levels in the cell suspension, though this failed to reach statistical significance. Neither PAF, FMLP or A23187 stimulated kinin production, though apparently, ECP did. However, upon further study, it was found that the diluent used for the ECP also caused significant kinin detection. ECP is a very 'sticky' protein, due to its large negative charge. Therefore the diluent contained N-cetyl-NNN-trimethylammonium bromide (CTAB), a detergent which may have interfered with the kinin assay (M. Huelskamp, Peninsula Laboratories, personal communication). Incubation of EA.hy926 endothelial cells with HMWK, followed by the addition of plasma kallikrein, generated large amounts of kinin, implying that the

HMWK was able to bind to EA.hy926 cells *in vitro*. This result is consistent with a similar study using freshly-isolated human umbilical vein endothelial cells (Nishikawa *et al.*, 1992). Unfortunately, it was not possible to measure the effect of icatibant on kinin production in this model, because the antagonist possessed a large degree of crossreactivity with the antibody used in the assay.

Addition of plasma kallikrein to the migration system, after pre-incubation of the endothelial cell monolayer with HMWK, significantly increased PAF-induced neutrophil transmigration. Furthermore, this effect was abolished by addition of icatibant. Since icatibant did not reduce the potentiation of PAF-induced neutrophil transmigration by bradykinin, this implies that icatibant was interfering with kinin generation, rather than the action of kinins in potentiating transmigration. This may, therefore, explain the ability of icatibant to reduce PAF-induced neutrophil and eosinophil recruitment, *in vivo*, in the human nasal airway, as follows: PAF induces both kinin release and eosinophil recruitment, the latter of which is potentiated by the kinins generated. Inhibition of kinin production by icatibant could, therefore, reduce the PAF-induced eosinophilia. The failure of [Ad]-BK to have a similar effect on the PAF-induced eosinophilia in the human nasal airway may be due to the different degrees to which icatibant and [Ad]-BK are able to inhibit kinin production (in addition to their property as antagonists at the bradykinin B_2 receptor).

However, there are differences in the mechanism of inflammatory cell recruitment *in vitro*, and *in vivo* in the human nasal airway. For example, *in vivo*, adhesion of inflammatory cells to the endothelium occurs under non-static conditions. There are also differences in the permeability characteristics of endothelial cells *in vitro* and *in vivo* (Albelda *et al.*, 1988). Even *in vitro*, the migration of neutrophils across monolayers of endothelial cells and airway epithelial cells occurs by different mechanisms, and also varies depending on whether the movement is in an apical-basolateral direction or vice versa (Liu *et al.*, 1996). Therefore, a certain amount of caution is required in extrapolating results from *in vitro* models to the recruitment of inflammatory cells *in vivo.*

In summary, the data presented in this chapter provide evidence for a role of kinins in the potentiation of inflammatory cell recruitment in response to PAF, both *in vivo*, in the human nasal airway, and *in vitro*, across endothelial cell monolayers. This may occur by a mechanism involving human neutrophil elastase and/or IL-8.

Summary

- Intranasal administration of platelet activating factor (PAF), 60 µg, resulted in a significant increase in the numbers of eosinophils and neutrophils in nasal lavage samples, obtained two and six hours later.

- Pretreatment with icatibant, 200 µg, a bradykinin B_2 receptor antagonist, significantly reduced the PAF-induced eosinophilia. PAF also failed to cause a neutrophilia after pretreatment with icatibant. The bradykinin B_2 receptor antagonist [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin ([Ad]-BK), also at 200 µg, did not alter PAF-induced eosinophil or neutrophil recruitment.

- Bradykinin, 500 µg, given repeatedly over 2 hours, did not cause a change in the number of eosinophils or neutrophils present in nasal lavage fluid.

- Bradykinin, 1 µM, potentiated neutrophil migration across an endothelial cell monolayer *in vitro*, stimulated by PAF, but not by FMLP. This effect was dependent on the presence of an endothelial barrier, and was not affected by the addition of icatibant or Lys-[des-Arg^9, Leu^8]-bradykinin, a bradykinin B_1 receptor antagonist.

- Bradykinin alone did not cause neutrophil migration. Similarly, icatibant did not reduce neutrophil migration.

- Bradykinin also potentiated PAF-induced eosinophil transmigration across an endothelial cell monolayer, but only in the presence of neutrophils. Icatibant alone had no effect on eosinophil transmigration induced by PAF.

- The kinin generated by the action of plasma kallikrein on high molecular weight kininogen bound to endothelial cells, also potentiated PAF-induced neutrophil transmigration. Addition of icatibant prevented this effect.

- The data indicates a role for kinins in the recruitment of inflammatory cells *in vivo*, in the human nasal airway, and also *in vitro*, across an endothelial cell monolayer. This effect may occur via an interaction between kinins and other inflammatory mediators, and be dependent on the presence of neutrophils.

CHAPTER 5

THE ROLE OF KININS IN ANTIGEN-INDUCED HYPERRESPONSIVENESS IN THE HUMAN NASAL AIRWAY

5.1 Introduction

Intranasal administration of platelet activating factor (PAF), in non-atopic subjects, causes a similar airway hyperresponsiveness (AHR) to that observed following nasal antigen challenge in subjects with allergic rhinitis. In both seasonal and perennial allergic rhinitis, antigen challenge results in an increase in eosinophils, bradykinin, albumin, eosinophil cationic protein (ECP) and major basic protein (MBP) in nasal lavage fluid (Svensson *et al.*, 1990; Bascom *et al.*, 1989; Knani *et al.*, 1992; Kato *et al.*, 1995; Nishioka *et al.*, 1995; Dear *et al.*, 1996b), and a similar profile of mediator release occurs following administration of PAF into the normal, non-atopic human nasal airway, as found in chapters 3 and 4. It is, therefore, possible that the mechanisms of antigen- and PAF-induced AHR in the human nasal airway are similar.

The data in the previous chapters indicates a role for kinins in PAF-induced AHR in the human nasal airway, and the associated eosinophilia. There is also evidence to suggest that kinins may have a similar role in the AHR induced by antigen. For example, bradykinin antagonists inhibit antigen-induced AHR in the lower airways of sheep (Abraham *et al.*, 1991) and the guinea pig (Farmer *et al.*, 1992; Featherstone *et al.*, 1996). Furthermore, Farmer and co-workers also found that the eosinophilia, induced by antigen, was markedly reduced by a number of different bradykinin receptor antagonists.

In man, the bradykinin B_2 receptor antagonist icatibant abolishes the nasal blockage induced by house dust mite antigen in subjects with perennial allergic rhinitis, as well

as the increase in kinin production following antigen challenge (Dear *et al.*, 1996b). However, icatibant does not have a similar effect on the early phase in subjects with seasonal allergic rhinitis (Akbary and Bender, 1993; Dear, 1996). Therefore, depending on the specific antigen, icatibant attenuates the immediate allergic response in the nose. The aim of this chapter was to investigate the effect of icatibant on antigen induced-AHR in the human nasal airway.

5.2 Experimental Protocol

In the following experiments, human subjects with a history of seasonal allergic rhinitis and a positive skin prick test to grass pollen were used.

5.2.1 The effect of nasal challenge with grass pollen antigen on the responsiveness of the human nasal airway

Initially, a baseline measure of Amin. was taken, following which subjects received a nasal spray of either diluent or grass pollen antigen, 100 IU or 500 IU. Administration of grass pollen antigen into the nasal airways of sensitive subjects induces an AHR 24 hours later (Klementsson *et al.*, 1990). Therefore, 24 hours after diluent or antigen challenge, subjects were challenged with histamine, 200 µg. Immediately prior to, and 2, 5 and 10 minutes after nasal challenge, the minimal cross-sectional area (Amin.) of the nasal airways was determined by acoustic rhinometry. The protocol is summarised in Figure 5.1.

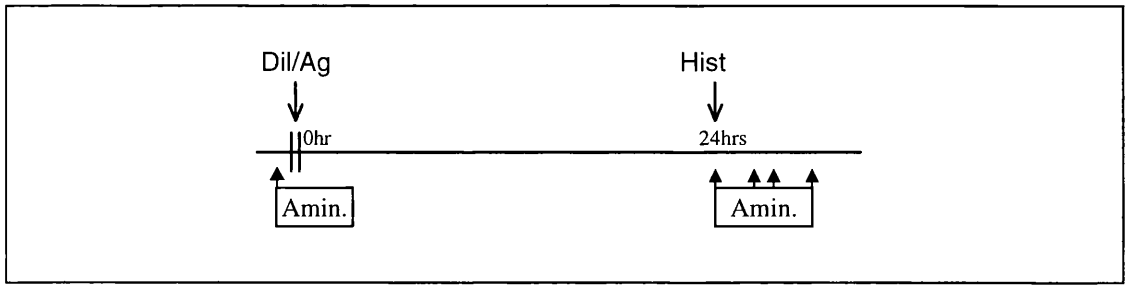

Figure 5.1: Protocol for investigating the ability of grass pollen antigen to induce a hyperresponsiveness to histamine, by measuring the change in nasal patency following intranasal histamine challenge. Ag = antigen, Dil = diluent, Hist = histamine.

In a cross-over design, subjects received all three doses of antigen (diluent/0 IU, 100 IU and 500 IU) on separate occasions at least one week apart, in a random order.

5.2.2 The duration of antigen-induce hyperresponsiveness in the human nasal airway

In order to establish a time course for the nasal AHR induced by antigen, subjects received a nasal spray of grass pollen antigen, 500 IU, or diluent as a control. Subjects then received, by intranasal challenge, histamine, 200 µg, at various time intervals: 6 hours, 24 hours, 48 hours and 7 days after nasal provocation with antigen. Histamine challenge was carried out 24 hours after diluent, since this timepoint was associated with the maximum increase in responsiveness after antigen challenge. Amin. was determined by acoustic rhinometry immediately prior to, and 2, 5 and 10 minutes after histamine challenge. The protocol is summarised in Figure 5.2. All subjects received antigen or diluent on separate occasions, in a random order.

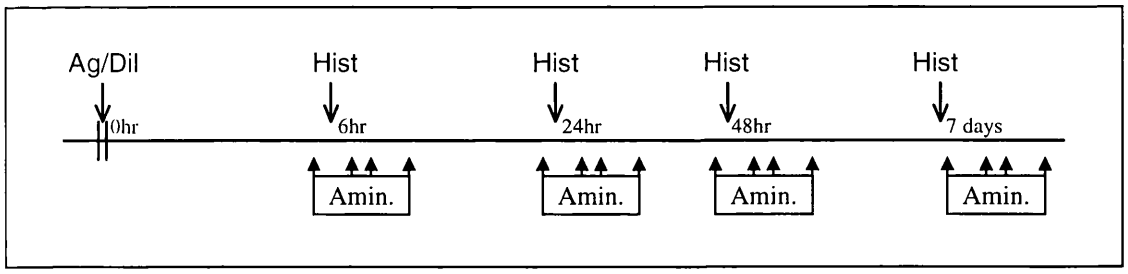

Figure 5.2: Protocol for investigating the duration of the hyperresponsiveness to histamine, induced by a single nasal challenge with grass pollen antigen. Ag = antigen, Dil = diluent, Hist = histamine.

5.2.3 The effect of icatibant on the early phase response and airway hyperresponsiveness following nasal antigen challenge

Initially, three nasal lavages were carried out, the third was retained for analysis as a baseline. The nasal cavity was allowed to dry, following which a baseline measurement of Amin. was taken using acoustic rhinometry. Subjects then received a

nasal spray of either icatibant, 200 µg, or a saline control. Two minutes later, a further spray was administered, containing either grass pollen antigen, 100 IU or 500 IU, or diluent as a control. The subsequent nasal response was monitored by measuring Amin. 15 minutes later. Since the duration of action of icatibant is approximately 2 hours (Dear, 1996), each subject received a further dose of icatibant or saline 2, 4 and 6 hours after challenge with antigen or diluent. Immediately prior to the final administration, the nasal cavity was again lavaged.

Twenty four hours after the start, subjects were challenged with histamine, 200 µg. Immediately prior to, and 2, 5 and 10 minutes after histamine challenge, Amin. was redetermined. In addition, a nasal lavage was carried out after the final measurement of Amin. The protocol is summarised in Figure 5.3.

The preliminary experiments found that the antigen-induced hyperresponsiveness was no longer present after 7 days. Therefore, each combination was administered on separate occasions at least 7 days apart. The order of treatments was determined using a randomised, balanced block design. All subjects received all four possible combinations of treatment, i.e.:

- Icatibant, 200 µg (four administrations) and antigen (once)
- Icatibant, 200 µg (four administrations) and saline (once)
- Saline (four administrations) and antigen (once)
- Saline (four administrations) and saline (once)

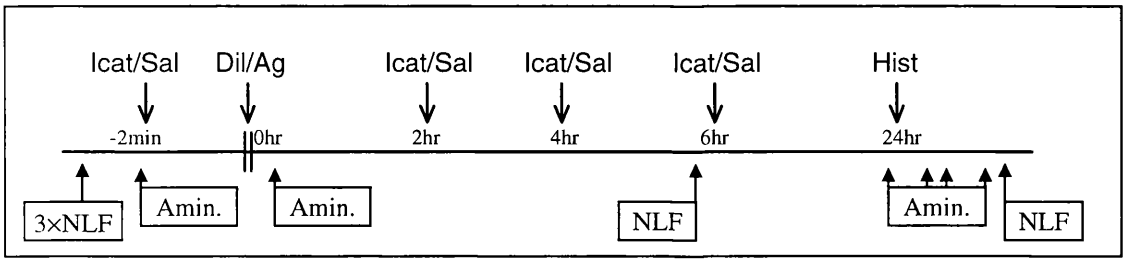

Figure 5.3: Protocol for investigating the effect of icatibant on the early phase response and airway hyperresponsiveness following nasal antigen challenge. NLF = nasal lavage, Ag = antigen, Icat = icatibant, Sal = saline, Dil = diluent, Hist = histamine.

The nasal lavage samples were processed as described, and cytological specimens prepared and examined for eosinophils and neutrophils. The remaining fluid from the samples collected at 0 and 6 hours was divided into aliquots and stored at -70°C, until assayed for their ECP, kinin, IL-5 and IL-8 content.

5.2.4 Data analysis

Values of Amin. following nasal challenge were normalised, as described in chapter 2. For each histamine challenge, a response-time curve was constructed using the percentage decrease in Amin. from baseline against time, and the area under curve (AUC) determined. The data are presented as mean values, together with s.e.mean. Data from nasal lavage experiments are expressed as medians, together with the interquartile range and 80% central range. The appropriate non-parametric statistical test is given with each data set. A value of $p<0.05$ is taken as significant.

5.3 Results

5.3.1 Preliminary investigations into the hyperresponsiveness to histamine challenge, induced by antigen in the human nasal airway

Challenge of the nasal cavity with histamine, 200 µg, after administration of the diluent control 24 hours earlier, caused a significant increase in AUC ($p=0.036$, Wilcoxon sign-rank test). Pretreatment with 100 IU of grass pollen antigen did not increase this response ($p>0.05$, Wilcoxon sign-rank test). However, administration of 500 IU grass pollen antigen resulted in a significant potentiation of the response to nasal histamine challenge, 24 hours after antigen provocation ($p=0.036$, Wilcoxon sign-rank test), as shown in Figure 5.4.

The next study investigated the duration of the antigen-induced nasal AHR. It was found that the increase in response to histamine peaked at 24 hours after antigen challenge, and declined thereafter (Figure 5.5).

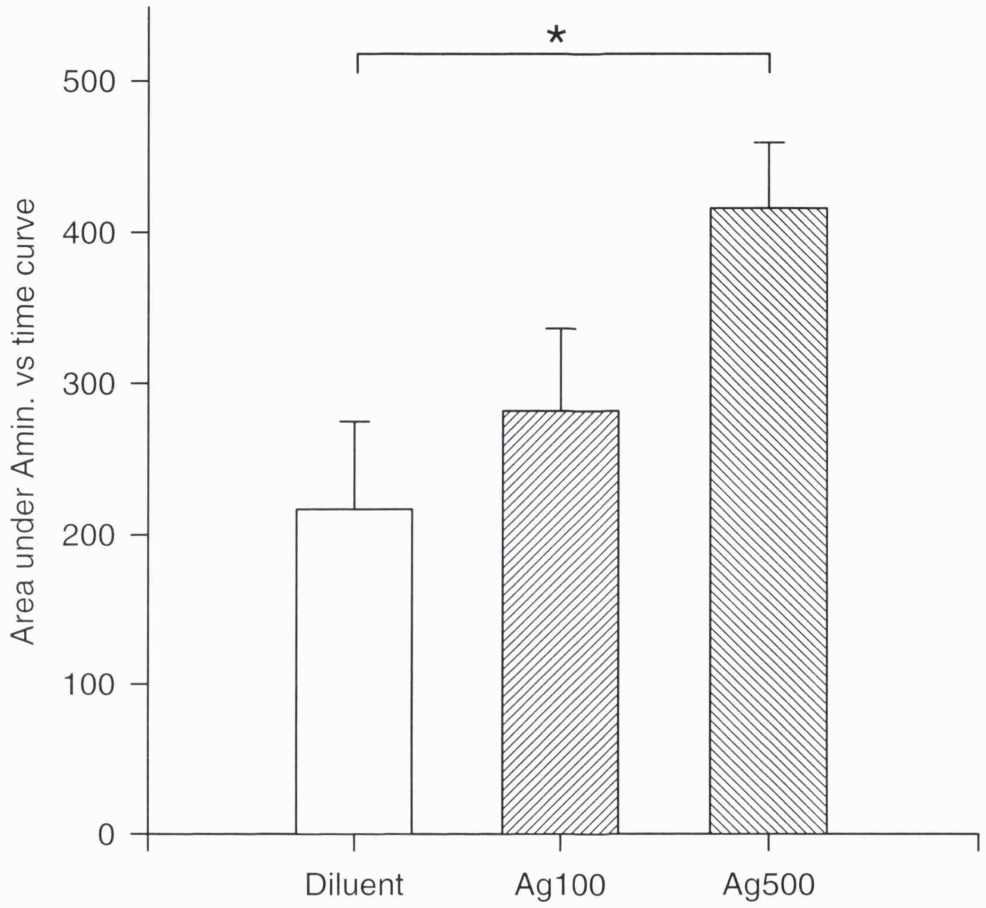

Figure 5.4: Effect of pretreatment with grass pollen antigen on the response to nasal challenge with histamine, 200 µg. The nasal cavity was pretreated with diluent or one of two doses of grass pollen antigen, either 100 IU (Ag100) or 500 IU (Ag500), as described in the experimental protocol. Amin. was measured immediately before, and 2, 5 and 10 minutes after histamine challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve (AUC) was determined. Data are means from 6 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value of Amin. was 0.58±0.07 cm^2. *Significant difference in AUC following histamine challenge after treatment of the nasal airway with antigen, 500 IU, compared to saline control ($p<0.05$, Wilcoxon sign-rank test).

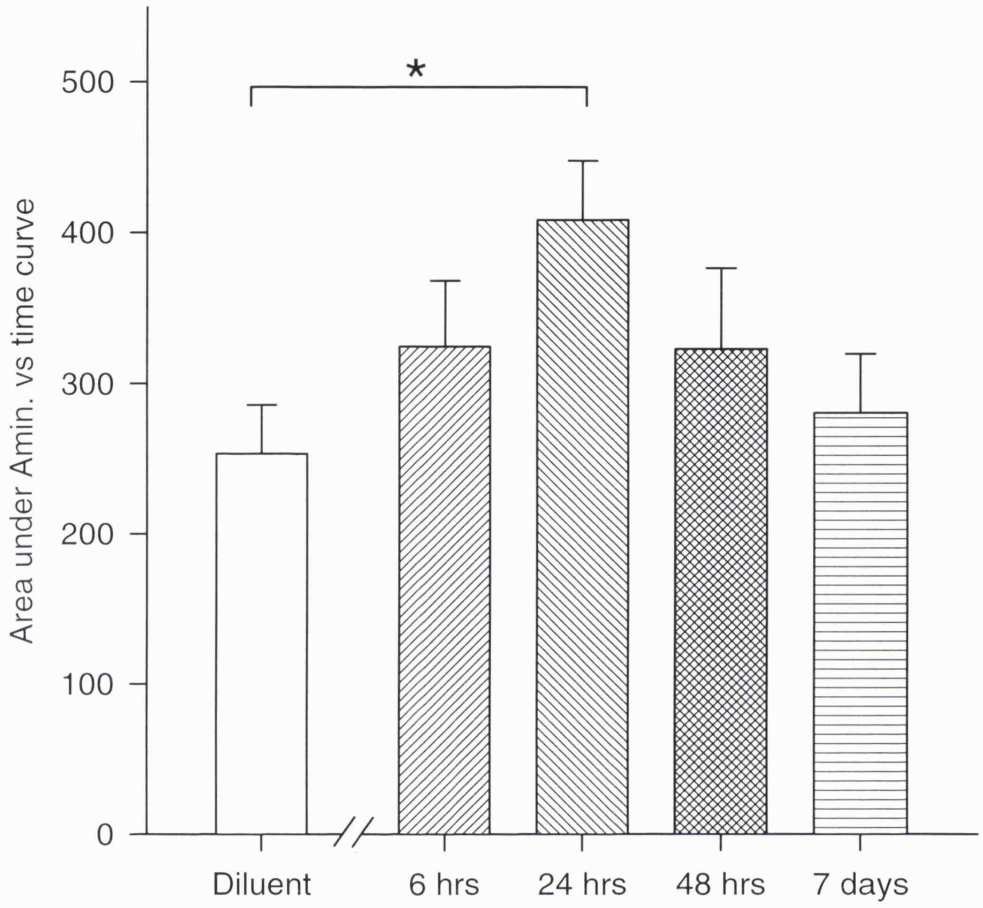

Figure 5.5: Time course for the duration of the hyperresponsiveness induced by grass pollen antigen to histamine, 200 µg. The nasal cavity was pretreated with diluent, and a histamine challenge administered 24 hours later, or with grass pollen antigen, 500 IU, with histamine challenge at 6 hours, 24 hours, 48 hours and 7 days after initial antigen provocation. Amin. was measured immediately before, and 2, 5 and 10 minutes after challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve (AUC) was determined. Data are means from 6 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value of Amin. was 0.67±0.06 cm^2. *Significant difference in AUC following histamine challenge, 24 hours after provocation with antigen, compared to saline control ($p<0.05$, Wilcoxon sign-rank test).

5.3.2 Effect of icatibant on the early phase response to nasal antigen challenge

Figure 5.6 shows the change in the patency of the nasal airways, 15 minutes after challenge with grass pollen antigen, following pretreatment with icatibant, 200 μg, or saline control. Nasal challenge with the diluent control, following pretreatment with saline or icatibant, did not cause a significant change in Amin. from baseline levels ($p>0.05$, Wilcoxon sign-rank test). Administration of grass pollen antigen caused a decrease in the patency of the nasal airways, with 500 IU and 100 IU of antigen causing a decrease in Amin. (from baseline) of 76.5±7.4% and 62.0±4.8%, respectively. These decreases were significantly greater than those following challenge with the diluent control ($p=0.022$, Wilcoxon sign-rank test). Pretreatment of the nasal airway with icatibant did not alter the antigen-induced reduction in nasal patency, at either dose of antigen used ($p>0.05$, Wilcoxon sign-rank test). There were no significant differences in the baseline values of Amin. between treatments ($p>0.05$, Friedman's test).

The absence of an effect of icatibant on the acute response to antigen challenge in seasonal allergic rhinitis is in agreement with the findings of Dear, 1996.

5.3.3 Effect of icatibant on airway hyperresponsiveness following nasal antigen challenge

Challenge with histamine, 200 μg, 24 hours after pretreatment with saline/diluent, caused a significant increase in AUC, corresponding to a decrease in nasal patency ($p=0.022$, Wilcoxon sign-rank test) (Figure 5.7). Pretreatment with icatibant, 200 μg, did not affect the response to histamine, 24 hours after administration of the diluent control ($p>0.05$, Wilcoxon sign-rank test).

Administration of grass pollen antigen, 500 IU, significantly potentiated the response to histamine challenge 24 hours later, compared to the saline/diluent control ($p=0.014$, Wilcoxon sign-rank test). This increase was abolished by treating the nasal airway with icatibant, 200 μg, every two hours for eight hours following antigen provocation ($p=0.03$, Wilcoxon sign-rank test). There were no significant differences in the baseline values of Amin. between treatments ($p>0.05$, Friedman's test).

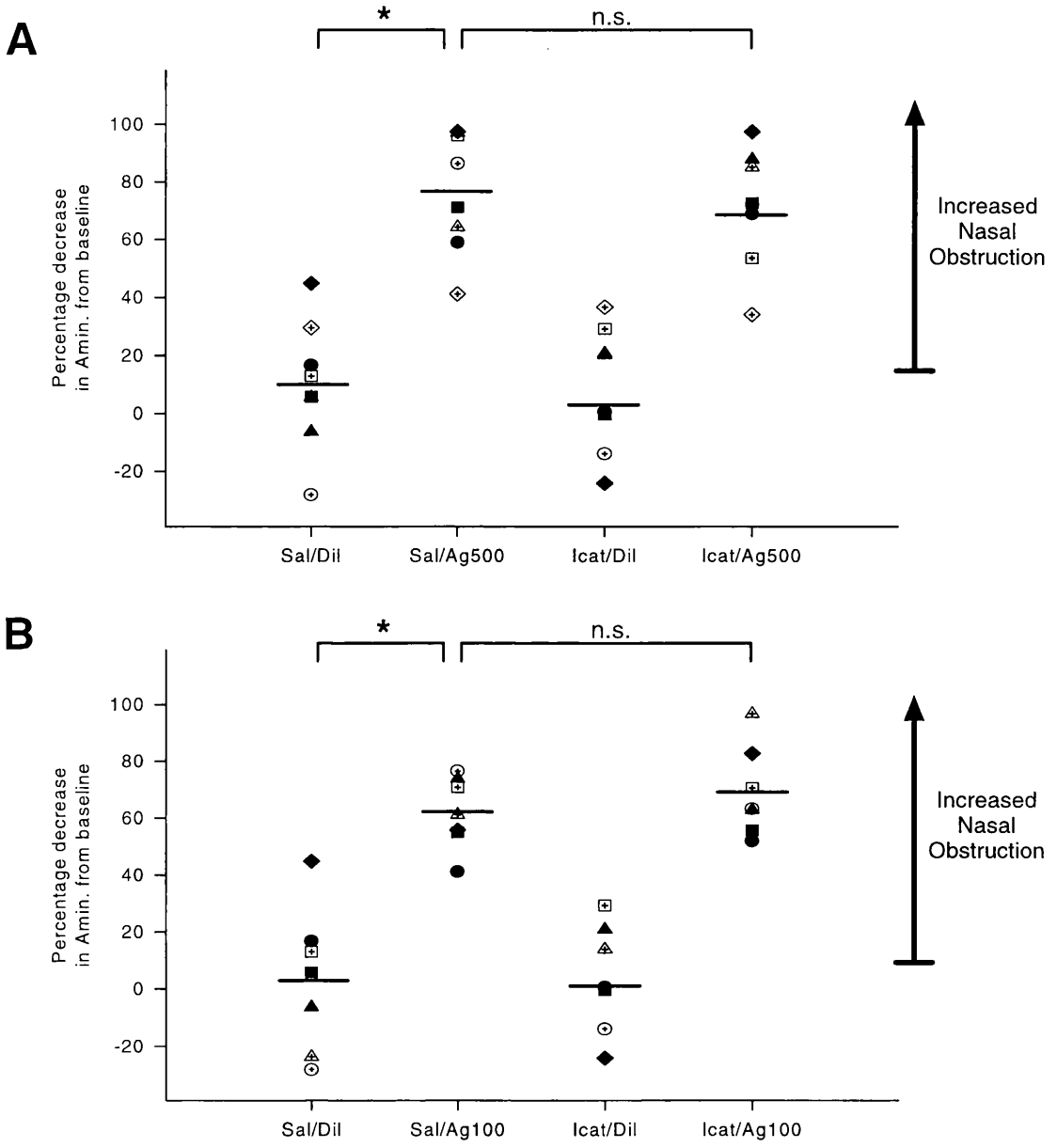

Figure 5.6: The effect of icatibant on nasal blockage induced by grass pollen antigen. The nasal airway was first pretreated with saline (Sal) or icatibant (Icat), 200 µg, following which subjects received a nasal challenge of either antigen or diluent (Dil) as a control. In graph A, the dose of antigen used was 500 IU (Ag500), while in graph B, the dose was 100 IU (Ag100). The Amin. was determined immediately prior to the pretreatment, and again 15 minutes after antigen. Each symbol represents a separate subject, horizontal bars represent the mean. Changes in Amin. were normalised by expressing them as a percentage change from baseline for each subject. The mean ± s.e.mean baseline value of Amin. was 0.56±0.04 cm^2. for graph A and 0.52±0.04 cm^2. for graph B. *Significant decrease in Amin. following antigen challenge, compared to saline control ($p<0.05$, Wilcoxon sign-rank test). n.s. = not significant.

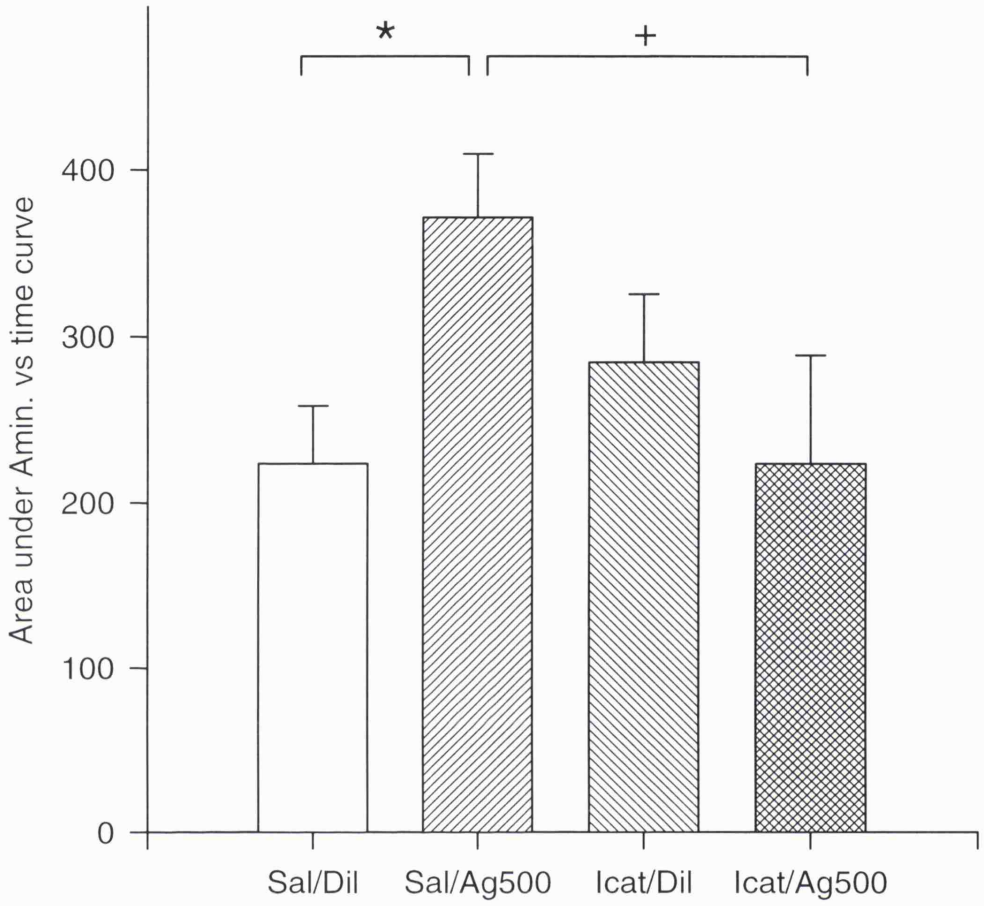

Figure 5.7: Effect of icatibant on the hyperresponsiveness to histamine, 200 µg, induced by grass pollen antigen after 24 hours. The nasal airway was first pretreated with saline (Sal) or icatibant (Icat), 200 µg, following which subjects received a nasal challenge of either grass pollen antigen, 500 IU (Ag500) or diluent (Dil) as a control. Amin. was measured immediately before, and 2, 5 and 10 minutes after histamine challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve (AUC) was determined. Data are means from 8 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value of Amin. was 0.58±0.04 cm^2. *Significant difference in AUC following histamine challenge, 24 hours after pretreatment with saline/Ag500 compared to control ($p<0.05$, Wilcoxon sign-rank test). +Significant difference in AUC following histamine challenge, after pretreatment with saline/Ag500 compared to icatibant/Ag500 ($p<0.05$, Wilcoxon sign-rank test).

5.3.4 Effect of icatibant on antigen-induced changes on nasal cytology

In the following experiments, nine subjects were recruited to the study. While eight received the higher dose (500 IU) of antigen, it was decided to use a lower dose (100 IU) in the ninth subject due to a medical history of asthma in that individual. In order to determine whether these two doses resulted in different effects, in some of the subjects, the experiment was repeated using the lower dose of antigen as well. There were no differences in the cytological profile of the lavage samples taken 6 and 24 hours after saline/diluent provocation, indicating that histamine challenge alone did not affect neutrophil or eosinophil counts. No significant differences were found in baseline values between treatment combinations ($p>0.05$, Friedman's test).

Antigen provocation resulted in an increase in the number of neutrophils (as a percentage of total cells isolated) in lavage samples six hours later, but this did not reach statistical significance. No other changes in neutrophil recruitment were observed (Figure 5.8). However, antigen challenge caused a significant increase in the differential and absolute eosinophil count, at both 6 and 24 hours later, compared to diluent control ($p=0.009$ for all cases, Wilcoxon sign-rank test), as shown in Figures 5.9 and 5.10. Pretreatment with icatibant, 200 µg, in the absence of antigen, did not cause an eosinophilia compared to saline control ($p>0.05$, Wilcoxon sign-rank test). The antigen-induced eosinophilia was still present after pretreatment with icatibant at both timepoints ($p=0.009$ to 0.024, Wilcoxon sign-rank test). However, the eosinophilia, 6 hours after antigen, was significantly less than that induced by antigen in the absence of icatibant pretreatment ($p=0.044$, Wilcoxon sign-rank test).

Figures 5.11 and 5.12 show the changes in nasal cytology following antigen challenge using both 100 IU and 500 IU in the same group of subjects. Antigen caused a significant eosinophilia following challenge, irrespective of the dose used, both with and without icatibant pretreatment ($p=0.006$ to 0.031, Friedman's test). Furthermore, the eosinophilia induced by 500 IU of antigen was comparable to that observed following provocation with 100 IU of antigen. The results confirm the inhibitory action of icatibant on the antigen-induced eosinophilia, 6 hours after exposure to antigen ($p=0.017$ to 0.05, Wilcoxon sign-rank test). There were no significant correlations between the magnitude of the nasal response to histamine and any cytological marker in nasal lavage fluid.

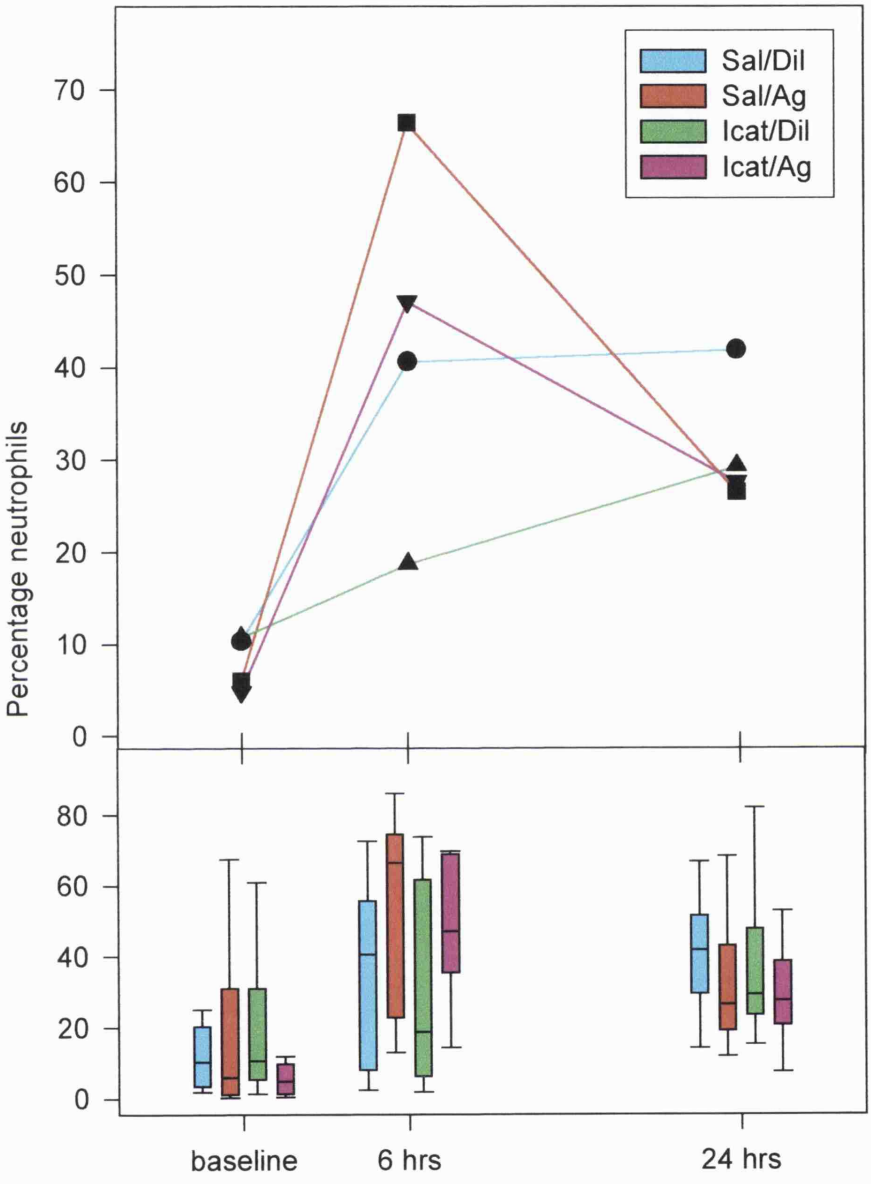

Figure 5.8: The change in the percentage neutrophils isolated from nasal lavage fluid, following treatment of the nasal cavity with saline (Sal) or icatibant (Icat), 200 µg, and grass pollen antigen (Ag) or diluent (Dil), as described in the experimental protocol. Data are medians from 9 subjects. The bottom window indicates the interquartile range of values for each median, with the horizontal line indicative of the median value. Vertical bars represent the 80% central range of values.

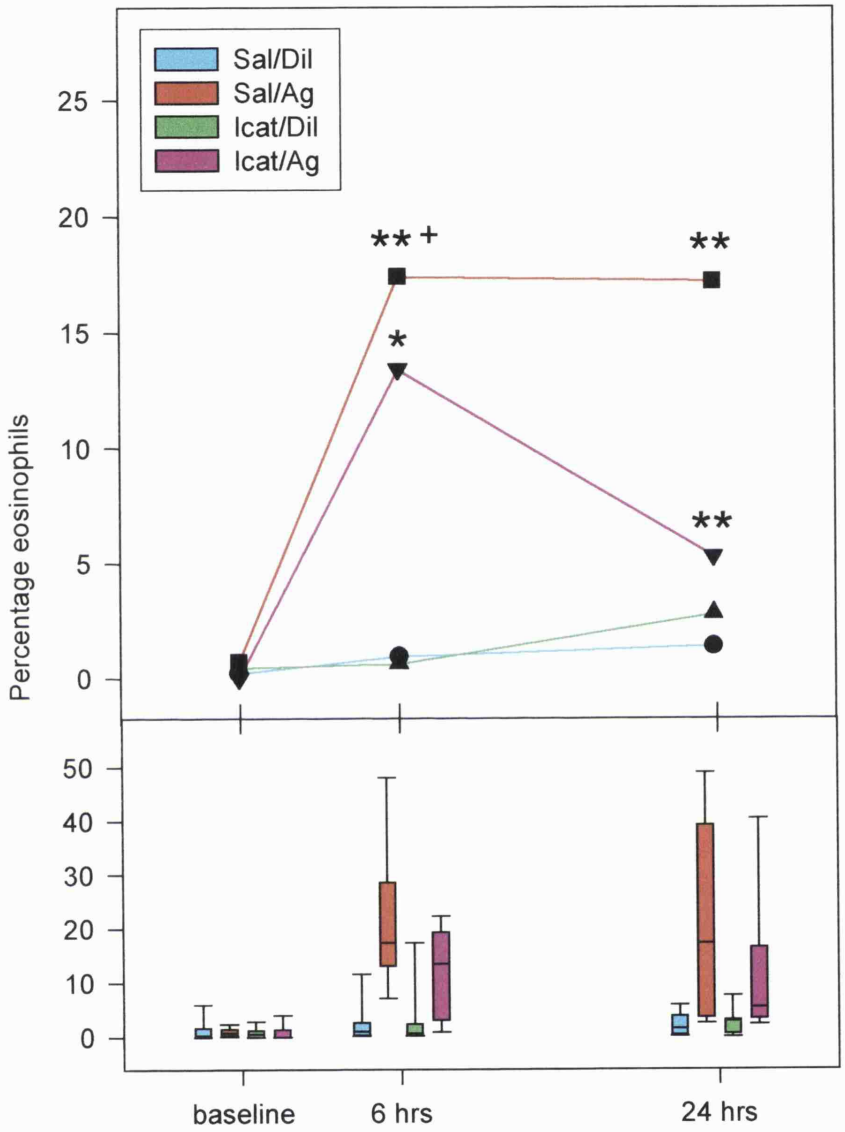

Figure 5.9: The change in the percentage eosinophils isolated from nasal lavage fluid, following treatment of the nasal cavity with saline (Sal) or icatibant (Icat), 200 µg, and grass pollen antigen (Ag) or diluent (Dil), as described in the experimental protocol. Data are medians from 9 subjects. The bottom window indicates the interquartile range of values for each median, with the horizontal line indicative of the median value. Vertical bars represent the 80% central range of values. */**Significant increase in the percentage of eosinophils isolated for the treatment shown, compared to treatment with saline/diluent control (*$p<0.05$, **$p<0.01$, Wilcoxon sign-rank test). +Significant difference in percentage of eosinophils isolated following treatment with saline/antigen, compared to icatibant/antigen ($p<0.05$, Wilcoxon sign-rank test).

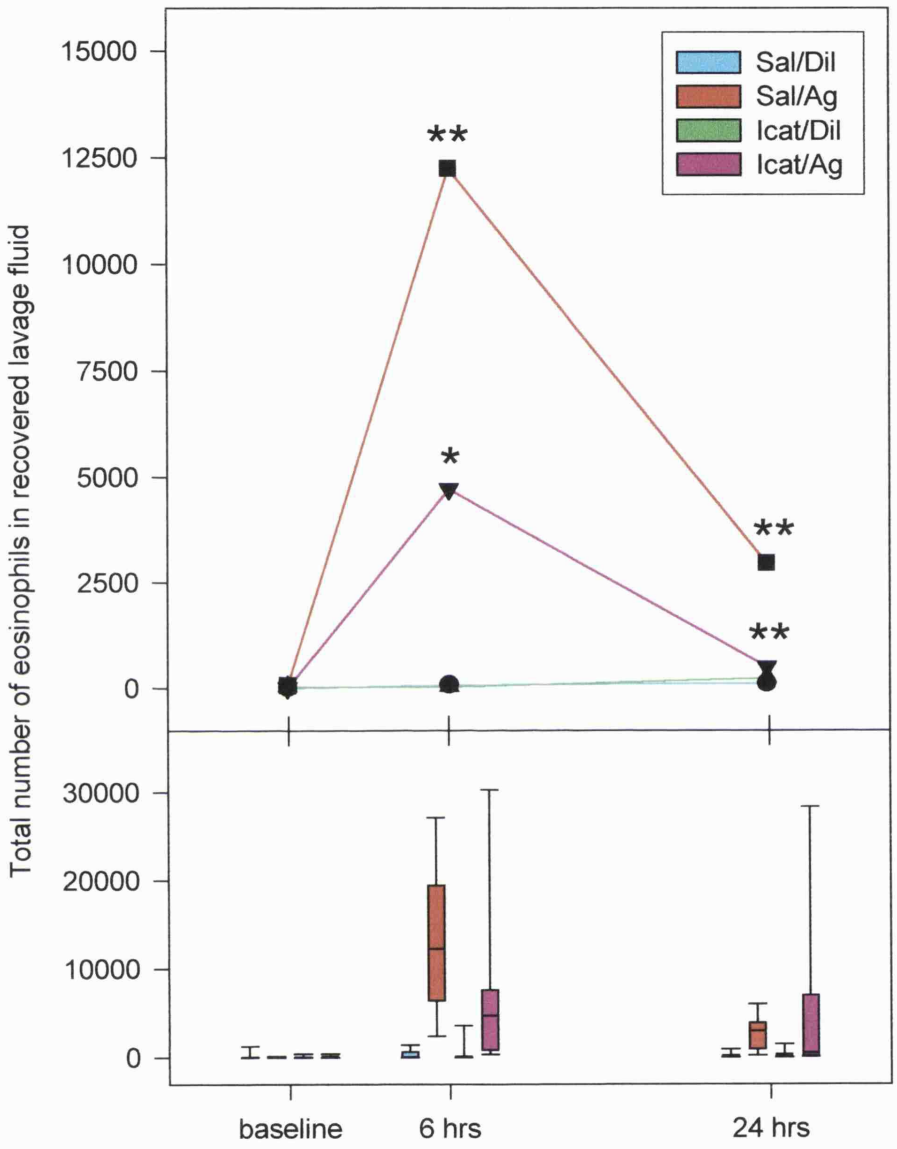

Figure 5.10: The change in the total number of eosinophils isolated in the recovered nasal lavage fluid, following treatment of the nasal cavity with saline (Sal) or icatibant (Icat), 200 µg, and grass pollen antigen (Ag) or diluent (Dil), as described in the experimental protocol. Data are medians from 9 subjects. The bottom window indicates the interquartile range of values for each median, with the horizontal line indicative of the median value. Vertical bars represent the 80% central range of values. */**Significant increase in the total number of eosinophils isolated for the treatment shown, compared to treatment with saline/diluent control (*$p<0.05$, **$p<0.01$, Wilcoxon sign-rank test).

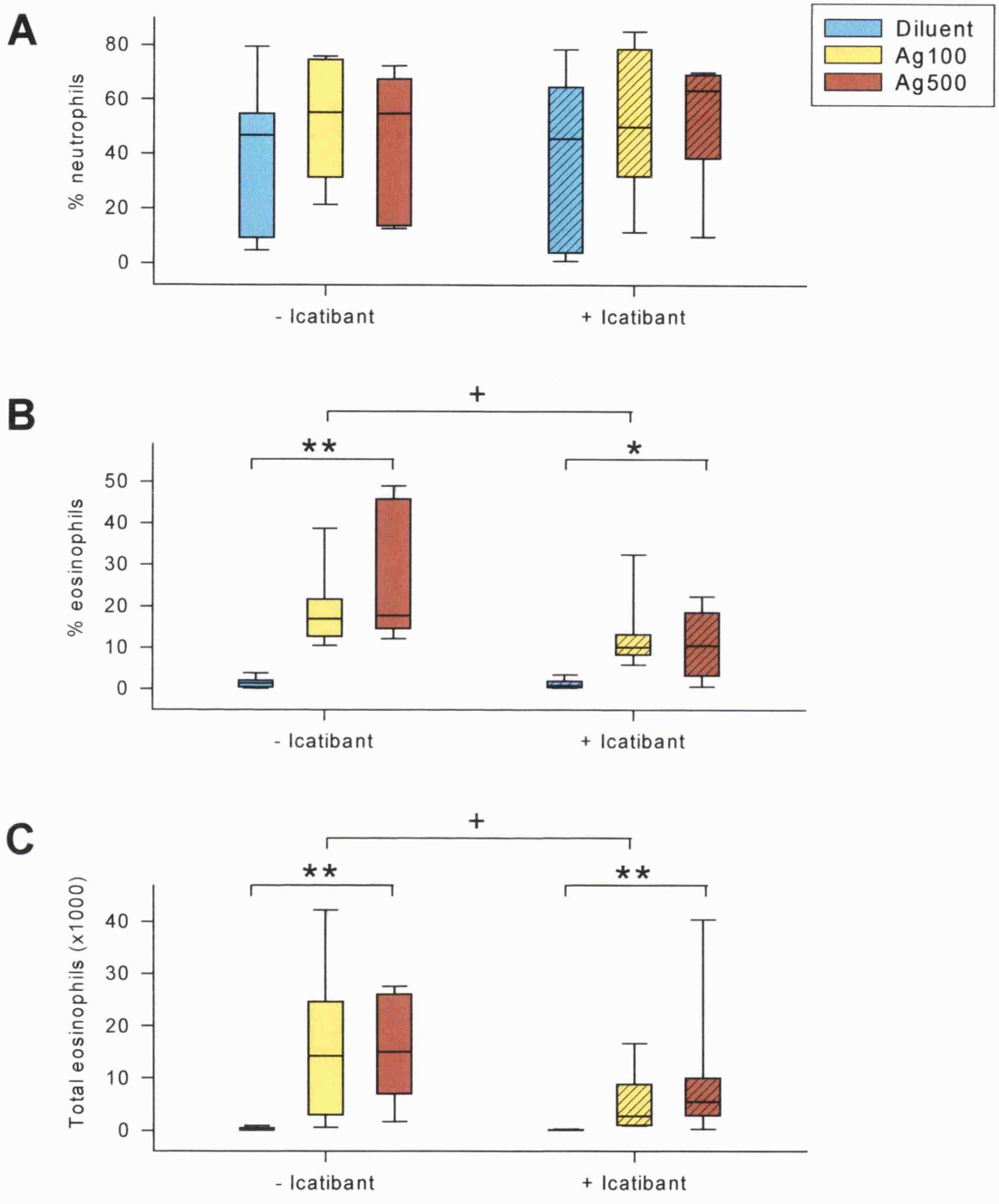

Figure 5.11: Changes in nasal cytology, six hours after treatment of the nasal cavity with saline or icatibant, 200 µg, and grass pollen antigen, 500 IU (Ag500) or 100 IU (Ag100), or diluent, as described in the experimental protocol. The changes shown are percentage neutrophils (graph A), percentage eosinophils (graph B) and total number of eosinophils in lavage sample (graph C). Data are medians from 6 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values. */**Significant increase in the cytological marker shown with increasing dose of antigen (*$p<0.05$, **$p<0.01$, Friedman's test). +Significant difference in cytological marker shown following antigen treatment with or without icatibant ($p<0.05$, Wilcoxon sign-rank test).

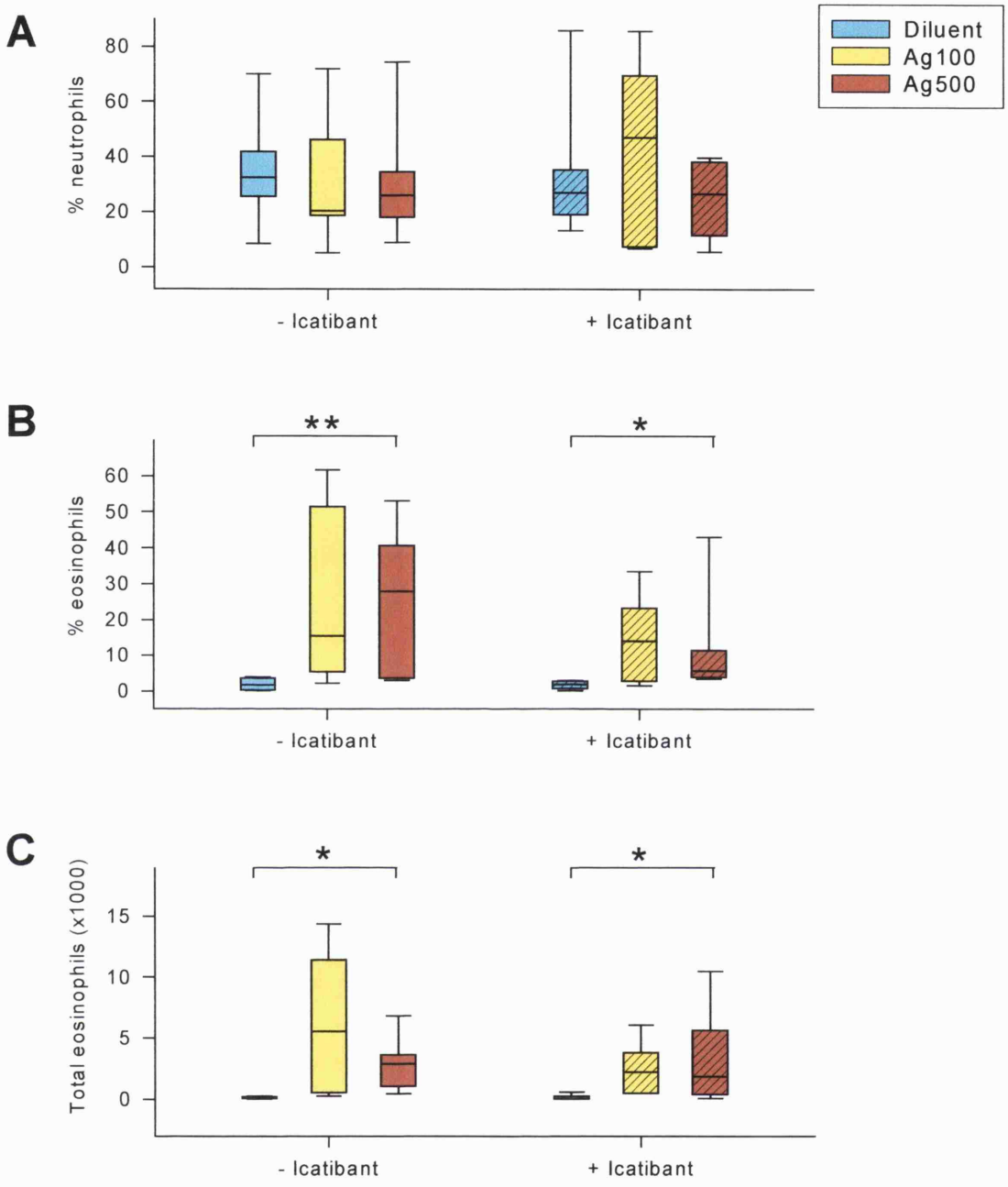

Figure 5.12: Changes in nasal cytology, twenty four hours after treatment of the nasal cavity with saline or icatibant, 200 µg, and grass pollen antigen, 500 IU (Ag500) or 100 IU (Ag100), or diluent, as described in the experimental protocol. The changes shown are percentage neutrophils (graph A), percentage eosinophils (graph B) and total number of eosinophils in lavage sample (graph C). Data are medians from 6 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values. */**Significant increase in the cytological marker shown with increasing dose of antigen (*$p<0.05$, **$p<0.01$, Friedman's test).

5.3.5 Effect of icatibant on antigen-induced changes in mediator release

Challenge with grass pollen antigen increased the levels of ECP (Figure 5.13) and kinin (Figure 5.14) detected in nasal lavage six hours later, compared to the diluent control ($p=0.044$ and 0.009 respectively, Wilcoxon sign-rank test). Following pretreatment with icatibant, antigen failed to induce a significant rise in ECP or kinins ($p>0.05$, Wilcoxon sign-rank test). Unfortunately, the levels of IL-5 in nasal lavage fluid were below the limit of detection of the assay used. However, provocation with antigen caused a significant increase in the IL-8 content of the lavage samples, compared to the diluent control ($p=0.033$, Wilcoxon sign-rank test) (Figure 5.15). Pretreatment with icatibant significantly reduced the antigen-induced increase in IL-8 release ($p=0.018$, Wilcoxon sign-rank test). Icatibant had no effect on the levels of these mediators in the absence of antigen ($p>0.05$, Wilcoxon sign-rank test). In addition, there were no differences in the baseline levels of these mediators, between treatment occasions ($p>0.05$, Friedman's test).

Figure 5.16 displays the effect of different doses of antigen, with or without icatibant, on ECP, kinin and IL-8 levels in nasal lavage fluid. No major differences were identified in the ranges of these mediators between the two doses of antigen used. Icatibant significantly reduced the ECP content of nasal lavage fluid following antigen administration ($p=0.045$, Wilcoxon sign-rank test).

A number of significant correlations were observed between neutrophil or eosinophil counts, and the increase in levels of ECP, kinins or IL-8 in the nasal lavage fluid samples collected six hours after the start ($p<0.05$, Spearman's Rank test):

	% neutrophils	% eosinophils	Total no. eosinophils
ECP	$r_s=0.716$	$r_s=0.728$	$r_s=0.884$
Kinin	$r_s=0.587$	$r_s=0.507$	$r_s=0.665$
IL-8	$r_s=0.612$	$r_s=0.543$	$r_s=0.703$

Furthermore, significant correlations were also identified between the levels of ECP, kinin and IL-8 in the lavage samples ($p<0.01$, Spearman's Rank test):

	ECP	Kinin
Kinin	$r_s=0.789$	
IL-8	$r_s=0.840$	$r_s=0.862$

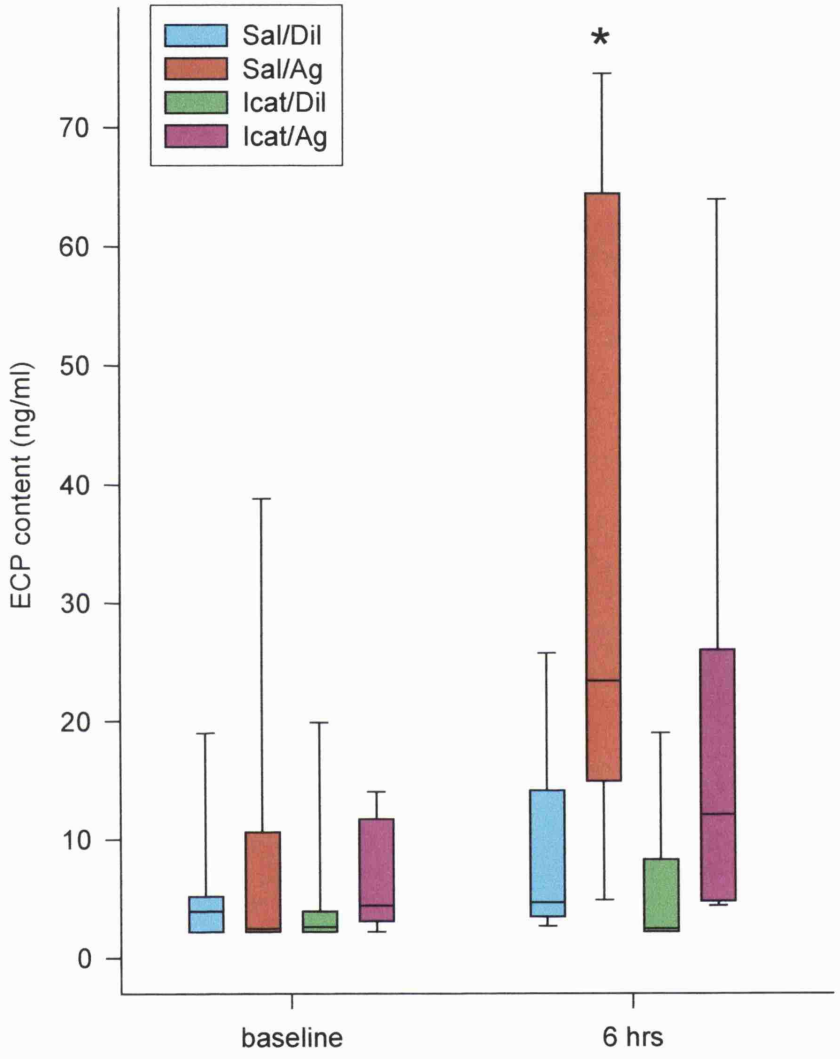

Figure 5.13: The change in ECP content of nasal lavage fluid following treatment of the nasal cavity with saline (Sal) or icatibant (Icat), 200 µg, and grass pollen antigen (Ag) or diluent (Dil), as described in the experimental protocol. Data are medians from 9 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values. *Significant increase in ECP content following antigen administration, compared to diluent control ($p<0.05$, Wilcoxon sign-rank test).

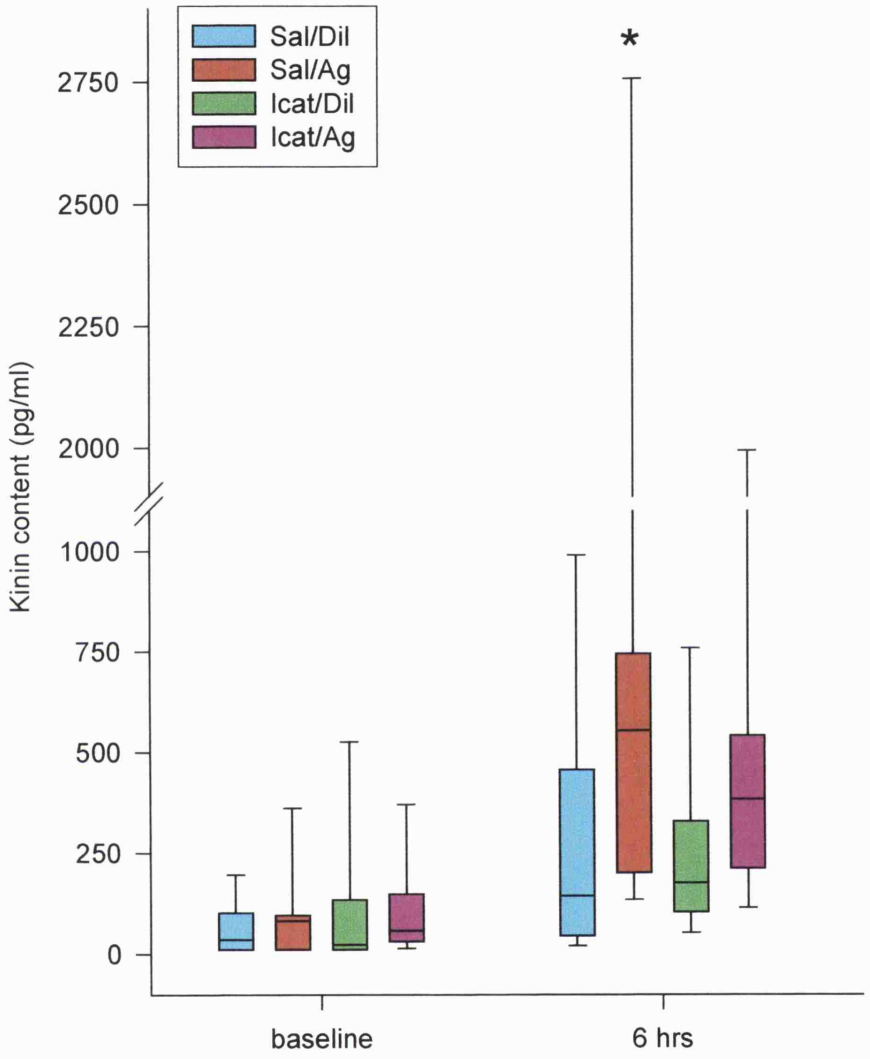

Figure 5.14: The change in kinin content of nasal lavage fluid following treatment of the nasal cavity with saline (Sal) or icatibant (Icat), 200 µg, and grass pollen antigen (Ag) or diluent (Dil), as described in the experimental protocol. Data are medians from 9 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values. *Significant increase in kinin content following antigen administration, compared to diluent control ($p<0.05$, Wilcoxon sign-rank test).

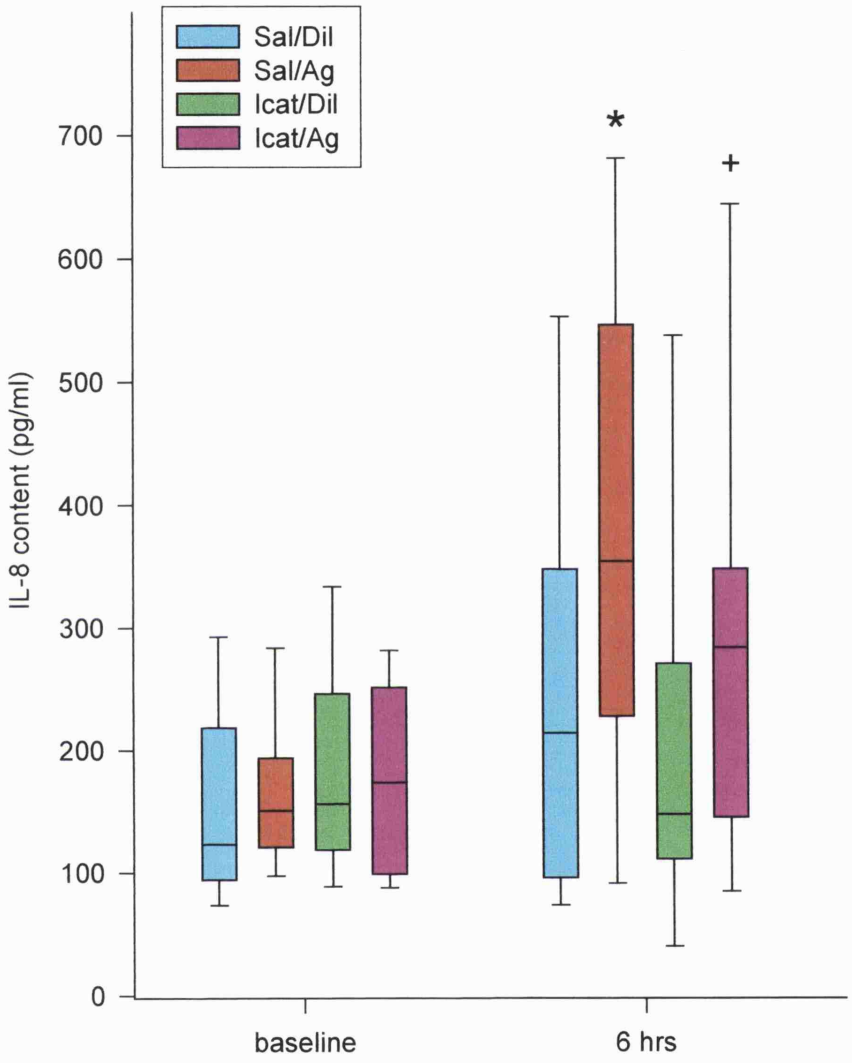

Figure 5.15: The change in IL-8 content of nasal lavage fluid following treatment of the nasal cavity with saline (Sal) or icatibant (Icat), 200 µg, and grass pollen antigen (Ag) or diluent (Dil), as described in the experimental protocol. Data are medians from 9 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values. *Significant increase in kinin content following antigen administration, compared to diluent control ($p<0.05$, Wilcoxon sign-rank test). +Significant difference in IL-8 content following treatment with saline/antigen, compared to icatibant/antigen ($p<0.05$, Wilcoxon sign-rank test).

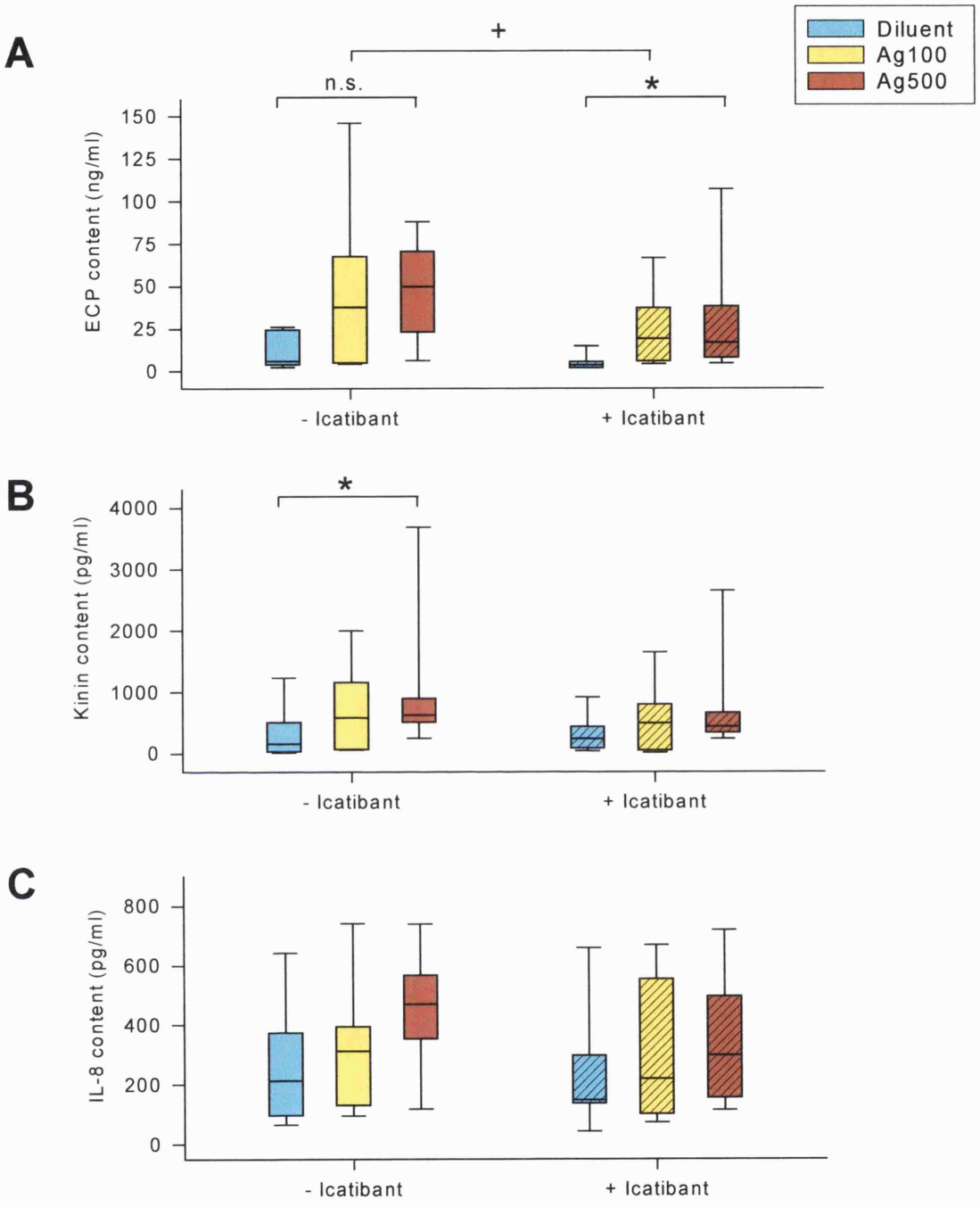

Figure 5.16: Changes in the levels of ECP (graph A), kinin (graph B) and IL-8 (graph C) in nasal lavage fluid, six hours after treatment of the nasal cavity with saline or icatibant, 200 µg, and grass pollen antigen, 500 IU (Ag500) or 100 IU (Ag100), or diluent, as described in the experimental protocol. Data are medians from 6 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values. *Significant increase in mediator shown with increasing dose of antigen ($p<0.05$, Friedman's test). +Significant difference in ECP levels following antigen treatment with or without icatibant ($p<0.05$, Wilcoxon sign-rank test). n.s. = not significant.

5.4 Discussion

Nasal challenge with grass pollen antigen, in sensitive subjects, resulted in the development of an AHR to histamine, present 24 hours after exposure to antigen. Antigen challenge also caused a nasal eosinophilia and increased the level of kinins, ECP and IL-8 in nasal lavage samples collected six hours after challenge. Unfortunately, the IL-5 in the lavage samples was below the limit of detection for the assay used. A number of other studies have also failed to detect IL-5 in nasal lavage fluid, after a single antigen challenge (Terada *et al.*, 1994; Proud *et al.*, 1998), though IL-5 can be measured in nasal secretions or concentrated nasal lavage fluid (Sim *et al.*, 1994; Dunnette *et al.*, 1997). For medical reasons, one subject was given a lower dose of antigen in the experiments which investigated nasal cytology and mediator release. Nonetheless, this does not appear to have affected the overall results obtained.

The antigen-induced AHR was abolished by pretreatment with icatibant, a bradykinin B_2 receptor antagonist. This implies that the AHR, induced by a single antigen challenge in subjects sensitive to grass pollen, is dependent upon kinin generation, and the subsequent activation of a kinin-dependent pathway. This hypothesis is further supported by the observation that antigen increased the release of kinins into the nasal cavity, an effect which was reduced by icatibant, which is known to inhibit kinin production in the human nasal cavity (Dear *et al.*, 1996b). It was not possible to distinguish between the relative roles of bradykinin and kallidin in the development of AHR, since the assay used was equally sensitive to both peptides.

The data also suggests that the generation of kinin is an early event in the mechanism causing AHR, since pretreatment with icatibant for the first 8 hours prevented the AHR, 24 hours later. Interestingly, bradykinin is one of the few mediators released in both the early and late allergic reactions. Furthermore, while systemic steroids inhibit mediator release associated with the late phase response of allergic rhinitis, they do not affect the release of mediators in the early phase, with the exception of kinins (Naclerio, 1988). Therefore, the ability of steroids to prevent antigen-induced AHR may be dependent on the inhibition of kinin production during the immediate allergic response.

It is unclear whether the AHR was dependent upon the activation of bradykinin B_2 receptors or, indeed, whether receptor activation was involved at all. While bradykinin B_1 receptors are expressed on nasal epithelial cells of subjects with untreated, active allergic rhinitis, they are not found in the normal, non-inflammed nasal airway (Woessner & Zuraw, 1998). Moreover, it is unlikely that B_1 receptors are present in atopics outside the pollen season, or that antigen could cause a significant upregulation of B_1 receptors in the timescale used in this experiment. There is little evidence to suggest that AHR is caused by the induction of B_1 receptors in perennial allergic rhinitis (Reynolds *et al.*, 1999). The ability of icatibant to prevent the AHR may have been due to an anti-kallikrein effect, rather than an action at bradykinin B_2 receptors. However, while icatibant did reduce kinin levels following antigen challenge, detectable levels of kinin were still present even after icatibant pretreatment. This implies that activation of bradykinin B_2 receptors is likely to be an important step in the development of antigen-induced AHR in the human nasal airway.

Icatibant inhibits the early phase response in perennial allergic rhinitis (using house dust mite as the allergen) (Austin *et al.*, 1994; Dear *et al.*, 1996b). However, in this study, icatibant failed to reduce the nasal obstruction induced by antigen in subjects with seasonal allergic rhinitis, confirming previous findings (Akbary and Bender, 1993; Dear, 1996). Therefore, it might have been prudent to use subjects sensitive to the house dust mite allergen to investigate antigen-induced AHR in the human nasal airway. Furthermore, it is been suggested that perennial allergic rhinitis may be more representative of chronic allergic airway disease, in which AHR has an important pathological role (van Wijk, 1998). However, the aim of this experiment was not to investigate the action of kinins in chronic allergy, but rather, their role in the development of antigen-induced AHR. Therefore, subjects with seasonal allergic rhinitis were used out of the pollen season, for a number of reasons. First, the induction of AHR may be dependent on the occurrence of an early phase allergic response (van Wijk *et al.*, 1992). Abolishing the early phase reaction, in subjects sensitive to house dust mite, might have affected the development, and subsequent pharmacological modulation, of the AHR. Second, subjects with seasonal allergic rhinitis are less likely to be using medication, out of season, which could interfere with the study. Finally, it would have been difficult to control for natural allergen exposure in subjects with perennial allergic rhinitis, in whom a pre-existing degree of AHR would probably be present. In contrast, the use of subjects sensitive to grass pollen

provides a model in which the allergic response can be measured from a resting state, with little evidence of pre-existing allergic inflammation (as confirmed by the lack of eosinophils in nasal lavage fluid following administration of the diluent control).

The mechanisms underlying the development of AHR following single antigen challenge may be different from those causing AHR in the chronic allergic state, following multiple exposure to antigen. Furthermore, there are differences in the underlying pathophysiology of perennial and seasonal allergic rhinitis, and this could include the process by which AHR is induced. Caution is therefore required in extrapolating the findings presented in this chapter to the mechanisms underlying the development of AHR in allergic rhinitis, although it is likely that kinins have a role.

Icatibant also significantly reduced the antigen-induced nasal eosinophilia. For example, the differential eosinophil count was reduced by an average of 30.7±10.2%. This result compares favourably with the anti-eosinophil activity of second generation, histamine H_1 receptor antagonists, such as cetirizine, terfenadine and azelastine (Klementsson *et al.*, 1990; Wang *et al.*, 1997). The ability of cetirizine to oppose eosinophil recruitment appears to be a direct effect on eosinophils (Leprevost *et al.*, 1988), possibly by inhibiting the adherence of eosinophils to endothelial cells (Kyan-Aung *et al.*, 1992). However, the experiments in chapter 4 provide no evidence for a similar action of icatibant. A number of different bradykinin receptor antagonists and inhibitors of tissue kallikrein reduce antigen-induced eosinophilia in the lower airway of the guinea pig (Farmer *et al.*, 1992; Evans *et al.*, 1996). This implies that the fall in the antigen-induced eosinophilia, after pretreatment with icatibant, is probably the result of an inhibition of kinin generation following antigen challenge.

The ability of icatibant to abolish the antigen-induced AHR could have been a consequence of the decrease in eosinophil recruitment. However, antigen-induced AHR can occur without a change in eosinophil recruitment or activation (Andersson *et al.*, 1989; Klementsson *et al.*, 1991). This will be discussed further in chapter 8. Although neutrophils have also been implicated in the development of AHR (Anticevich *et al.*, 1996), there is no evidence for this in the current study, confirming similar results, *in vivo*, in the lower human airway (Frangova *et al.*, 1996). Icatibant also reduced the antigen-induced increase in ECP levels in nasal lavage fluid. This may have been the result of an inhibition of eosinophil activation or, more likely, a

consequence of the reduction in eosinophil recruitment into the nasal mucosa. The decrease in ECP release may have reduced kinin generation (if kinins are produced via a process dependent on cationic proteins, as described in chapter 3), but the current data cannot confirm this.

Therefore, the data provide evidence for a role for kinins in the recruitment of inflammatory cells. This may be due to the kinin-mediated release of cytokines which can potentiate this process, as discussed in chapter 4. Bradykinin stimulates the release of a number of cytokines in the rat hind-paw model of inflammation, including TNF-α, IL-1β and IL-8 (Ferreira *et al.*, 1993), and the release of TNF-α and IL-1 from human macrophages *in vitro* (Tiffany & Burch, 1989). These cytokines are also released following antigen challenge in allergic rhinitis (Sim *et al.*, 1995). Both TNF-α and IL-1 increase the expression of the adhesion molecule ICAM-1 on human airway epithelial cells (Tosi *et al.*, 1992). Also, the expression of ICAM-1 and VCAM-1 is upregulated in allergic rhinitis (Bradding *et al.*, 1993), Therefore, icatibant may have decreased the production of kinins which stimulate the release of cytokines and, in turn, upregulate the expression of adhesion molecules. The upregulation of ICAM-1 expression by cytokines can occur within hours on endothelial cells (Wheller & Perretti, 1997), so such a mechanism would be feasible in the timescale of this experiment.

Alternatively, the anti-eosinophil action of icatibant may have been the result of a reduction in the kinin-dependent release of IL-8. Bradykinin stimulates the release of a range of chemotactants for eosinophils and neutrophils, including IL-8, from alveolar macrophages isolated from atopic individuals (Sato *et al.*, 1996). Recently, it was found that bradykinin stimulates IL-8 production in cultured human airway muscle cells (Pang and Knox, 1998) and airway epithelial cells (Koyama *et al.*, 1998). In addition, the expression of mRNA for IL-8 is increased in subjects with active allergic rhinitis (KleinJan *et al.*, 1999). IL-8 is a potent stimulus of neutrophil recruitment *in vivo* and *in vitro* (Peveri *et al.*, 1988), and can also act as a stimulus for eosinophil recruitment (Shute, 1994). For example, the high expression of IL-8 in nasal polyps tissue may explain the local eosinophilia associated with this condition (Allen *et al.*, 1997). Douglass *et al.* (1994) reported that administration of human recombinant IL-8 into the nasal cavity of both normal and atopic subjects induced both a significant neutrophilia and eosinophilia. However, the eosinophilia observed was very mild (up to about 2-3% of total cells in nasal smears), generally limited to certain atopic

individuals, and only significant when analysed over all time points (Douglass *et al.*, 1994). IL-8 probably acts in conjunction with other factors to cause the inflammatory cell recruitment seen after antigen challenge (Gosset *et al.*, 1997).

Interestingly, icatibant significantly reduced levels of IL-8 in nasal lavage fluid obtained after antigen challenge. Furthermore, there was a high correlation between the levels of kinins and IL-8 in the lavage samples. Therefore, the kinin generated following antigen challenge may have released IL-8 which could, in turn, contribute to eosinophil recruitment. The removal of this component, by icatibant, could explain the observation that icatibant reduced, but did not abolish, the antigen-induced nasal eosinophilia. Human eosinophils can produce IL-8 (Simon *et al.*, 1995; Yousefi *et al.*, 1995), so the decrease in IL-8 may have been a consequence of the reduction in eosinophil recruitment by icatibant. However, IL-8 is also synthesised by a number of other cells, including monocytes (Peveri *et al.*, 1988), mast cells (Moller *et al.*, 1993), neutrophils (Yousefi *et al.*, 1995), endothelial cells (Compton *et al.*, 1998) and airway epithelial cells (Calderon *et al.*, 1997). In particular, nasal epithelial cells secrete high levels of interleukin-8 (Calderon *et al.*, 1997), so it is possible that the reduction in IL-8 levels was independent of the decrease in eosinophil recruitment.

Although IL-8 is a potent neutrophil chemotactant, icatibant did not reduce the recruitment of neutrophils following antigen challenge. It has been proposed that IL-8 may not be a major stimulus of neutrophil accumulation in allergic inflammation (Shute, 1994). Neutrophils are more abundant than eosinophils in the peripheral circulation, and their recruitment is stimulated by a number of different mechanisms (Walsh *et al.*, 1993). In contrast, the selective recruitment of eosinophils in allergy may be a consequence of a VCAM-1/VLA-4 interaction, which is modulated by only a few cytokines, such as IL-4 (Walsh *et al.*, 1993). Therefore, a reduction in IL-8-mediated cell recruitment may not affect the overall degree of neutrophil infiltration. Alternatively, the priming of eosinophils can alter their chemotactic response. For example, priming eosinophils with IL-5 significantly increases the recruitment of eosinophils, but not neutrophils, to a range of stimuli (Sehmi *et al.*, 1992). A number of cytokines potentiate the chemotactic response of eosinophils to IL-8, including GM-CSF and IL-3 (Warringa *et al.*, 1991), IL-4 (Villar *et al.*, 1993) and IL-5 (Sehmi *et al.*, 1993). Therefore, the priming of eosinophils, by cytokines released during the allergic response, may explain the preferential action of IL-8 on eosinophils in allergic inflammation.

While there is no evidence for a link between IL-8 and AHR in man, IL-8 induces an AHR in the lower airways of the guinea pig (Medhurst *et al.*, 1991; Fujimura *et al.*, 1997). The AHR was not associated with the infiltration of eosinophils or neutrophils, but was abolished by pretreatment with thromboxane A_2 receptor antagonists (Fujimura *et al.*, 1997). Theoretically, icatibant may have prevented the antigen-induced AHR by inhibiting the release of IL-8. However, this is unlikely, since levels of IL-8 in nasal lavage fluid remained elevated even after icatibant pretreatment. It is more plausible that the decrease in IL-8 might contribute to the prevention of the AHR by reducing inflammatory cell recruitment and/or activation. Interestingly, a number of different mediators are generated during natural and experimentally-induced rhinovirus colds, including kinins (Naclerio *et al.*, 1988; Proud *et al.*, 1990) and IL-8 (Noah *et al.*, 1995; Grunberg *et al.*, 1997). Furthermore, the increase in bronchial hyperresponsiveness observed in rhinovirus-induced exacerbations of asthma is associated with an increase in IL-8 (Grunberg *et al.*, 1997). This provides further evidence for an interaction between kinins, IL-8 and the development of AHR.

The nasal hyperresponsiveness induced by PAF is also associated with eosinophil recruitment, the release of kinins and ECP, and the activation of bradykinin B_2 receptors (chapters 3 and 4). PAF is released following nasal antigen challenge in subjects with seasonal allergic rhinitis (Miadonna *et al.*, 1989; Shin *et al.*, 1994). Therefore, the AHR following antigen exposure could be the result of the release of PAF. However, antigen-induced AHR in the human nasal airway was not abolished by the PAF antagonists UK 74,505 (Kuitert *et al.*, 1993) or WEB 2086 (Shin *et al.*, 1994). Furthermore, the novel PAF antagonist SR 27417A, which exhibits a high potency at the PAF receptor on human polymorphonuclear leukocytes (Herbert *et al.*, 1993), was ineffective against nasal symptoms in subjects with combined asthma and allergic rhinitis to grass pollen (M. Cluzel, personal communication). These findings imply that the development of antigen-induced AHR, in the human nasal airway, is not dependent on the production of PAF. Unfortunately, it was not possible to obtain a PAF antagonist, suitable for use in human subjects, to confirm this.

In summary, this chapter has provided evidence for the involvement of kinins, and the bradykinin B_2 receptor, in the development of antigen-induced AHR and the associated eosinophilia in the human nasal airway. These processes may, in part, be dependent upon the kinin-mediated release of inflammatory cytokines, such as IL-8.

Summary

- Nasal provocation with grass pollen antigen, 500 IU, caused a significant decrease in nasal patency. This obstruction was not altered by pretreatment with icatibant, 200 µg.

- A single administration of grass pollen antigen, 500 IU, induced a hyperresponsiveness to histamine in the nasal airways of sensitive subjects. This hyperresponsiveness was present 24 hours after antigen exposure.

- Pretreatment with icatibant, 200 µg, during the first 8 hours after antigen challenge, abolished the antigen-induced hyperresponsiveness.

- Icatibant also significantly reduced the antigen-induced nasal eosinophilia, six hours after antigen challenge.

- Antigen challenge caused an increase in the levels of kinin, ECP and IL-8 in nasal lavage samples, obtained 6 hours after challenge. The levels of these mediators were significantly reduced if the nasal airway was first pretreated with icatibant.

- The data indicate a role for kinins in the development of AHR, but not in the early phase allergic response, following exposure to grass pollen antigen in subjects with seasonal allergic rhinitis.

CHAPTER 6

THE CONTRIBUTION OF NEUROPEPTIDES TO HYPERRESPONSIVENESS IN THE HUMAN NASAL AIRWAY

6.1 Introduction

The results presented in chapters 3 and 5 indicate a role for kinins, acting on the bradykinin B_2 receptor, in the development of both antigen- and PAF-induced hyperresponsiveness (AHR) in the human nasal airway.

One action of bradykinin is to cause the release of the tachykinins, substance P (sub P) and neurokinin A (NK-A), from sensory nerves in a variety of tissues, and these may mediate some of the actions of bradykinin (Geppetti, 1993). For example, antagonists at the tachykinin NK_1 receptor inhibit bradykinin-mediated extravasation in the lower airway of guinea pigs (Nakajima *et al.*, 1994) and bradykinin-induced bronchoconstriction in man (Ichinose *et al.*, 1992). In the human nasal airway, challenge with bradykinin causes the release of immunoreactive substance P (Baumgarten *et al.*, 1997). Both PAF and antigen cause an AHR in the human nasal airway, which is abolished by bradykinin B_2 receptor antagonists. The release of tachykinins from airway sensory nerves *in vitro*, by bradykinin, is mediated via the bradykinin B_2 receptor (Miura *et al.*, 1992). Therefore, kinins generated following administration of PAF or antigen may cause AHR by a tachykinin-dependent mechanism. Indeed, it has been demonstrated that the AHR induced by cationic proteins in the rat lower airway, a process which is kinin-dependent, is abolished by NK_1 receptor antagonists (Coyle *et al.*, 1994).

NK-A induces AHR in the lower airways of rats (Chiba & Misawa, 1995), sheep (Abraham *et al.*, 1991) and monkeys (Tamura *et al.*, 1989). In the latter two studies,

no AHR was observed following treatment with sub P, although it has been reported that sub P increases the response to methacholine challenge in the lower airways of asthmatics (Cheung *et al.*, 1995). Antagonists at the tachykinin receptors inhibit both antigen- and PAF-induced AHR in a number of animal models, although it is not clear which receptor sub-type is involved (Perretti *et al.*, 1995; Mizuguchi *et al.*, 1996; Schuiling *et al.*, 1999).

Further evidence for the involvement of tachykinins in AHR can be found in studies investigating the effect of capsaicin on AHR. Capsaicin, the pungent component of chilli peppers, prevents the release of neuropeptides from "capsaicin-sensitive" sensory nerves. Application of capsaicin to the lower airway in the guinea pig abrogates antigen-induced AHR (Ladenius & Biggs, 1989; Matsuse *et al.*, 1991). Although capsaicin abolished the AHR in the latter study, it did not affect the antigen-induced eosinophilia, implying that neuropeptides cause AHR independent of eosinophil activation. Spina and co-workers reported that capsaicin abolished PAF-induced bronchial AHR in the rabbit, suggesting a role for tachykinins (Spina *et al.*, 1991). These data imply a role for tachykinins in the development of AHR in animal models. Unfortunately, no similar studies, using capsaicin or tachykinin antagonists, have been carried out in the human nasal airway.

The ability of kinins or the tachykinins to cause AHR, may be enhanced by a decrease in their metabolism. The tachykinins are metabolised by a number of different enzymes, but in particular, by neutral endopeptidase-24.11 (NEP). Bradykinin is metabolised by both NEP and angiotensin converting enzyme (ACE). Both these enzymes are present on the human nasal mucosa (Ohkubo *et al.*, 1994a & 1994b). Inhibition of NEP, pharmacologically or as a result of epithelial damage, potentiates the airway response to tachykinins *in vitro* (Kohrogi *et al.*, 1991) and *in vivo* in the human nasal airway (Chatelain *et al.*, 1995). This implies that a reduction in NEP activity may contribute to nasal AHR in man.

Therefore, in this chapter, the ability of phosphoramidon, an inhibitor of NEP, to potentiate the nasal response to histamine and bradykinin is investigated. In addition, the role of tachykinins in AHR in the human nasal airway has been explored.

6.2 Experimental Protocol

In the following experiments, healthy, non-atopic, human volunteers were used as subjects.

6.2.1 The effect of phosphoramidon, an inhibitor of neutral endopeptidase, on the nasal response to histamine and bradykinin

Initially, the nasal cavity was lavaged three times, and the third lavage sample was retained for analysis. Five minutes later, an initial value of Amin. was determined by acoustic rhinometry. A nasal spray was then administered, delivering either 92 nmol of phosphoramidon, an NEP inhibitor, or saline as a control. The dose of phosphoramidon used has previously been shown to inhibit tachykinin metabolism in the human nasal airway (Chatelain *et al.*, 1995). After ten minutes, Amin. was again measured, following which subjects received a nasal challenge with histamine, 2 mg/ml. Three further values of Amin. were determined 2, 5 and 10 min later. The nasal cavity was lavaged for a second time, and the samples assayed for their albumin content, as described in chapter 2. The protocol is summarised in Figure 6.1.

In a cross-over study, each subject received saline and phosphoramidon pretreatments on separate occasions, at least 48 hours apart. The order of treatments was randomised, and followed a double-blind protocol.

In a second experiment, the above protocol was repeated, using bradykinin, 200 µg, as the challenge agent, instead of histamine.

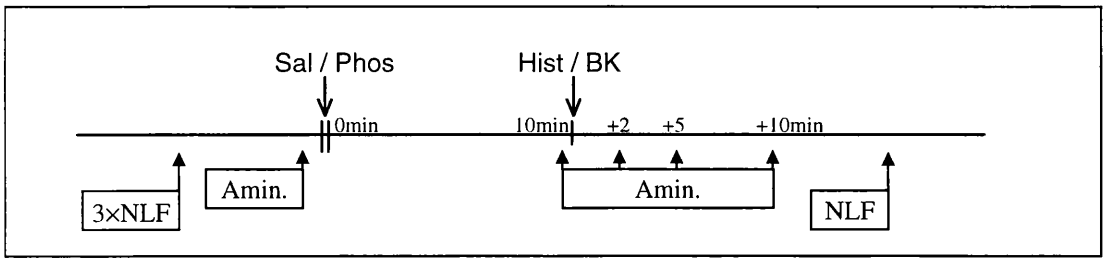

Figure 6.1: Protocol for investigating the effect of phosphoramidon on the response of the human nasal airway to histamine and bradykinin. NLF = nasal lavage, Phos = phosphoramidon, Sal = saline, Hist = histamine, BK = bradykinin.

6.2.2 The effect of repeated administration of substance P and neurokinin-A on the responsiveness of the human nasal airway to histamine

In this study, three nasal lavages were carried out, and the third lavage sample was retained for analysis. Five minutes later, an initial value of Amin. was determined by acoustic rhinometry. Subjects then received, by intranasal spray, 10 µg (per nostril) of either sub P, NK-A or saline as a control. This administration was repeated every 30 minutes, for 4 hours. Two hours after the final administration of tachykinin or control, a second value of Amin. was measured. This value was used to assess the change in nasal patency caused by administration of neuropeptides to the nasal mucosa. Subjects then received a nasal challenge with histamine, 2 mg/ml. Three further values of Amin. were taken 2, 5 and 10 min after histamine challenge, following which another nasal lavage was carried out. The lavage samples were processed as described in chapter 2, and cytological specimens prepared and examined for eosinophils and neutrophils. The protocol is summarised in Figure 6.2.

Subjects received all three pretreatments on separate occasions, at least 7 days apart, in order to prevent the pretreatment from affecting the results of subsequent experiments. The order of treatments was determined using a randomised, balanced-block design. Subjects were randomly assigned to the treatment protocols.

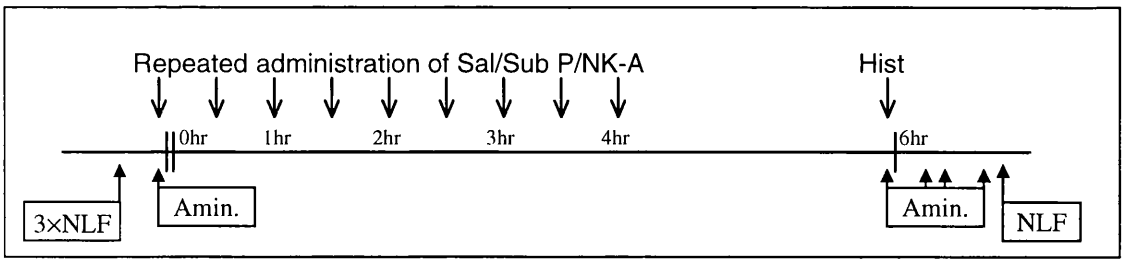

Figure 6.2: Protocol for investigating the effect of repeated administration of substance P or neurokinin-A on the response of the human nasal airway to histamine. NLF = nasal lavage, Sub P = substance P, NK-A = neurokinin A, Sal = saline, Hist = histamine.

6.2.3 The effect of PAF on substance P release in the nasal cavity

Subjects received either PAF, 60 µg, or saline by intranasal spray. Immediately prior to treatment, three nasal lavages were performed, the third being retained as a baseline. At 2, 4 and 6 hours later, a further nasal lavage was carried out. The lavage samples were processed as described in chapter 2, and assayed for their sub P content. Subjects received both treatments on separate occasions, in a random order, at least 48 hours apart. The protocol is summarised in Figure 6.3.

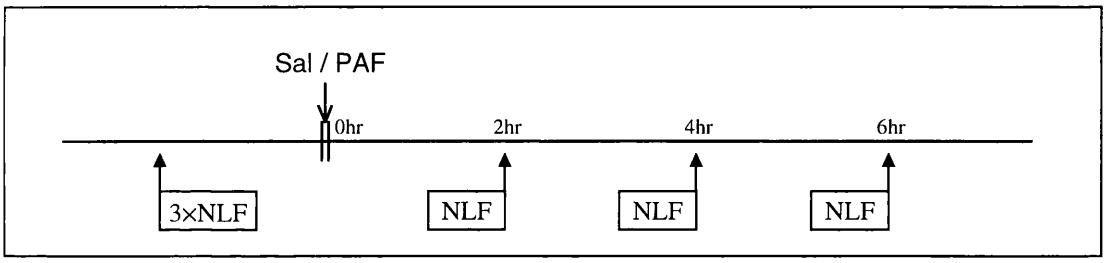

Figure 6.3: Protocol for investigating the effect of PAF on the release of substance P into the nasal cavity. NLF = nasal lavage, Sal = saline.

6.2.4 Data analysis

Values of Amin. following nasal challenge were normalised, as described in chapter 2. For each challenge, a response-time curve was plotted using the percentage decrease in Amin. from baseline against time, and the area under the curve (AUC) determined. The data are presented as mean values, together with s.e.mean. Data from nasal lavage are expressed as medians, together with the interquartile range and 80% central range. The appropriate non-parametric statistical test is given with each data set. A value of $p<0.05$ is taken as significant.

6.3 Results

6.3.1 Effect of phosphoramidon on the nasal response to histamine and bradykinin

Nasal challenge with either histamine or bradykinin, 200 µg, caused a significant increase in AUC, corresponding to an increase in the obstruction of the nasal airway, after pretreatment with the saline control ($p=0.019$, Wilcoxon sign-rank test). Pretreatment of the nasal cavity with phosphoramidon, 92 nmol, did not affect the baseline value of Amin. ($p>0.05$, Wilcoxon sign-rank test, data not shown). The baseline value of Amin. was not significantly different between pretreatments ($p>0.05$, Wilcoxon sign-rank test).

Pretreatment of the nasal cavity with phosphoramidon, 92 nmol, did not alter the decrease in nasal patency induced by histamine challenge, 200 µg, ten minutes later, compared to saline control ($p>0.05$, Wilcoxon sign-rank test). However, phosphoramidon did potentiate the response of the nasal cavity to bradykinin challenge ($p=0.04$, Wilcoxon sign-rank test), as shown in Figure 6.4. Phosphoramidon did not significantly alter the amount of albumin detected in nasal lavage fluid following challenge with either histamine or bradykinin ($p>0.05$, Wilcoxon sign-rank test) (Figure 6.5).

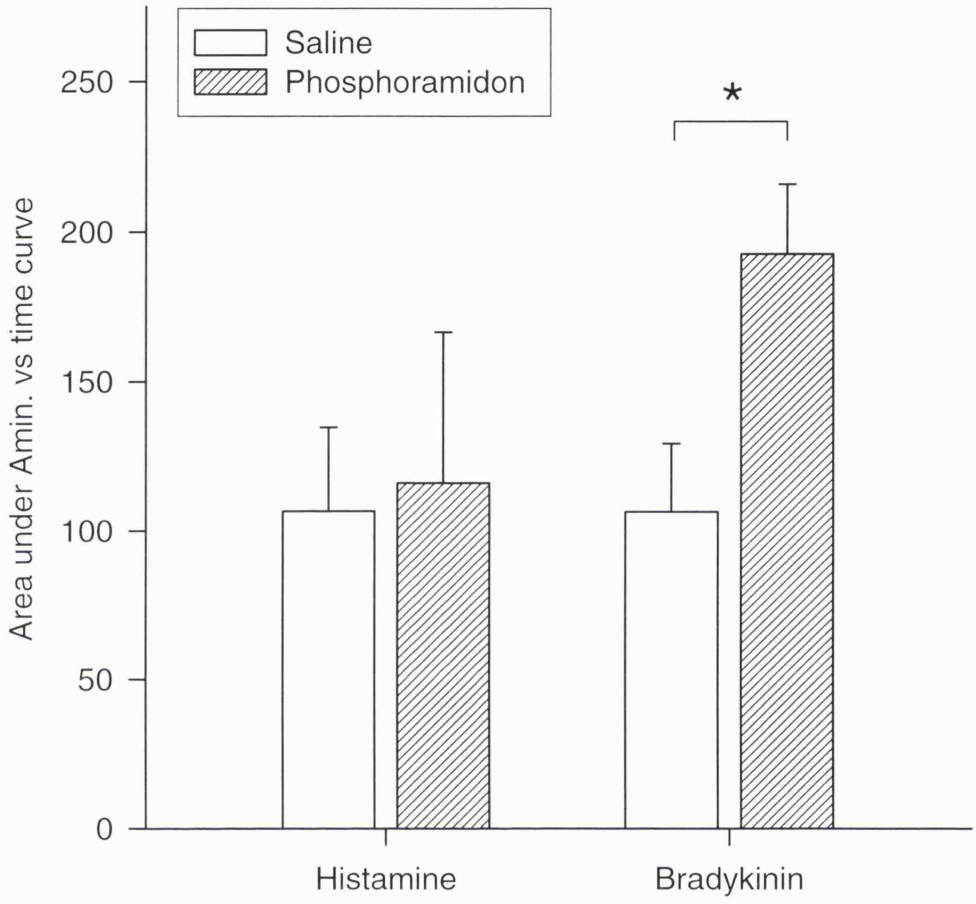

Figure 6.4: Effect of phosphoramidon (a neutral endopeptidase inhibitor), 92 nmol, or saline control, on the response to nasal challenge with histamine or bradykinin, both at 200 µg. Amin. was measured immediately before, and 2, 5 and 10 minutes after challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve (AUC) was determined. Data are means from 10 subjects. The mean ± s.e.mean baseline value of Amin. was 0.59±0.03 cm^2. *Significant difference in AUC following pretreatment with phosphoramidon compared to saline control ($p<0.05$, Wilcoxon sign-rank test).

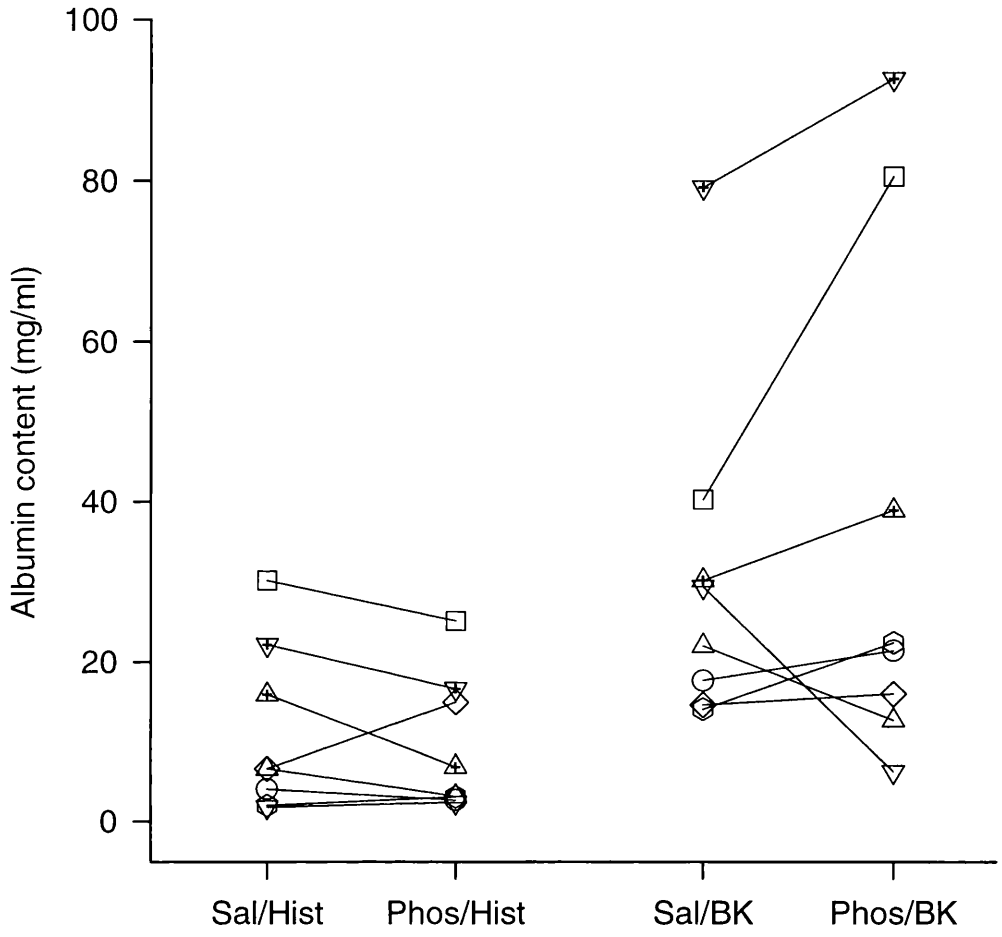

Figure 6.5: The albumin content of nasal lavage fluid following challenge with histamine (Hist) or bradykinin (BK), both at 200 µg. The nasal cavity was pretreated with the neutral endopeptidase inhibitor phosphoramidon (Phos), 92 nmol, or a saline control (Sal), ten minutes prior to challenge with histamine or bradykinin. Data are means from 8 subjects. Each symbol represents an individual subject.

6.3.2 Effect of repeated administration of substance P and neurokinin-A on the responsiveness of the human nasal airway to histamine

Administration of histamine, 200 µg, caused a significant increase in AUC, corresponding to an increase in nasal airway obstruction, after pretreatment with saline control only ($p=0.01$, Wilcoxon sign-rank test). Administration of NK-A, 10 µg, every thirty minutes for four hours, significantly increased the nasal response to histamine, compared to pretreatment with the saline control ($p=0.019$, Wilcoxon sign-rank test) (Figure 6.6). Although pretreatment with sub P also caused a similar increase in AUC, this was not significant compared to the saline control ($p=0.221$, Wilcoxon sign-rank test). Pretreatment of the nasal cavity with either NK-A or sub P did not alter the resting Amin. ($p>0.05$, Friedman's test, data not shown), nor were there any differences in baseline values of Amin. between the different pretreatments ($p>0.05$, Friedman's test).

Figures 6.7 to 6.9 display the cytological profile of the nasal lavage samples before and after pretreatment. There were no significant differences in the initial levels of eosinophils or neutrophils in the lavage samples between pretreatments ($p>0.05$, Friedman's test). Histamine challenge alone did not cause a change in any cytological marker, compared to baseline levels ($p>0.05$, Wilcoxon sign-rank test). Neither sub P nor NK-A caused a significant increase in the percentage of neutrophils or eosinophils obtained after pretreatment, compared to saline control ($p>0.05$, Wilcoxon sign-rank test). However, treatment with NK-A, but not sub P, induced a small but statistically significant increase in the total number of eosinophils isolated in the lavage samples, compared to pretreatment with saline control, as shown in Figure 6.9 ($p=0.05$, Wilcoxon sign-rank test).

6.3.3 Effect of PAF on substance P release in the nasal cavity

Intranasal administration of PAF, 60 µg, did not cause an increase in the amount of sub P detected in nasal lavage fluid over the ensuing six hours ($p>0.05$, Wilcoxon sign-rank test), as shown in Figure 6.10. There was no significant difference in the baseline level of sub P between treatments ($p>0.05$, Wilcoxon sign-rank test).

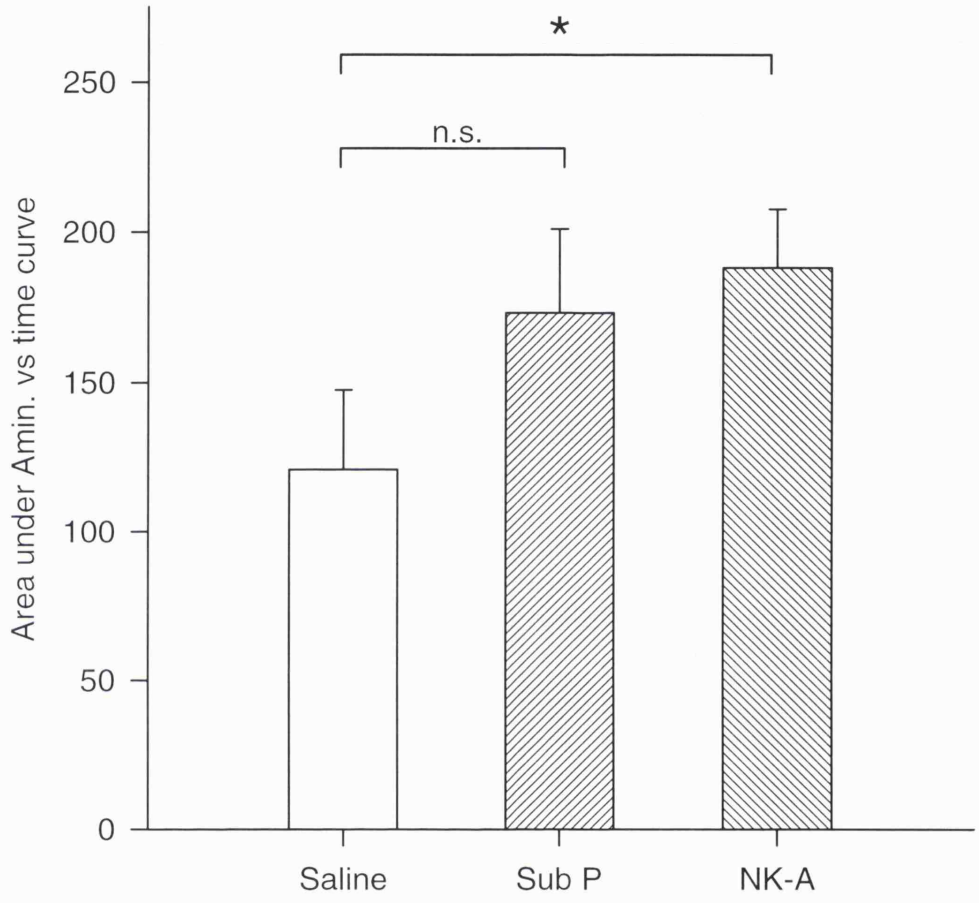

Figure 6.6: Effect of substance P (Sub P) and neurokinin A (NK-A) on the response to nasal challenge with histamine, 200 µg. The nasal cavity was pretreated with saline, substance P or neurokinin-A, as described in the experimental protocol. Amin. was measured immediately before, and 2, 5 and 10 minutes after histamine challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve (AUC) was determined. Data are means from 10 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value of Amin. was 0.64±0.03 cm^2. *Significant difference in AUC following pretreatment with NK-A compared to saline control ($p<0.05$, Wilcoxon sign-rank test). n.s. = not significant.

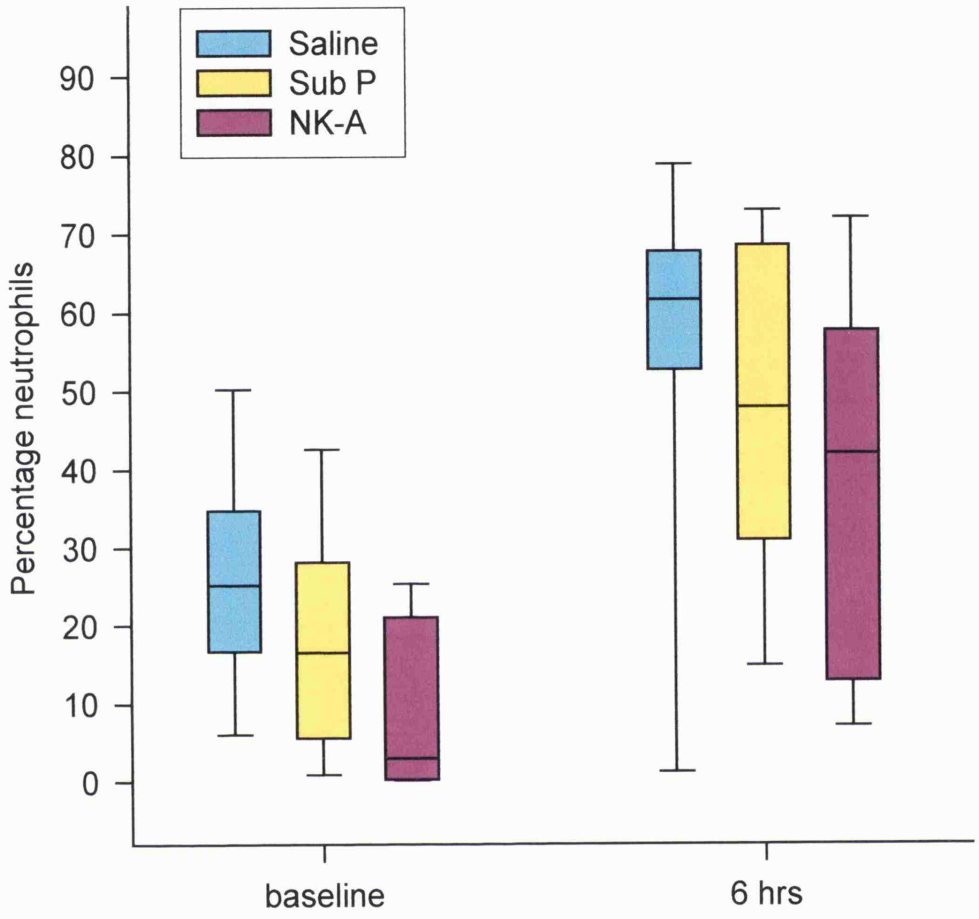

Figure 6.7: The change in the percentage neutrophils isolated from nasal lavage fluid following pretreatment of the nasal cavity with saline, substance P (Sub P) or neurokinin-A (NK-A), as described in the experimental protocol. Data are medians from 10 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values.

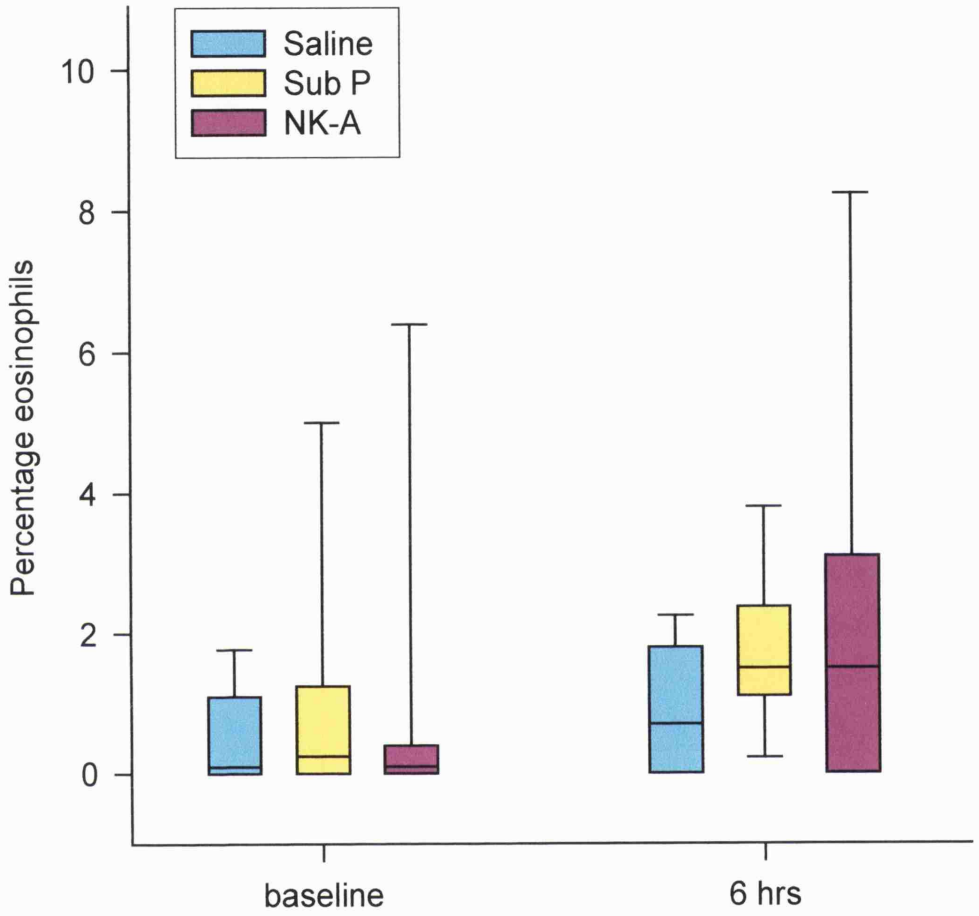

Figure 6.8: The change in the percentage eosinophils isolated from nasal lavage fluid following pretreatment of the nasal cavity with saline, substance P (Sub P) or neurokinin-A (NK-A), as described in the experimental protocol. Data are medians from 10 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values.

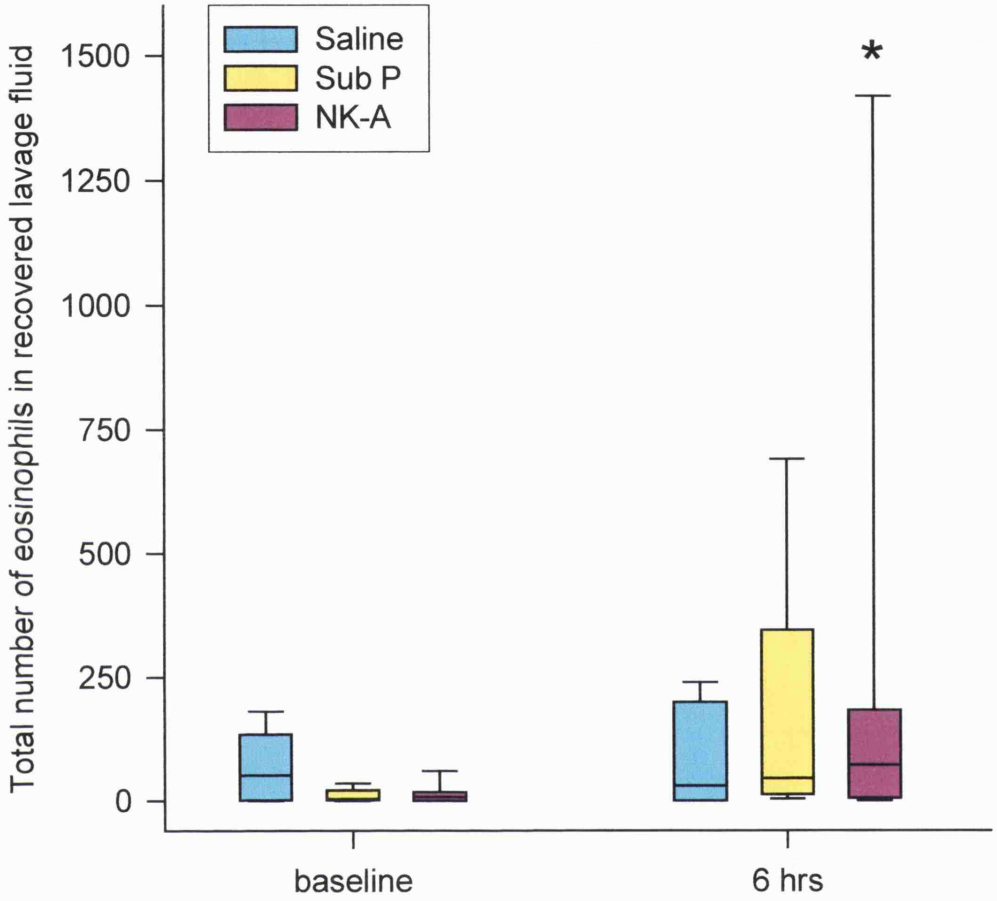

Figure 6.9: The change in the total number of eosinophils isolated in the nasal lavage fluid following pretreatment of the nasal cavity with saline, substance P (Sub P) or neurokinin-A (NK-A), as described in the experimental protocol. Data are medians from 10 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values. *Significant increase in the total number of eosinophils isolated following pretreatment with NK-A, compared to pretreatment with saline ($p<0.05$, Wilcoxon sign-rank test).

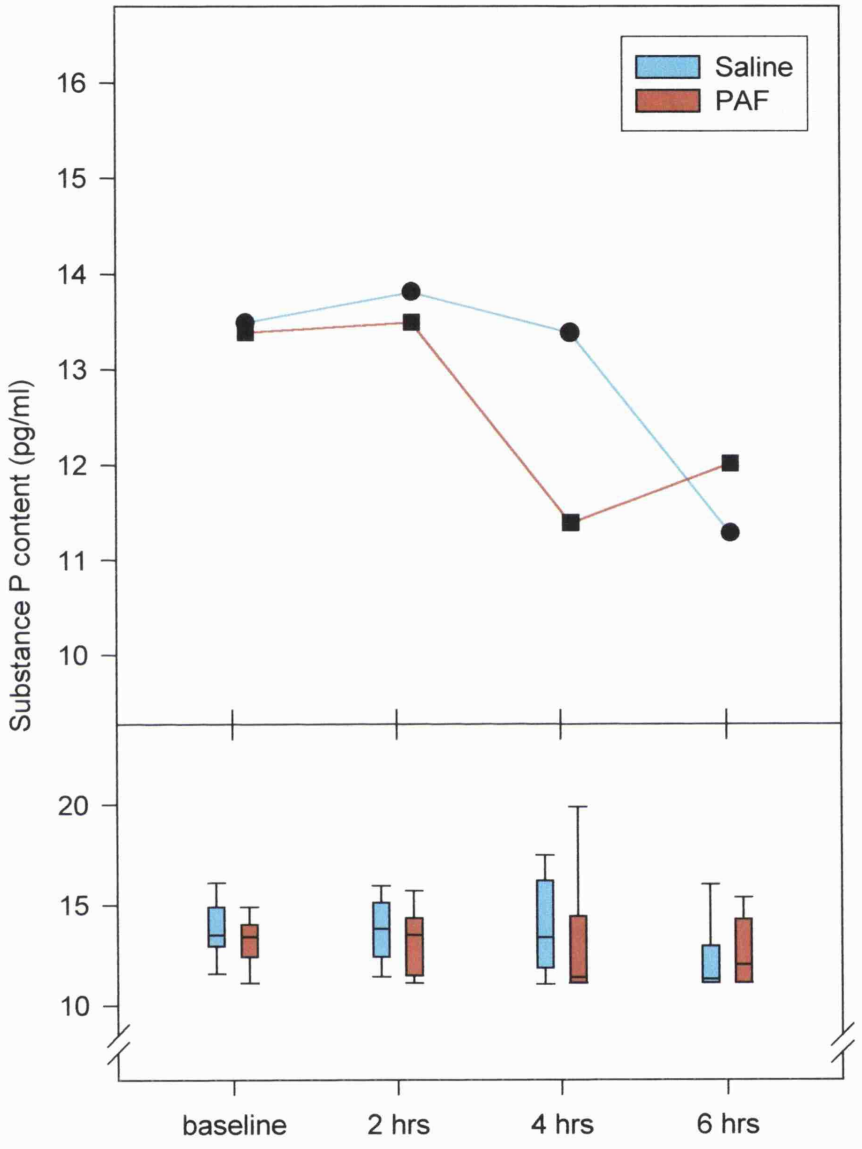

Figure 6.10: The effect of PAF on the concentration of substance P in nasal lavage fluid. The nasal airway was challenged with either saline or PAF, 60 µg, and the nasal cavity lavaged at 2, 4 and 6 hours later. Following lavage, a peptidase inhibitor was added to prevent degradation of substance P prior to assay. Data are medians from 9 subjects. The bottom window indicates the interquartile range of values for each median, with the horizontal line indicative of the median value. Vertical bars represent the 80% central range of values.

6.4 Discussion

Phosphoramidon, an inhibitor of NEP, potentiated the nasal response to bradykinin, but not to histamine. One explanation for this could be that bradykinin, but not histamine, caused the release of tachykinins, which are predominantly metabolised by NEP. Therefore, phosphoramidon may have prevented the metabolism of these tachykinins, increasing the nasal response to bradykinin. Indeed, while bradykinin causes substance P release in the human nasal airway (Baumgarten *et al.*, 1997), histamine does not (Mosimann *et al.*, 1993). However, NEP is also implicated in the metabolism of a number of different peptides, including bradykinin (Erdos & Skidgel, 1997). Thus, the increase in response bradykinin, following phosphoramidon treatment, may have been a consequence of an inhibition of bradykinin degradation by NEP, without tachykinin involvement. Although both NEP and ACE are involved in the metabolism of bradykinin in the human nasal airway (Ohkubo *et al.*, 1994a), ACE has an affinity for bradykinin almost one hundred times greater than that of NEP for bradykinin (the K_m for ACE and NEP are 0.18 μM and 120 μM respectively, in man) (Erdos & Skidgel, 1997). Although the metabolism of bradykinin by NEP may be insignificant in the presence of a high density of bradykinin receptors (Solan *et al.*, 1998), the density of bradykinin B_2 receptors on human nasal membrane preparations is low (Dear *et al.*, 1996b). In reality, phosphoramidon probably potentiated the action of bradykinin by both mechanisms i.e. inhibition of bradykinin degradation, and also by inhibiting the metabolism of tachykinins released by bradykinin. Although histamine causes the release of tachykinins in human lung tissue *ex vivo* (Saria *et al.*, 1988), the data in the present study provides no evidence for the release of tachykinins by histamine in the normal, non-atopic human nasal airway.

Administration of NK-A, but not sub P, resulted in an increased nasal response to histamine, measured using acoustic rhinometry. This finding is consistent with data from animal models (Tamura *et al.*, 1989; Abraham *et al.*, 1991; Chiba & Misawa, 1995). The different abilities of the tachykinins to cause AHR may be due to their action as agonists at different receptors. Sub P causes greater activation of NK_1 receptors, while NK-A acts preferentially at the NK_2 receptor, so it is possible that tachykinin-induced AHR is mediated only through the NK_2 receptor. A role for NK_2

receptors in AHR has been implicated in a number of animal models of airway allergy. Mazuguchi *et al.* (1996) observed that FK-224, a non-selective NK receptor antagonist, inhibited ovalbumin-induced AHR in the lower airway of the guinea pig, while FK-888, a selective NK_1 receptor antagonist, did not. These results were corroborated by a related study, using the NK_2 receptor antagonist SR 48968, in the same experimental model (Boichot *et al.*, 1995). In a later study, it was found that if sub P was used to induce AHR in phosphoramidon-treated guinea pigs, rather than ovalbumin, SR 140333, a NK_1 receptor antagonist, also abolished the secretory hyperresponsiveness observed (Boichot *et al.*, 1996). NK_1 receptor antagonists were also found to be effective in preventing AHR to histamine induced by antigen (Schuiling *et al.*, 1999) and PAF (Perretti *et al.*, 1995) in other studies. Furthermore, cationic protein-induced AHR to methacholine in the rat is also abolished by pretreatment with NK_1 receptor antagonists (Coyle et al., 1994).

These conflicting reports may be the result of differences in the antagonists and stimuli used to induce AHR, their route of delivery, the challenge agent (methacholine in some models, histamine in others), or simply variation in the mechanism of AHR between species (Schuiling *et al.*, 1999). However, there is an alternative explanation, which may also account for the different abilities of sub P and NK-A to cause AHR in man. In the human nasal airway, NK_1 receptors are localised to the epithelium, glands and blood vessels, while NK_2 receptors are limited to arterial vessels (Baraniuk *et al.*, 1991). A similar distribution exists in the lower airways in man, where NK_2 receptors are mainly present on airway smooth muscle (Barnes *et al.*, 1998). Therefore, the increased ability of histamine to induce nasal obstruction, following NK-A, may be dependent on both NK_1 and NK_2 receptors; while NK_1 receptors may be involved in a secretory hyperresponsiveness. Some challenge agents, such as methacholine, act mainly on secretory glands, and cause little nasal obstruction compared to histamine (Doyle *et al.*, 1990). Therefore, the above hypothesis may explain why sub P causes AHR to methacholine in the lower airways in man (Cheung *et al.*, 1995), and also the observation that NK_1 receptor antagonists abolished the secretory hyperresponsiveness in the study by Boichot *et al.* (1996). The ability of sub P to induce a secretory hyperresponsiveness was not investigated in this chapter.

The different actions NK-A and sub P on tachykinin receptors may also explain why NK-A, but not sub P, caused a mild eosinophilia in some subjects, since the effects of the tachykinins on inflammatory cells is thought to be mediated through NK_2 receptors (Braunstein *et al.*, 1991). It is unknown whether the eosinophilia observed in this study was a direct result of NK-A administration, or a consequence of the hyperresponsive state. The tachykinins are chemotactic for eosinophils *in vitro* (Wiedermann *et al.*, 1993), and activate both eosinophils and neutrophils (Iwamoto *et al.*, 1993). However, in addition to a chemotactic action, the tachykinins can also potentiate the ability of other chemotactants to cause eosinophil recruitment, by priming the cells (Numao & Agrawal, 1992). This effect may be mediated through the NK_1 receptor, since antagonists of this receptor reduced both antigen-induced eosinophilia *in vivo* in the guinea pig (Schuiling *et al.*, 1999) and the potentiation of PAF-induced chemotaxis by sub P *in vitro* (El-Shazly *et al.*, 1996). Fajac *et al.* (1995) found that administration of sub P into the nasal cavity induced a selective eosinophilia in subjects with active seasonal allergic rhinitis. Unfortunately, no controls were included in the protocol. These results are compatible with a role for sub P in the modulation of eosinophil recruitment. Interestingly, stimulation of nasal mucosal tissue *ex vivo*, from both atopics and non-atopics, with sub P results in the increased expression of IL-1β, IL-3, IL-5, IL-6, TNFα and IFNγ (Okamoto *et al.*, 1993). A number of these cytokines can prime eosinophils and contribute to their accumulation in allergic disease. Therefore, the observation that NK-A caused an eosinophilia in some individuals, but not in others, may reflect differing degrees to which NK-A can cause NK_1 and NK_2 receptor activation, or different levels of receptor density, between subjects.

The results presented above indicate that the development of AHR in the human nasal airway may be dependent on the release of NK-A from sensory nerves. However, intranasal administration of PAF did not cause an increase in the levels of sub P detected in nasal lavage fluid. Although only low levels of sub P are released in the human nose, the levels obtained were within the detection range of the assay used, and comparable to the amount of sub P detected by Baumgarten *et al.* (1997). Theoretically, since AHR may be associated with NK_2 receptor activation, it is possible that PAF causes the selective release of NK-A. However, there is no

evidence for the selective release of sensory neuropeptides in any tissue (Geppetti, 1993). Therefore, PAF may cause AHR via a process independent of neuropeptide release. Alternatively, PAF may potentiate the subsequent release of neuropeptides from sensory nerves, and not cause neuropeptide release *per se*, so an increase in sub P would only occur in the presence of a further stimulus, such as histamine. However, the data in this chapter imply that histamine does not cause tachykinin release in the absence of PAF. In order to ascertain whether tachykinins are involved in PAF-induced AHR, it would be necessary to investigate the effect of specific tachykinin receptor antagonists on the actions of PAF. Unfortunately, it was not possible to obtain such antagonists suitable for use in the human nasal airway.

If bradykinin causes the release of sensory neuropeptides in the human nasal airway (Baumgarten *et al.*, 1997), and NK-A can induce a nasal AHR to histamine, then it is somewhat of an anomaly that exogenous bradykinin does not itself induce AHR, as observed in chapter 3. Furthermore, although PAF causes kinin production and AHR via a kinin-dependent process, PAF did not cause the release of sub P in this experiment, as mentioned before. One explanation might be that the dose of bradykinin, used in the experiment described in this chapter, did not cause the release of a sufficient amount of NK-A to induce AHR or result in a nasal eosinophilia. This would also explain the absence of a nasal eosinophilia, after repeated administration of bradykinin, found in chapter 4. Alternatively, NK-A may induce AHR by a mechanism dependent on the presence of other mediators and/or inflammatory cells. Again, the amount of NK-A released by bradykinin could be insufficient to provide these other components required for AHR.

Finally, there is evidence for an upregulation in the expression of both NK_1 receptors (Adcock *et al.*, 1993) and NK_2 receptors (Bai *et al.*, 1995) in asthma, and the same may happen in allergic rhinitis. Interestingly, glucocorticoids inhibit the expression of the mRNA for the NK_1 receptor (Ihara & Nakanishi, 1990), and this may contribute to their anti-inflammatory action. Therefore, it is possible that neurogenic inflammation, through the release of tachykinins, may contribute to the development of AHR in the human nose, particularly in subjects in whom there is an underlying degree of allergic inflammation.

Summary

- Intranasal challenge using histamine or bradykinin, both at 200 µg, caused a significant decrease in the patency of the human nasal airway.

- Administration of the neutral endopeptidase inhibitor, phosphoramidon, 92 nmol, potentiated the nasal response to bradykinin, 200 µg, but not to histamine.

- Pretreatment with neurokinin A (NK-A), 10 µg every thirty minutes for four hours, significantly increased the response to histamine, 200 µg, administered six hours late. Repeated administration of substance P did not cause an increased response to histamine challenge.

- NK-A pretreatment also caused a mild nasal eosinophilia in some subjects.

- Intranasal administration of platelet activating factor (PAF), 60 µg, did not cause the release of substance P into the nasal cavity.

- The results indicate that NK-A, and neurogenic inflammation in general, may contribute to nasal AHR; however, no evidence for the involvement of tachykinins in PAF-induced nasal AHR was identified.

CHAPTER 7

THE ROLE OF NITRIC OXIDE IN THE INDUCTION OF HYPERRESPONSIVENESS IN THE HUMAN NASAL AIRWAY

7.1 Introduction

In the lower airways of the guinea pig, inhibitors of nitric oxide synthase (NOS) induce an airway hyperresponsiveness (AHR) to histamine (Nijkamp *et al.*, 1993; Schuiling *et al.*, 1998), bradykinin (Ricciardolo *et al.*, 1994b) and antigen in ovalbumin-sensitised animals (Persson *et al.*, 1993). Ricciardolo *et al.* (1996) reported that the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) caused an AHR to both bradykinin and methacholine, in the lower airways of asthmatics. However, no similar studies have been carried out in the human nasal airway.

There is evidence of increased nitric oxide (NO) production in both perennial (Garrelds *et al.*, 1995) and seasonal allergic rhinitis (Kharitonov *et al.*, 1997b), and this may contribute to the AHR observed in allergic rhinitis. For example, increasing NO production in the murine airway potentiates the airway response to allergen (Takano *et al.*, 1998).

All three isoforms of NOS are present in the human nasal mucosa (Kulkarni *et al.*, 1994; Furukawa *et al.*, 1996). The plasma extravasation induced by challenge with histamine, bradykinin or antigen in the human nasal airway, is attenuated by the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (Dear *et al.*, 1995 & 1996a), implying a role for NO in the physiology of the nasal airway in human subjects.

The aim of this chapter was to examine the role of NO in the induction of hyperresponsiveness in the non-atopic, human nasal airway.

7.2 Experimental Protocol

In the following studies, healthy non-atopic human volunteers were used as subjects. In all experiments, a double-blind, balanced randomised-block, cross-over design was used. Subjects were assigned randomly to the treatment protocol. Subjects received all treatments on separate occasions, at least 72 hours apart. The doses used were based on previous studies (Dear *et al.*, 1996a) and pilot experiments carried out in the laboratory.

7.2.1 Characterisation of the nasal hyperresponsiveness induced by inhibitors of nitric oxide synthase

The minimal cross-sectional area of the nasal airway (Amin.) was determined by acoustic rhinometry. Subjects then received one of the following combinations of treatment by nasal aerosol: saline (control), L-NAME (1 µmol), L-NMMA (1 µmol), D-NAME (1 µmol) or L-NAME (1 µmol) plus L-arginine (30 µmol) combined. The duration of action of L-NAME and L-NMMA in the human nasal airway is about 30 minutes (Dear *et al.*, 1996a). Therefore, this pretreatment was repeated every 30 minutes for 5½ hours. Six hours after the first administration, a second baseline value of Amin. was determined, followed by challenge with either histamine or bradykinin, 200 µg. Amin. was then redetermined 2, 5 and 10 minutes after challenge. The protocol used is summarised in Figure 7.1.

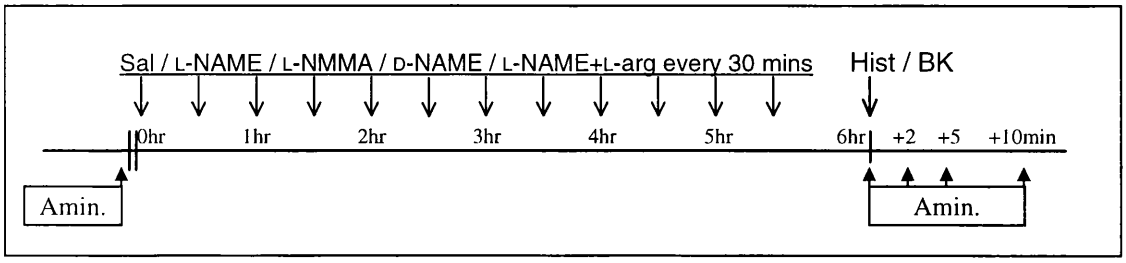

Figure 7.1: Protocol for investigating the effect of NOS inhibitors on the nasal response to histamine or bradykinin challenge. Sal = saline, L-arg = L-arginine, Hist = histamine, BK = bradykinin.

The above protocol was then modified in order to further investigate the action of L-NAME on nasal AHR. This involved using 3 doses of L-NAME (0.1, 1 and 10 µmol) and determining the response to histamine (50, 200 or 500 µg) at various time points after initial administration of the NOS inhibitor (2, 6, 24 and 48 hours later). The effect of a single administration of L-NAME on the nasal response to histamine was also studied.

7.2.2 The effect of L-NAME on the cytological profile of the human nasal airway

Initially, a nasal lavage was carried out three times per nostril, and the third lavage sample was retained. The nasal cavity was then allowed to dry, following which subjects received, by intranasal spray, L-NAME (1 µmol) or a saline control. This administration was repeated every 30 minutes, for 5½ hours. In addition, some subjects also received a single dose of PAF, 60 µg, instead of repeated administrations of L-NAME, as a positive control. Two and six hours after the start, a nasal lavage was again carried out. The lavage samples were processed as described, and cytological specimens prepared and examined for eosinophils and neutrophils. The protocol used is shown in Figure 7.2.

In order to investigate the effect of L-arginine on L-NAME-induced changes in nasal cytology, a separate experiment was carried out, utilising a similar protocol. Subjects received either L-NAME (1 µmol), L-NAME (1 µmol) plus L-arginine (30 µmol) combined, or a saline control. Nasal lavage was carried out at the start, and at six hours later, and cytological specimens prepared from the lavage samples as before.

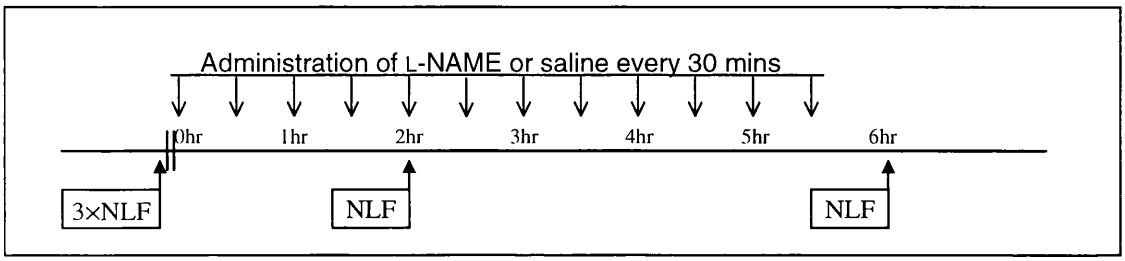

Figure 7.2: Protocol for investigating the effect of L-NAME on nasal cytology. NLF = nasal lavage.

7.2.3 The role of nitric oxide in PAF-induced nasal airway hyperresponsiveness

In a separate study, L-NMMA (1 µmol), L-arg (30 µmol) or a saline control was administered into each nostril. After two minutes, a second aerosol of either PAF, 60 µg, or vehicle (saline) was given. The first administration of L-NMMA, L-arginine or saline was then repeated every 30 minutes, for 5½ hours, as before. Six hours after the start, a nasal challenge of histamine, 200 µg, was carried out and the response assessed as before, as shown in Figure 7.3. Each subject received the following combinations of treatment, on separate occasions:

- Saline, followed by saline, then saline every 30 minutes
- Saline, followed by PAF, then saline every 30 minutes
- L-NMMA, followed by PAF, then L-NMMA every 30 minutes
- L-arginine, followed by PAF, then L-arginine every 30 minutes

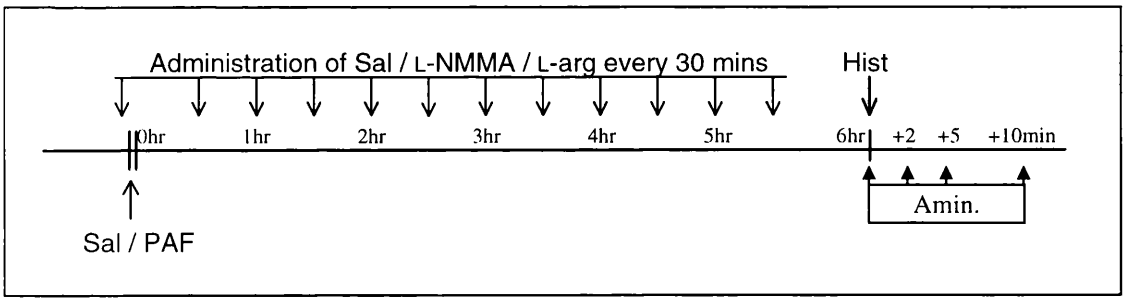

Figure 7.3: Protocol for investigating the role of nitric oxide on PAF-induced nasal hyperresponsiveness. Sal = saline, L-arg = L-arginine, Hist = histamine.

7.2.4 The action of L-NAME and PAF on nitric oxide production in the human nasal airway

In this study, the amount of NO in the nasal airway (NO_{nasal}) was measured using a chemiluminescence detector, as described in chapter 2. Nasal lavage was then carried out three times, the third sample was retained as the baseline for analysis. Subjects then received either L-NAME, 1 µmol/nostril, or saline control, every 30 minutes for 5½ hours. At two and six hours after the start, NO_{nasal} was again measured, and a nasal lavage carried out. The protocol used is shown in Figure 7.4.

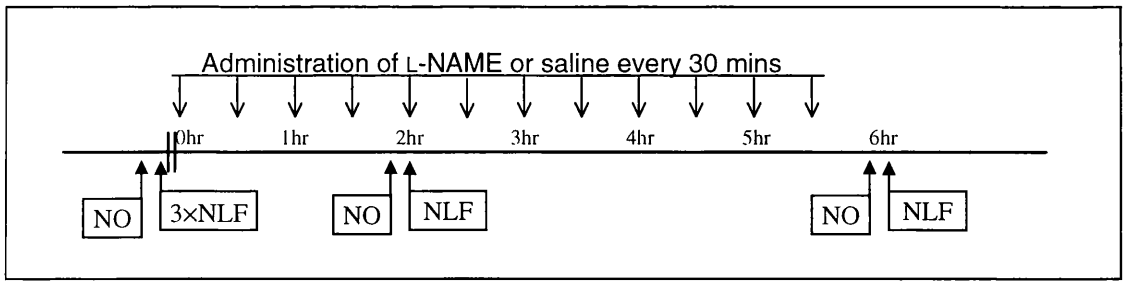

Figure 7.4: Protocol for investigating the effect of L-NAME on NO production in the human nasal airway. NO = nasal NO measurement, NLF = nasal lavage.

In a further experiment, the above protocol was adapted, using a single dose of PAF, 60 µg, or saline control as the active treatment, instead of L-NAME. NO_{nasal} was determined and a nasal lavage carried out at two and six hours after the start, as before.

The lavage samples were centrifuged to remove any cells present, and the supernatants were stored at -70°C until assay for the total nitrite+nitrate content, as described in chapter 2.

7.2.5 The effect of icatibant, a bradykinin B_2 receptor antagonist, on L-NAME-induced nasal hyperresponsiveness

In order to investigate whether kinins are involved in the AHR induced by NOS inhibitors, the following study was carried out. Subjects were given a nasal spray of either icatibant, 200 µg, or vehicle (saline). Two minutes later, a further spray was administered, containing either L-NAME (1 µmol) or a saline control. This second spray was then administered every 30 minutes, for 5½ hours, as before. At two and four hours after the start, the spray containing icatibant or saline was also given, since the duration of action of icatibant in the human nasal airway is about two hours (Dear, 1996). Finally, six hours after the first spray, a baseline measurement of Amin. was taken, followed by challenge with histamine, 200 µg. Amin. was then redetermined at 2, 5 and 10 minutes after challenge. The protocol is shown in Figure 7.5.

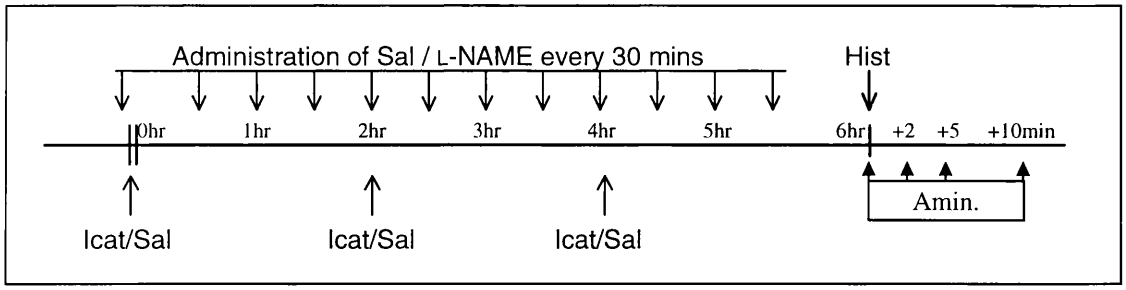

Figure 7.5: Protocol to study the role of kinins in the nasal hyperresponsiveness induced by inhibitors of nitric oxide synthase. Sal = saline, Icat = icatibant, Hist = histamine.

Each subject received the following combinations of treatment, on separate occasions:

- Saline (two hourly), followed by saline every 30 minutes.
- Saline (two hourly), followed by L-NAME every 30 minutes
- Icatibant, followed by L-NAME every 30 minutes

7.2.6 Data analysis

Values of Amin. following nasal challenge were normalised, as described in chapter 2. For each challenge, a response-time curve was plotted using the percentage decrease in Amin. from baseline against time, and the area under the curve (AUC) determined. The data are presented as mean values, together with s.e.mean. Data from nasal lavage are expressed as medians, together with the interquartile range and 80% central range. The appropriate non-parametric statistical test is given with each data set. A value of $p<0.05$ is taken as significant.

7.3 Results

7.3.1 Characterisation of the nasal hyperresponsiveness induced by inhibitors of nitric oxide synthase

Figure 7.6 shows that the nitric oxide synthase inhibitors, L-NAME and L-NMMA, at a concentration of 1 µmol and given repeatedly for 6 hours, increased the nasal response to histamine, 200 µg, compared to treatment with saline control (p=0.008 and p=0.032 respectively, Wilcoxon sign-rank test). L-NAME also induced a hyperresponsiveness to bradykinin, 200 µg (p=0.025, Wilcoxon sign-rank test) (Figure 7.7).

To test the specificity of the ability of L-NAME to induce AHR, a further study was carried out to investigate whether this action of L-NAME could be prevented by using L-arginine. Administration of L-arginine (30 µmol), together with L-NAME, abolished the ability of L-NAME to induce hyperresponsiveness to histamine (p>0.05, Wilcoxon sign-rank test). Furthermore, D-NAME (1 µmol) failed to induce hyperresponsiveness to histamine (p>0.05, Wilcoxon sign-rank test). None of the pretreatments caused a significant change in the resting Amin. in any of the experiments (p>0.05, Friedman's test, data not shown).

Figures 7.8 and 7.9 show the effects of changing the dose of L-NAME. Pretreatment of the nasal airway with L-NAME, at a concentration of 0.1 µmol, failed to induce AHR (p>0.05, Wilcoxon sign-rank test). Higher concentrations of L-NAME, at a dose of 1 mol and 10 µmol, did induce an AHR to histamine (p=0.036, Wilcoxon sign-rank test). No difference was observed in the degree of hyperresponsiveness between these two doses (p>0.05, Friedman's test).

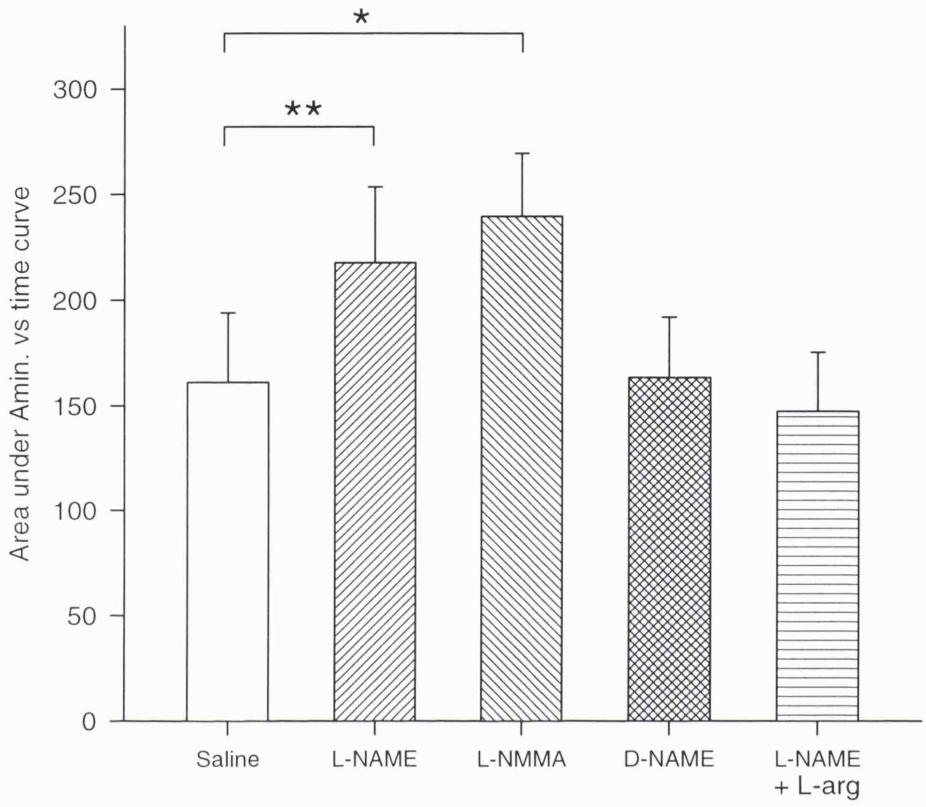

Figure 7.6: Effect of inhibitors of nitric oxide synthase on the response to nasal challenge with histamine, 200 µg. The nasal cavity was pretreated with one of the following: saline, L-NAME, 1 µmol; L-NMMA, 1 µmol; D-NAME, 1 µmol; L-NAME, 1 µmol, plus L-arginine (L-arg), 30 µmol; as described in the experimental protocol. Amin. was measured immediately before, and 2, 5 and 10 minutes after histamine challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve (AUC) was determined. Data are means from 10 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value of Amin. was 0.60±0.02 cm^2. */**Significant difference in AUC following histamine challenge after pretreatment with L-NAME, compared to saline control (*$p<0.05$, **$p<0.01$, Wilcoxon sign-rank test).

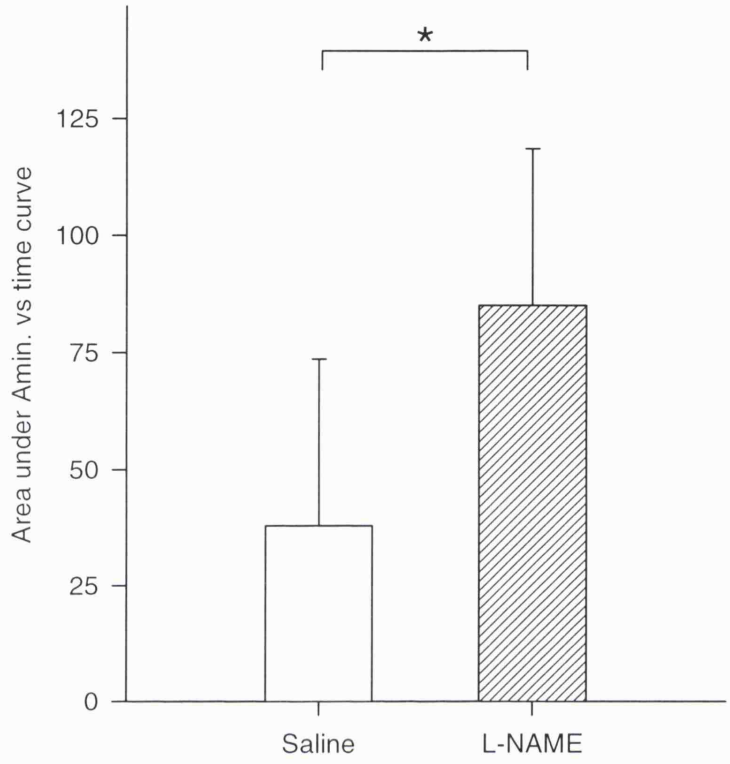

Figure 7.7: Effect of inhibitors of nitric oxide synthase on the response to nasal challenge with bradykinin, 200 µg. The nasal cavity was pretreated with either saline or L-NAME, 1 µmol, as described in the experimental protocol. Amin. was measured immediately before, and 2, 5 and 10 minutes after bradykinin challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve (AUC) was determined. Data are means from 10 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value of Amin. was 0.58±0.03 cm^2. *Significant difference in AUC following histamine challenge after pretreatment with L-NAME, compared to saline control ($p<0.05$, Wilcoxon sign-rank test).

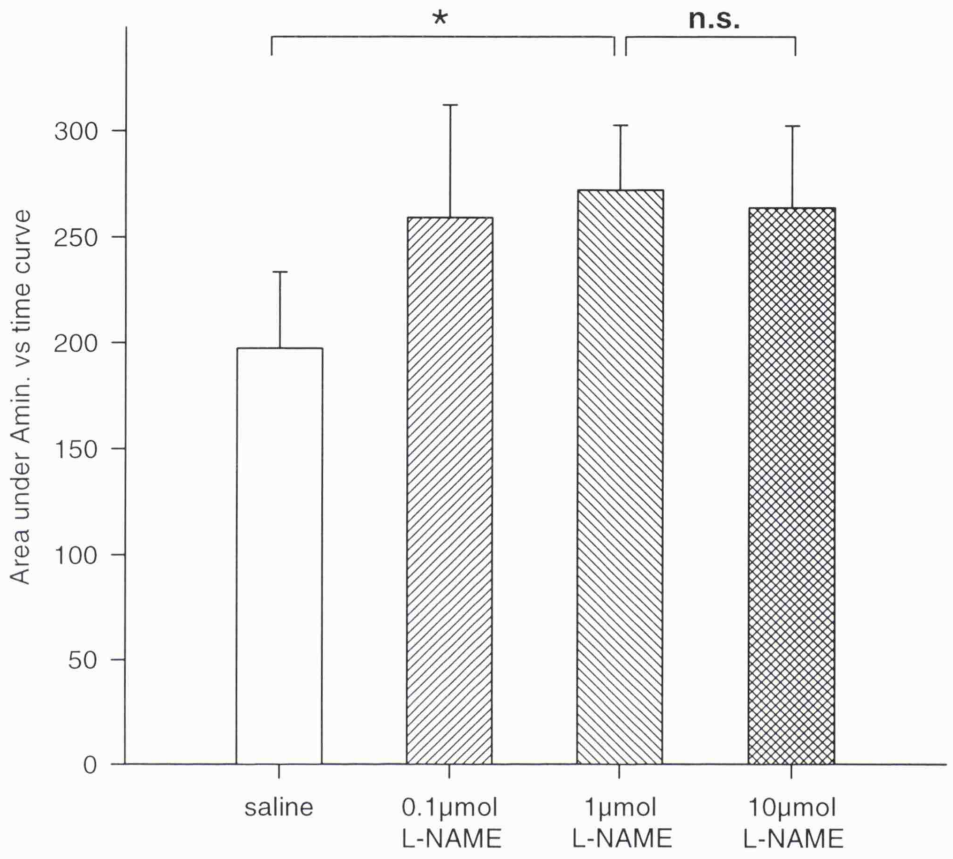

Figure 7.8: Effect of different concentrations of L-NAME on the response to nasal challenge with histamine, 200 µg. The nasal cavity was pretreated with repeated administrations of L-NAME, at the doses shown, every 30 minutes, as described in the experimental protocol. Amin. was measured immediately before, and 2, 5 and 10 minutes after histamine challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve (AUC) was determined. Data are means from 6 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value of Amin. was 0.56±0.04 cm^2. *Significant difference in AUC following histamine challenge after pretreatment with L-NAME, 1 µmol, compared to saline ($p<0.05$, Wilcoxon sign-rank test). n.s. = no significant difference in AUC between treatments shown ($p>0.05$, Wilcoxon sign-rank test).

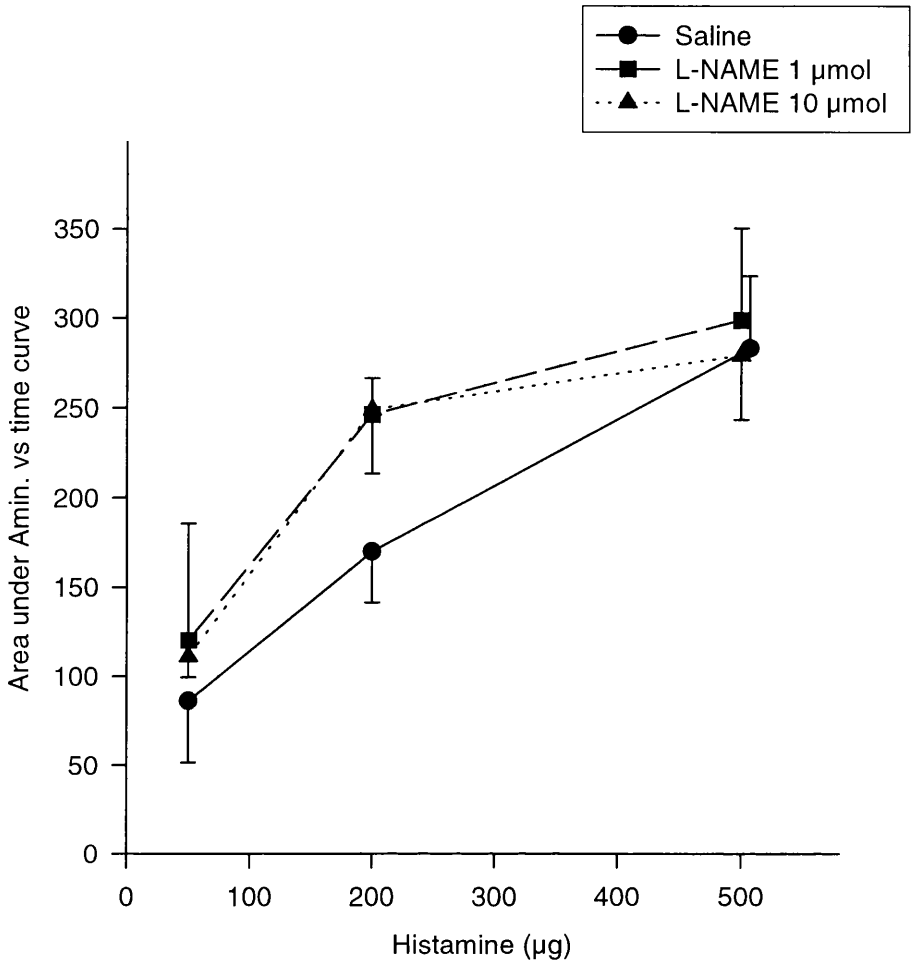

Figure 7.9: Dose-response curve for histamine, following pretreatment of the nasal cavity with repeated administrations of L-NAME, 1 µmol, or a saline control, as described in the experimental protocol. Amin. was measured immediately before, and 2, 5 and 10 minutes after histamine challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve (AUC) was determined. Data are means from 5 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value of Amin. was 0.58±0.03 cm^2.

When the nasal airway was treated with a single dose of L-NAME, 1 µmol, no hyperresponsiveness to histamine was observed ($p>0.05$, Wilcoxon sign-rank test) (Figure 7.10), implying that the AHR was due to a prolonged inhibition of NOS by L-NAME. This is further supported by the observation that the AHR was only present after 6 hours of L-NAME administration, and not after 2 hours of treatment, as shown in Figure 7.11. The hyperresponsiveness was not present 24 hours after initial pretreatment ($p>0.05$, Wilcoxon sign-rank test). However, when the nasal airway was pretreated with a higher dose of L-NAME (10 µmol), the duration of the hyperresponsiveness was prolonged ($p=0.048$, Wilcoxon sign rank test).

7.3.2 Effect of L-NAME on the cytological profile of the human nasal airway

Neither PAF, 60 µg, nor L-NAME, 1 µmol, given repeatedly, caused a change in the percentage of neutrophils isolated in nasal lavage fluid ($p>0.05$, Wilcoxon sign-rank test) (Figure 7.12). PAF caused a significant eosinophilia into the nasal cavity, observed as an increase in the number of eosinophils in the lavage samples ($p=0.014$, Wilcoxon sign-rank test), shown in Figures 7.13 and 7.14. This influx of eosinophils peaked at two hours after PAF administration. Interestingly, administration of L-NAME for six hours also caused a small, but significant, increase in both the differential and absolute eosinophil count ($p=0.036$ and $p=0.014$ respectively, Wilcoxon sign-rank test). This eosinophilia was not detected at two hours after the first treatment. Furthermore, the eosinophilia was not reduced when L-NAME was given together with L-arginine, 30 µmol ($p>0.05$, Wilcoxon sign-rank test) (Figure 7.15)

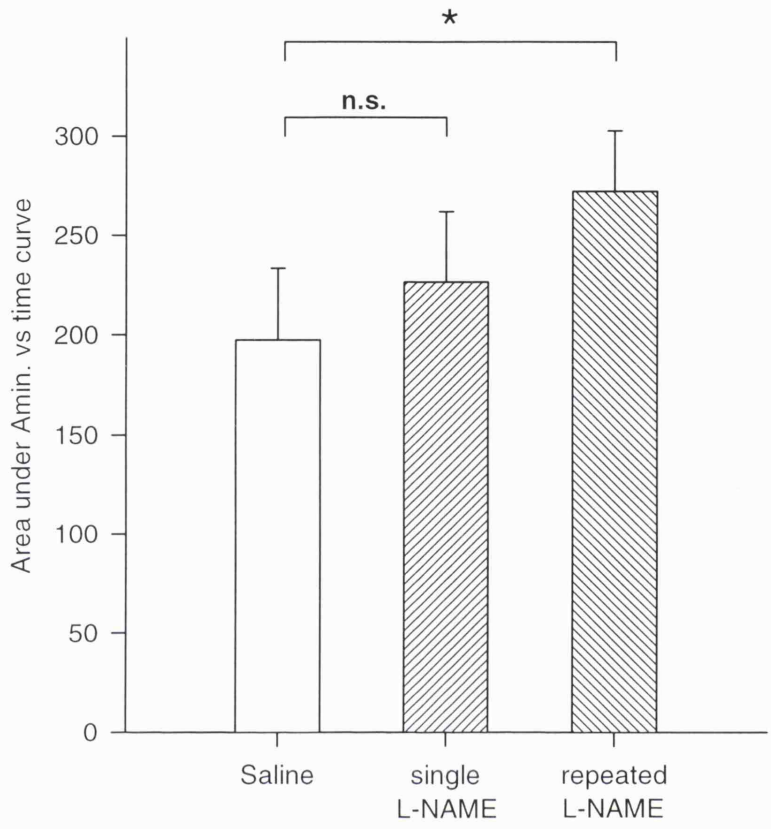

Figure 7.10: Effect of changes in the dosing regimen of L-NAME on the response to nasal challenge with histamine, 200 µg. The nasal cavity was pretreated either saline, a single dose of L-NAME, 1 µmol, or repeated administrations of L-NAME, 1 µmol, every 30 minutes, as described in the experimental protocol. Amin. was measured immediately before, and 2, 5 and 10 minutes after histamine challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve (AUC) was determined. Data are means from 6 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value of Amin. was 0.57±0.04 cm^2. *Significant difference in AUC following histamine challenge after repeated pretreatment with L-NAME, compared to saline control ($p<0.05$, Wilcoxon sign-rank test). n.s. = no significant difference in AUC between treatments shown ($p>0.05$, Wilcoxon sign-rank test).

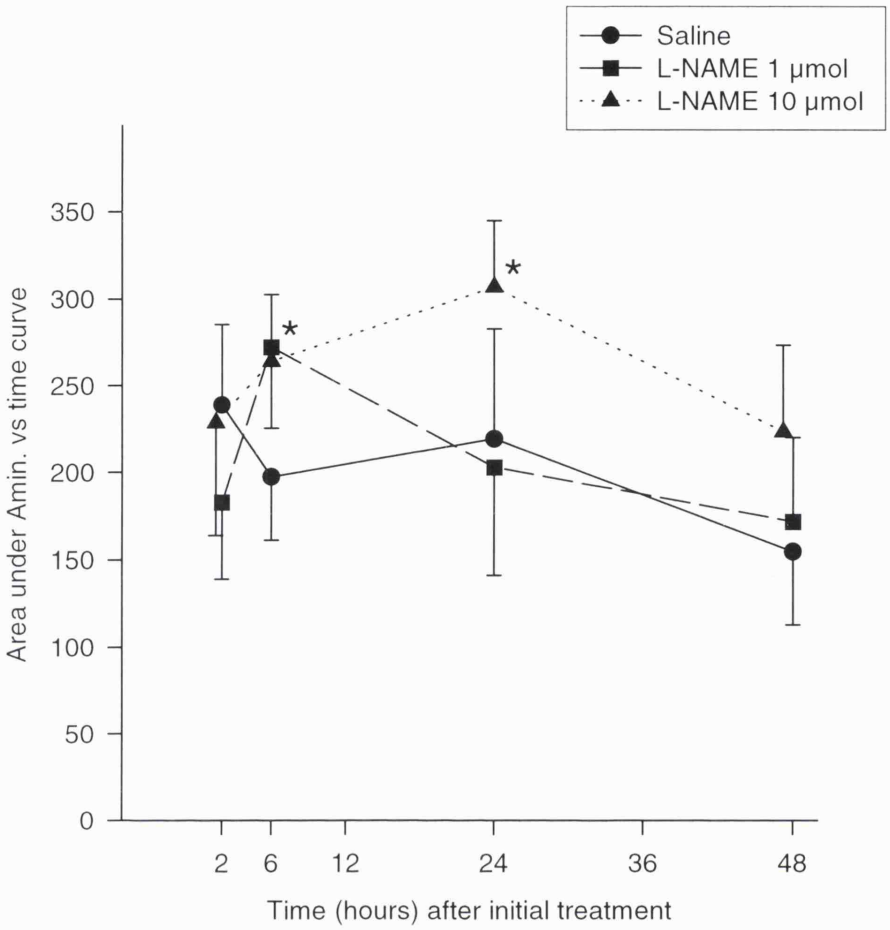

Figure 7.11: Time course for the duration of the hyperresponsiveness, induced by L-NAME, to histamine, 200 µg. The nasal cavity was pretreated with repeated administrations of L-NAME, at the doses shown, every 30 minutes, as described in the experimental protocol. Amin. was measured immediately before, and 2, 5 and 10 minutes after histamine challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve (AUC) was determined. Data are means from 6 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value of Amin. was 0.56±0.03 cm^2. *Significant difference in AUC following histamine challenge after pretreatment with L-NAME, compared to saline control ($p<0.05$, Wilcoxon sign-rank test).

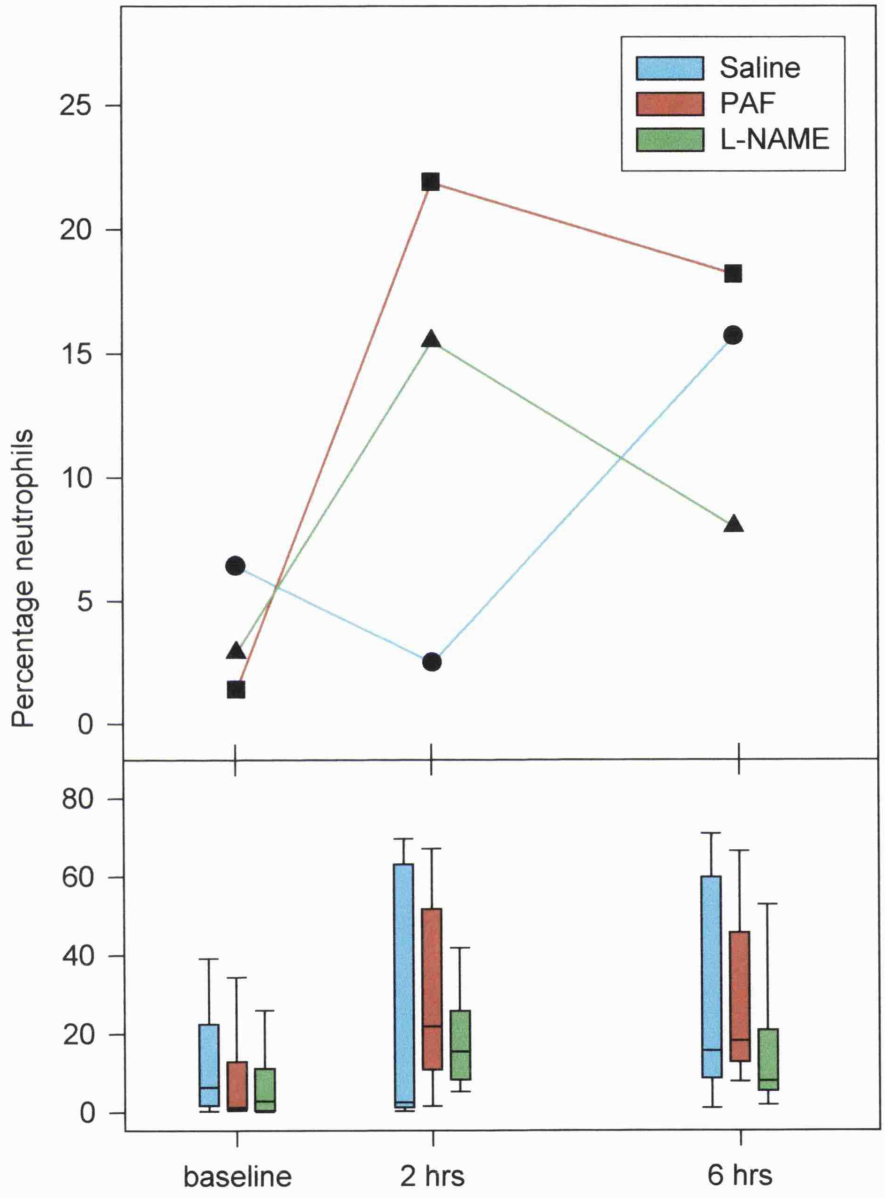

Figure 7.12: The change in the percentage neutrophils isolated from nasal lavage fluid, following treatment of the nasal cavity with saline, PAF, 60 µg, or L-NAME, 1 µmol, as described in the experimental protocol. Data are medians from 9 subjects. The bottom window indicates the interquartile range of values for each median, with the horizontal line indicative of the median value. Vertical bars represent the 80% central range of values.

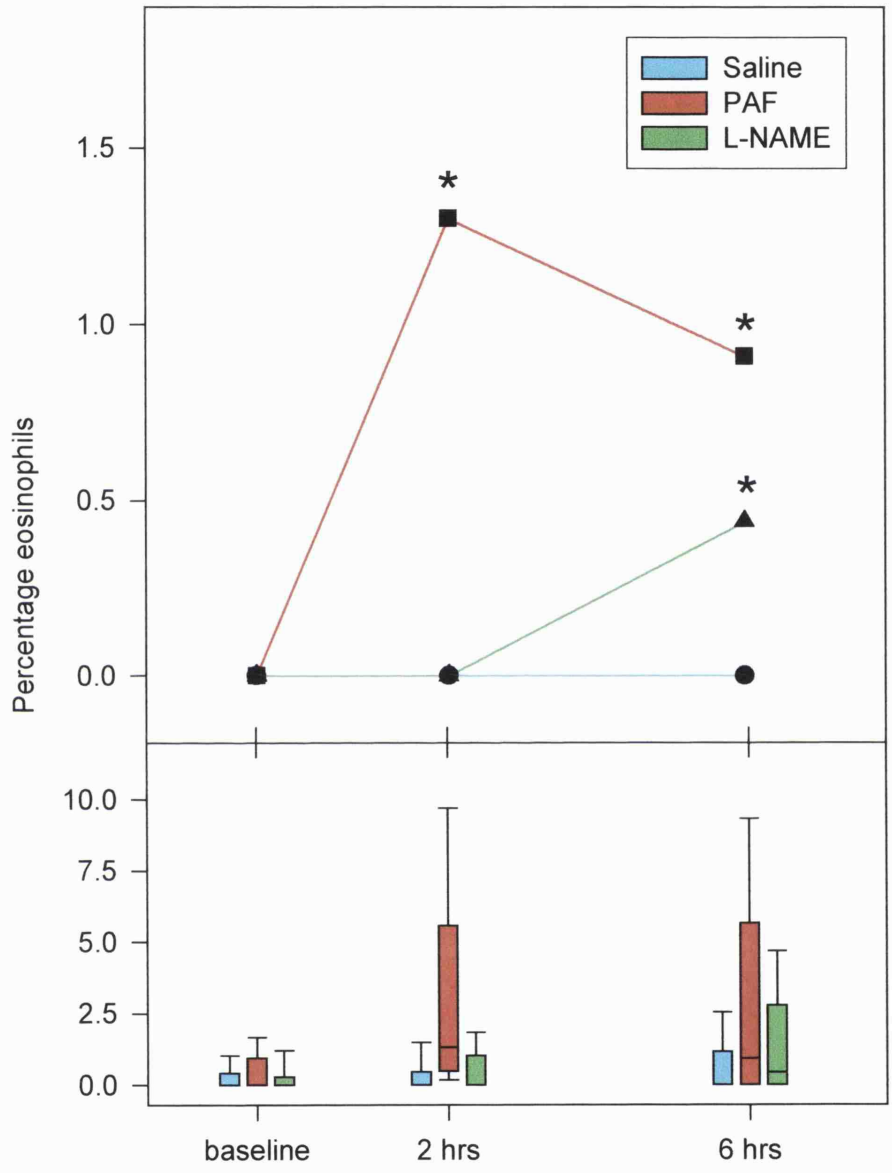

Figure 7.13: The change in the percentage eosinophils isolated from nasal lavage fluid following treatment of the nasal cavity with saline, PAF, 60 µg, or L-NAME, 1 µmol, as described in the experimental protocol. Data are medians from 9 subjects. The bottom window indicates the interquartile range of values for each median, with the horizontal line indicative of the median value. Vertical bars represent the 80% central range of values. *Significant increase in the percentage of eosinophils isolated for the treatment shown, compared to treatment with saline control ($p<0.05$, Wilcoxon sign-rank test).

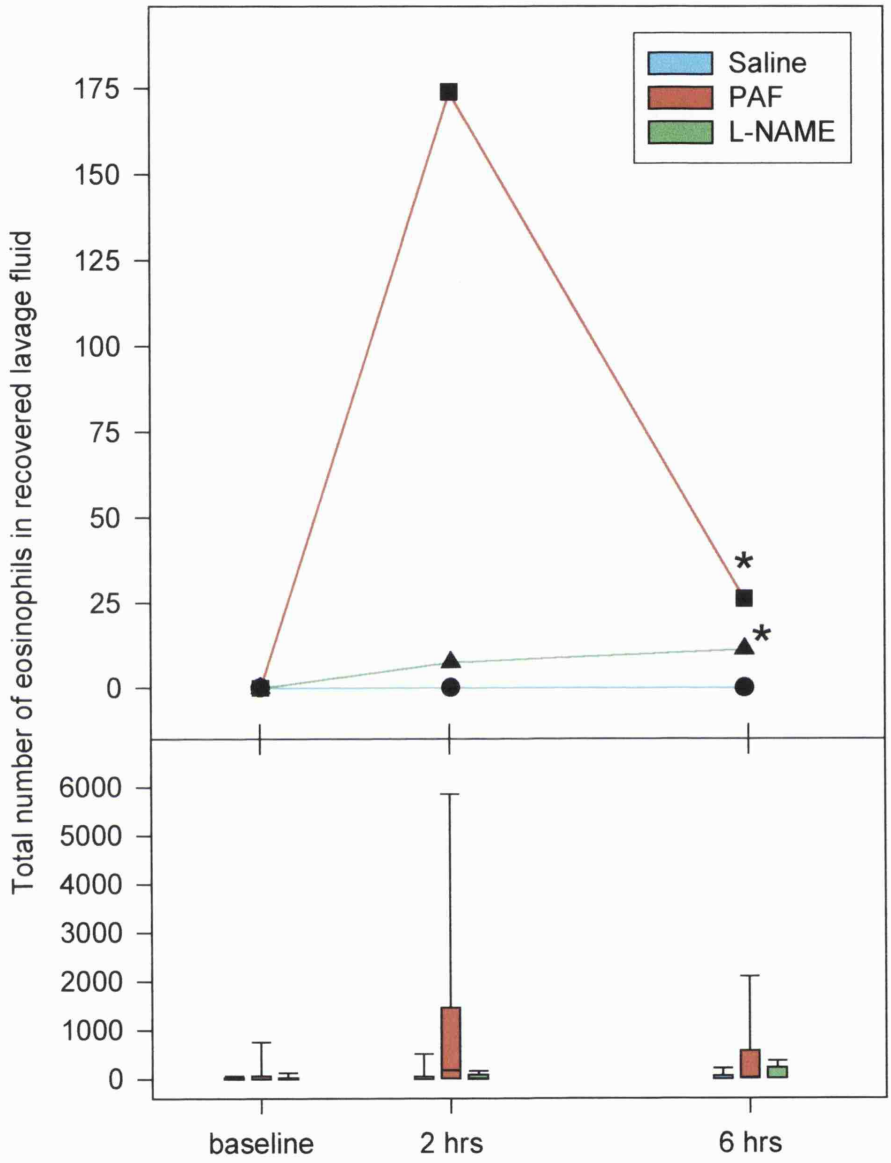

Figure 7.14: The change in the total number of eosinophils isolated in the recovered nasal lavage fluid, following treatment of the nasal cavity with saline, PAF, 60 µg, or L-NAME, 1 µmol, as described in the experimental protocol. Data are medians from 9 subjects. The bottom window indicates the interquartile range of values for each median, with the horizontal line indicative of the median value. Vertical bars represent the 80% central range of values. *Significant increase in the percentage of eosinophils isolated for the treatment shown, compared to treatment with saline control ($p<0.05$, Wilcoxon sign-rank test).

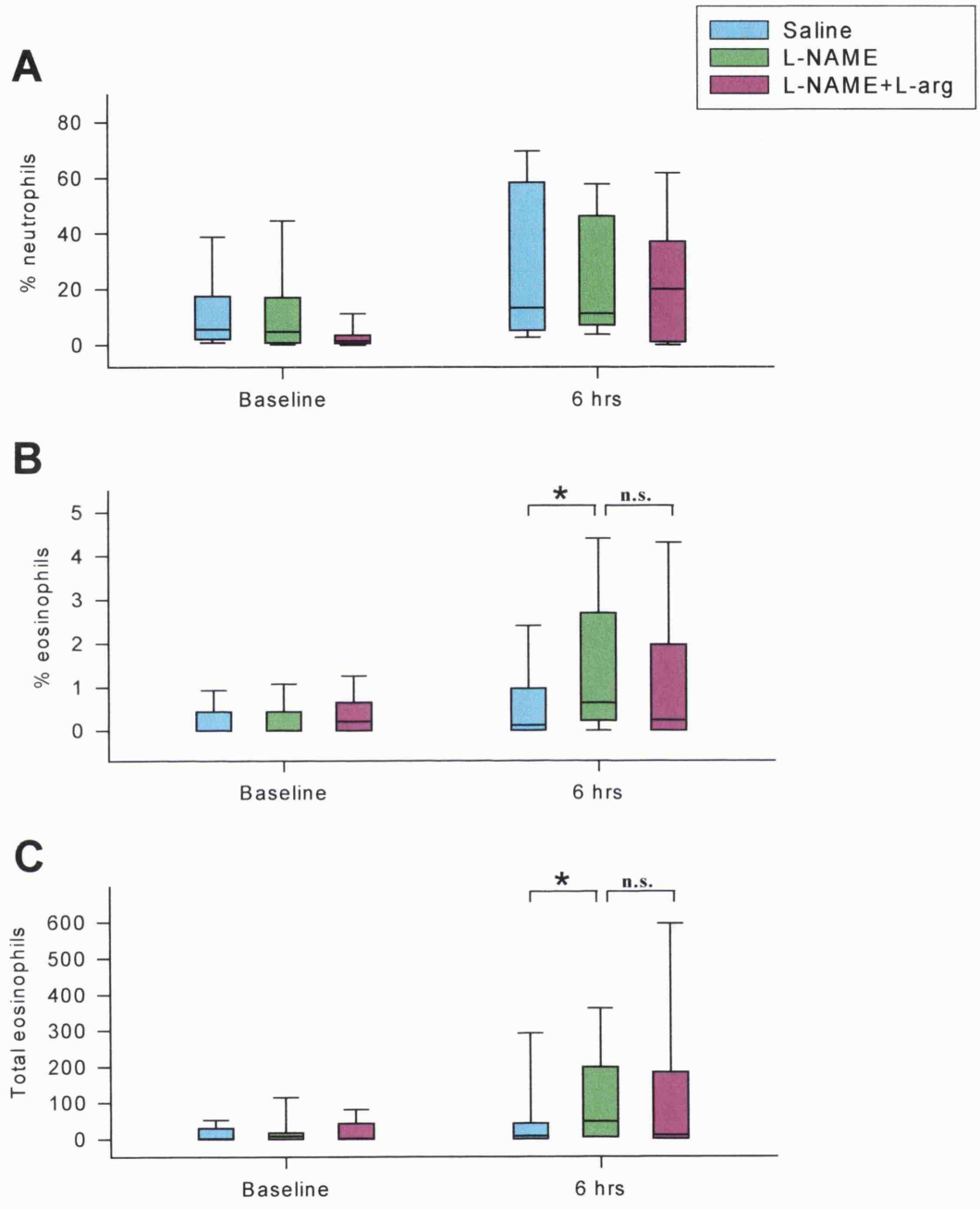

Figure 7.15: Changes in the cytological profile of nasal lavage fluid, following treatment of the nasal cavity with saline, L-NAME, 1 µmol, or L-NAME, 1 µmol, plus L-arginine (L-arg), 30 µmol, as described in the experimental protocol. The changes shown are percentage neutrophils (graph A), percentage eosinophils (graph B) and total number of eosinophils in the lavage sample (graph C). Data are medians from 9 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values. *Significant increase in the cytological marker shown for the treatment described, compared to saline control ($p<0.05$, Wilcoxon sign-rank test). n.s. = not significant.

7.3.3 The role of nitric oxide in PAF-induced nasal airway hyperresponsiveness

PAF, 60 µg, caused a nasal hyperresponsiveness to histamine compared to the saline control (p=0.006, Wilcoxon sign-rank test), which was not altered by treatment with L-NMMA (1 µmol every 30 minutes) nor L-arginine (30 µmol every 30 minutes) (p>0.05, Friedman's test), as shown in Figure 7.16.

7.3.4 The action of L-NAME and PAF on nitric oxide production in the human nasal airway

Treatment of the nasal cavity with L-NAME, 1 µmol every 30 minutes, caused a significant reduction in the amount of NO in the nasal airway (NO_{nasal}) 2 and 6 hours later, compared to the saline control (p=0.006 and p=0.019 respectively, Wilcoxon sign-rank test) (Figure 7.17a). This reduction was not present when NO was measured indirectly by determining the concentration of metabolites of NO in the nasal lavage samples (p>0.05, Wilcoxon sign-rank test) (Figure 7.17b). PAF, 60 µg, did not alter nasal NO levels, measured directly as NO_{nasal} or indirectly in nasal lavage samples, compared to the saline control (p>0.05, Wilcoxon sign-rank test) (Figure 7.18). There were no significant differences in the baseline levels of NO between treatments, in either experiment (p>0.05, Friedman's test).

7.3.5 Effect of icatibant, a bradykinin B_2 receptor antagonist, on L-NAME-induced nasal hyperresponsiveness

The repeated administration of L-NAME, 1 µmol, caused a significant increase in the response to challenge with histamine, 200 µg (p=0.014, Wilcoxon sign-rank test). Pretreatment of the nasal airway with icatibant, 200 µg, failed to prevent the hyperresponsiveness induced by L-NAME (p>0.05, Wilcoxon sign-rank test), as shown in Figure 7.19.

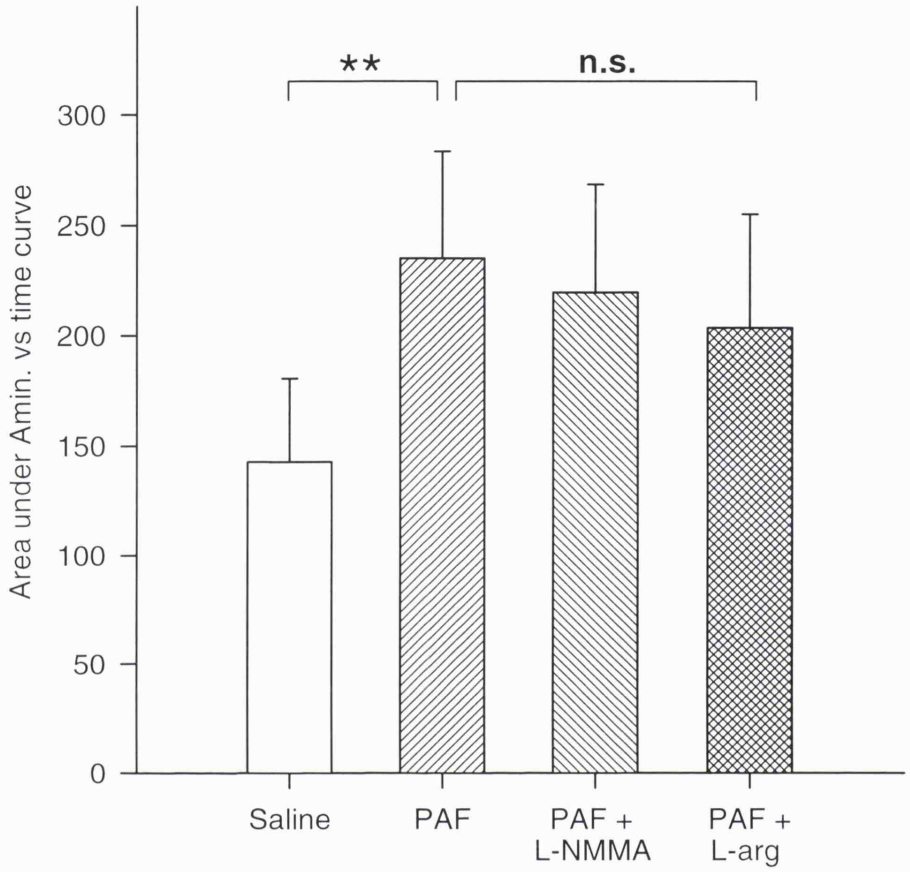

Figure 7.16: Effect of L-NMMA and L-arginine (L-arg) on PAF-induced hyperresponsiveness to histamine, 200 µg. The nasal cavity was pretreated with one of the following: saline, PAF, 60 µg; PAF, 60 µg, plus L-NMMA, 1 µmol; PAF, 60 µg, plus L-arginine (L-arg), 30 µmol, as described in the experimental protocol. Amin. was measured immediately before, and 2, 5 and 10 minutes after histamine challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve (AUC) was determined. Data are means from 10 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value of Amin. was 0.63±0.02 cm^2. **Significant difference in AUC following histamine challenge after pretreatment with PAF, compared to saline control ($p<0.01$, Wilcoxon sign-rank test). n.s. = no significant difference in AUC between treatments shown ($p>0.05$, Friedman's test).

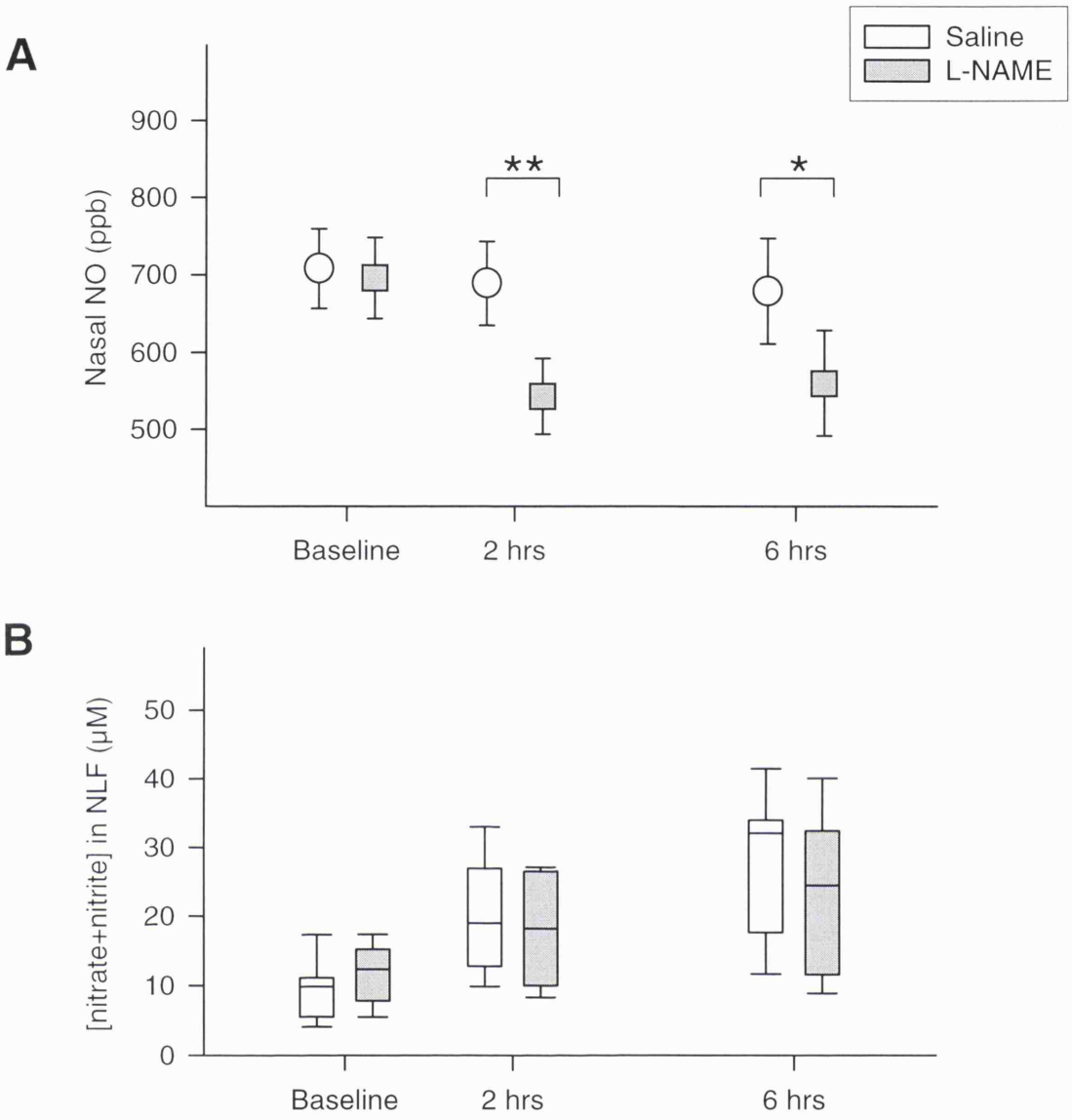

Figure 7.17: Generation of nitric oxide (NO) in the human nasal airway following treatment with L-NAME, 1 µmol, or saline control, every 30 minutes for 5½ hours. In graph A, nasal NO was determined by direct chemiluminescence, and the data is presented as means from 8 subjects. Vertical bars represent s.e.mean. In graph B, NO was measured indirectly, by measuring NO metabolites in nasal lavage fluid (NLF). Data are medians from 8 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values. */**Significant difference in nasal NO following treatment with L-NAME, compared to saline control (*$p<0.05$, **$p<0.01$, Wilcoxon sign-rank test).

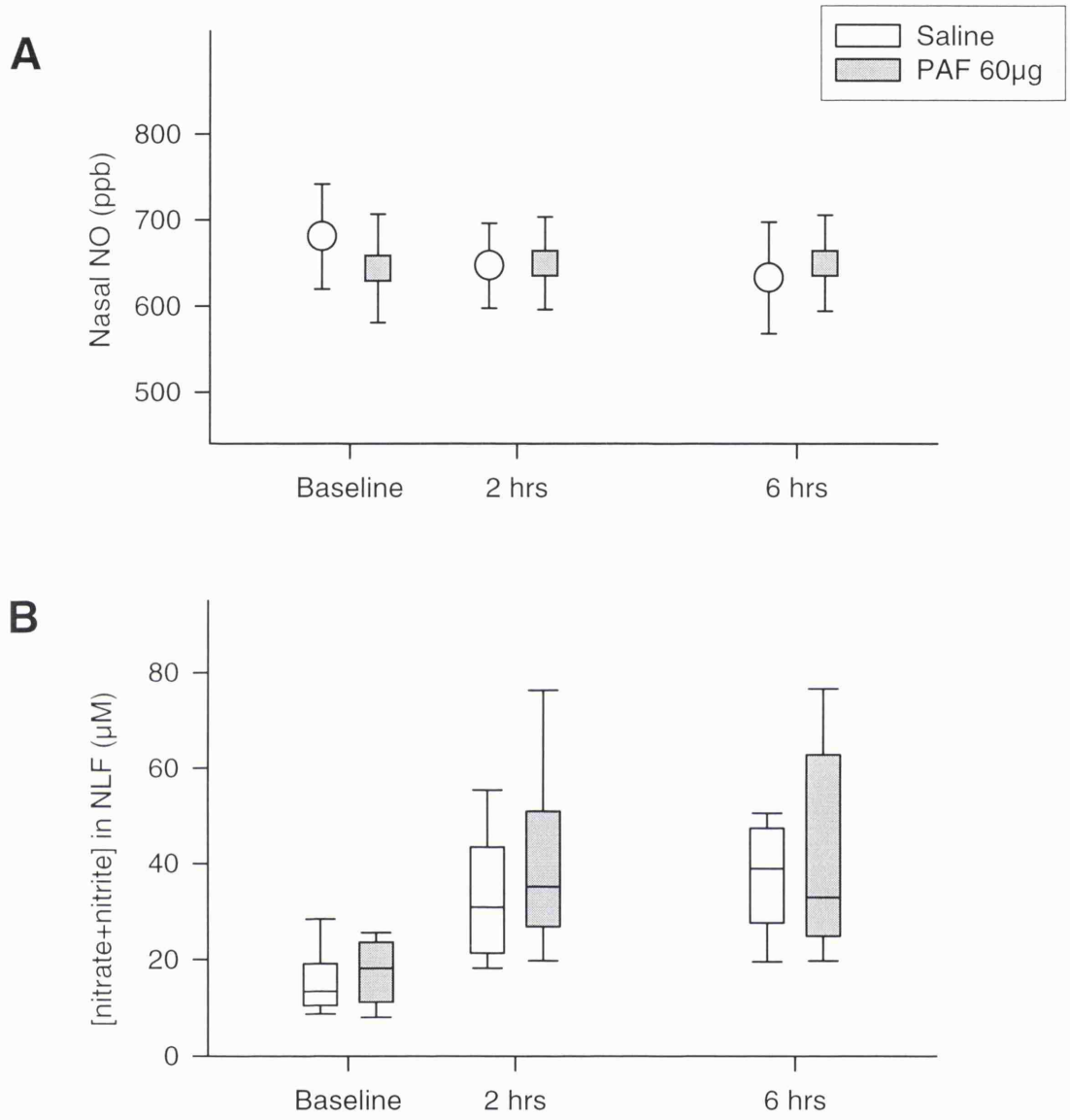

Figure 7.18: Generation of nitric oxide (NO) in the human nasal airway following treatment with PAF, 60 µg, or saline control. In graph A, nasal NO was determined by direct chemiluminescence, and the data is presented as means from 8 subjects. Vertical bars represent s.e.mean. In graph B, NO was measured indirectly, by measuring NO metabolites in nasal lavage fluid (NLF). Data are medians from 8 subjects, indicated by a horizontal line within the interquartile range of values for each median. Vertical bars represent the 80% central range of values.

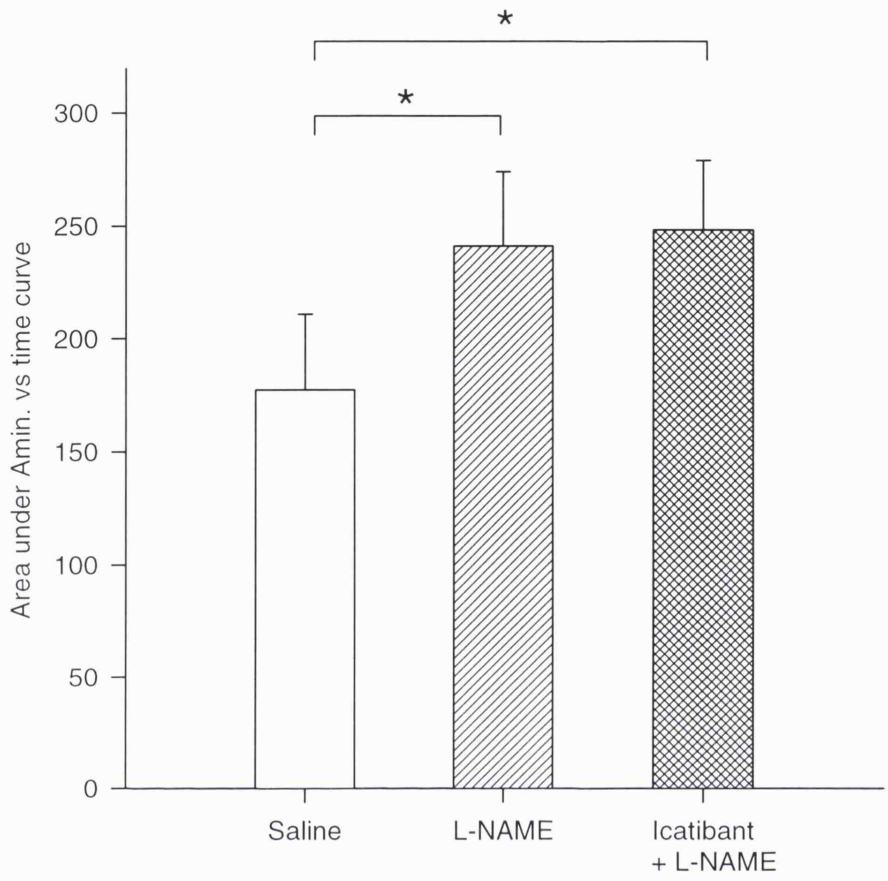

Figure 7.19: Effect of pretreatment with icatibant, a bradykinin B_2 receptor antagonist, on L-NAME-induced hyperresponsiveness to histamine, 200 µg. The nasal cavity was pretreated with saline, icatibant, 200 µg, and/or L-NAME, 1 µmol, as described in the experimental protocol. Amin. was measured immediately before, and 2, 5 and 10 minutes after histamine challenge. Values of Amin. were normalised by expressing them as a percentage decrease in Amin. from baseline for each subject, following which the area under each Amin. against time curve (AUC) was determined. Data are means from 8 subjects. Vertical bars represent s.e.mean. The mean ± s.e.mean baseline value of Amin. was 0.58±0.04 cm^2. *Significant difference in AUC following histamine challenge after treatment shown, compared to saline control ($p<0.05$, Wilcoxon sign-rank test).

7.4 Discussion

In this chapter, it was demonstrated that the NOS inhibitor L-NAME induced a hyperresponsiveness to both histamine and bradykinin, in the human nasal airway. This effect was stereo-selective and abolished by the administration of L-arginine, suggesting that a decrease in the production of basal levels of nitric oxide may induce AHR in the non-allergic, human nasal airway. This hypothesis is further supported by the observation that L-NAME decreased the amount of NO detected in the human nasal airway. Nasal NO production was determined by two methods, either directly, using a chemiluminescence detector, or indirectly, by measuring the concentration of metabolites of NO in nasal lavage fluid. The former measures NO production in both the nasal sinuses and the nasal cavity (Kharitonov *et al.*, 1997a), while the latter is a indirect marker of NO production (Moshage *et al.*, 1995). The decrease in NO levels following treatment with L-NAME was only apparent when measuring NO by chemiluminescence. Since this technique also measures NO from the nasal sinuses, the different results between the two methods may have been due to an inhibition of NO production in the sinuses, but not in the nasal cavity. Indeed, Lundberg *et al.* (1995) found that a single nebulised dose of L-NAME reduced NO production in the sinuses, but not in the nasal cavity. However, it is unlikely that any L-NAME reached the nasal sinuses using the method of administration described in the current study. It is more feasible that measurement by direct chemiluminescence is more sensitive to a decrease in NO production than the other method, which measures metabolites of NO which are chemically stable, and therefore less likely to be altered by L-NAME.

The ability of L-NAME to induce AHR may have been due its action as a weak antagonist at muscarinic receptors (Buxton *et al.*, 1993). However, this is unlikely,

because the L-NAME-induced AHR was prevented by concomitant administration of L-arginine, implying a reversal of NOS inhibition. Furthermore, L-NMMA, a NOS inhibitor with no affinity for the muscarinic receptor, also resulted in AHR to histamine.

Dear *et al.* (1996a) demonstrated that the acute administration of a single dose of L-NAME, at the same concentration used in this study, had no effect on the histamine-induced reduction in Amin., but inhibited the reduction in Amin. induced by bradykinin. The same dose of L-NAME inhibited both histamine- and bradykinin-induced plasma extravasation. In the current study, repeated administration of a NOS inhibitor, over six hours, induced an AHR to both histamine and bradykinin. Therefore, while the normal nasal response to histamine and bradykinin may be mediated, in part, by the generation of NO, chronic inhibition of NOS potentiates the nasal response to these stimuli. The expression of inducible NOS (iNOS) in the nasal airway is strongly linked to the degree of inflammation present (Furukawa *et al.*, 1996), so it is probable that the activity of iNOS in the normal, non-inflamed airway is negligible. Therefore, the AHR may be due to inhibition of one of the constitutive isoforms of NOS present in the nose, and possibly the prolonged inhibition of one of these enzymes. It is possible that the contrasting effects of NOS inhibitors observed in this study and the study by Dear *et al.* (1996a) is due to the inhibition of different isoforms of NOS, though this cannot be confirmed.

Nitric oxide can act as a scavenger of oxidative free radicals, including superoxide (Muijsers *et al.*, 1997). Superoxide and other reactive oxygen species (ROS) have been implicated in the development of AHR in the lower airways of sheep (Lansing *et al.*, 1991), guinea pigs (Ikuta *et al.*, 1992) and cats (Takahashi *et al.*, 1993). In man, exposure to ozone (also a ROS) has been found to cause AHR in the both the lower

and upper nasal airways (Hilterman *et al.*, 1998; Michelson *et al.*, 1999). The majority of NOS is associated with the epithelium in both the nasal (Furukawa *et al.*, 1996) and lower airways (Kobzik *et al.*, 1993) in man. ROS can induce AHR by causing epithelial damage in isolated human bronchi *in vitro*, in the absence of inflammatory cells (Hulsmann *et al.*, 1994). The production of basal levels of NO, by the epithelium, could represent a defence mechanism, conferring a degree of protection against free radical-induced damage to unsensitised airways (Nijkamp & Folkerts, 1995). Therefore, inhibition of epithelium-associated NOS might increase the susceptibility of the epithelium to oxidative damage, resulting in AHR (Nijkamp *et al.*, 1993). Alternatively, inhibiting NOS decreases the production of cyclic GMP (cGMP), which could cause AHR (Sadeghi-Hashjin *et al.*, 1996a). Such a mechanism could result in an increased contractile response of airway muscle to stimuli, since cGMP causes muscle relaxation. However, in the human nasal airway, there is little airway smooth muscle present. Furthermore, a decrease in cGMP production would cause contraction of the nasal vascular smooth muscle, resulting in a fall in obstruction and a reduced response to stimulants such as histamine. Therefore, this mechanism cannot explain the development of AHR in the human nasal airway.

NO may also regulate the responsiveness of the airways, by its action as an inhibitory neurotransmitter of non-adrenergic non-cholinergic (NANC) transmission (Li & Rand, 1991). Neuronal NOS (nNOS) has been identified in the nerves of the human nasal mucosa (Kulkarni *et al.*, 1994). A reduction in NO production by nNOS may reverse NO-mediated inhibition of the NANC system, thus increasing the response of the nasal mucosa to stimuli such as histamine and bradykinin. In allergic rhinitis, the increased response to bradykinin is partly mediated by neuronal reflexes (Riccio and Proud, 1996). Certainly, NO is important in the neuronal control of blood flow in the

nasal mucosa in the guinea pig (Rinder, 1996), and NOS inhibitors alter plasma extravasation in the human nasal airway (Dear *et al.*, 1996a), implying a role for NO in the regulation of the vasculature in the human nasal airway. The study by Dear *et al.* also found that local anaesthetics do not alter the responses to histamine or bradykinin in the normal nasal airway, implying that these actions are not mediated by neuronal activation. However, the study did not look at hyperresponsiveness, where nerves could be involved.

A third mechanism, proposed by Folkerts *et al.* (1995), is that inhibition of NOS causes a shift in the metabolism of arachidonic acid, from products of cyclooxygenase to 5-lipoxygenase, producing leukotrienes which induce AHR. The study found that AA-861, an inhibitor of 5-lipoxygenase, reversed the AHR induced by L-NAME in guinea pig isolated tracheas. However, there are no other reports of the leukotrienes mediating the AHR induced by NOS inhibitors, in either animals or in man. It would be interesting to investigate the effect of a leukotriene antagonist, such as montelukast, on L-NAME-induced AHR in the human nasal airway.

Few studies have investigated the effect of L-NAME on eosinophil recruitment. L-NAME inhibits antigen-induced eosinophilia in the lower airway of the guinea pig (Iijima *et al.*, 1998), mice (Feder *et al.*, 1997) and in rats (Ferreira *et al.*, 1998), and the ability of allergen to cause eosinophil recruitment is significant reduced in iNOS-deficient mice (Xiong *et al.*, 1999). Ferreira *et al.* (1996) demonstrated that eosinophil chemotaxis was significantly lower when the eosinophils were isolated from rats which had received chronic treatment with L-NAME, compared to untreated controls. It is, therefore, surprising that in this study, chronic treatment with L-NAME caused a small, but statistically significant, nasal eosinophilia. This effect is unlikely to be due to any

chemotactic action of L-NAME, since the nasal eosinophilia was not apparent two hours after initial treatment, while PAF, which is chemotactic for eosinophils, did cause an eosinophilia at 2 hours. The eosinophilia may have been caused by the induction of AHR by L-NAME. Alternatively, the eosinophilia, induced by L-NAME, could have been a consequence of an increase in leukotriene production following NOS inhibition as mentioned above, since the leukotrienes, and in particular LTB_4, stimulate eosinophil recruitment (Wardlaw *et al.*, 1986). However, if the eosinophilia was a consequence of NOS inhibition, it is unclear as to why treatment with L-arginine failed to prevent it. L-arginine can potentiate eosinophil recruitment, possibly by increasing IL-5 secretion in the murine airway (Takano *et al.*, 1998), and this could explain the lack of effect of L-arginine in reversing the L-NAME-induced eosinophilia.

Therefore, inhibition of NOS can cause AHR in both the nasal airway, and the lower airways in man (Ricciardolo *et al.*, 1996). Paradoxically, there is a correlation between the amount of NO detected in the airways, and the presence of AHR in both asthmatics and subjects with allergic rhinitis (Kharitonov and Barnes, 1996). The production of NO by the airways in raised in both asthma and allergic rhinitis (Garrelds *et al.*, 1995; Martin *et al.*, 1996; Kharitonov *et al.*, 1997b), and this increase is probably caused by an increase in the expression of iNOS (Kharitonov and Barnes, 1996). NO readily reacts with the superoxide anion to form peroxynitrite (Nijkamp and Folkerts, 1995). This cytotoxic product induces AHR in the guinea pig lower airway both *in vivo* and *in vitro* (Sadeghi-Hashjin *et al.*, 1996b), and its formation in the asthmatic airway is strongly associated with AHR to methacholine (Saleh *et al.*, 1998). Therefore, while a basal level of NO production may be protective against AHR, excessive NO release, possibly mediated by an upregulation of iNOS, may be destructive and cause AHR.

PAF may induce a AHR in the human nasal airway by a mechanism dependent on the generation of free radicals (Austin & Foreman, 1993). Therefore, a NOS inhibitor could, theoretically, potentiate PAF-induced AHR. Alternatively, PAF may upregulate the expression of iNOS, generating higher levels of NO which can combine with superoxide and free radicals to form peroxynitrite, causing AHR. However, in this study, modulating NOS activity, with L-NMMA or L-arginine, had no effect on PAF-induced nasal AHR, nor was an increase in nasal NO production observed after administration of PAF. This is unlikely to be a consequence of the administration of an insufficient amount of L-NMMA and L-arginine, since the same doses modulated the responsiveness of the nasal airway in the absence of PAF. The results therefore suggest that PAF causes AHR via a mechanism independent of NOS activity in the human nasal airway. This is further supported by the finding that icatibant, a bradykinin B_2 receptor antagonist, abolishes PAF-induced nasal AHR, but not the AHR observed following inhibition of NOS.

Using the lower airway of the guinea pig as an experimental model, a number of studies have found that L-NAME does not modulate allergen-induced AHR (De Boer *et al.*, 1996; Schuiling *et al.*, 1998), nor the AHR caused by cationic proteins (Meurs *et al.*, 1999). These results imply that antigen-induced AHR cannot be reduced by inhibiting NOS and therefore, it is unlikely that antigen causes AHR by increasing NO production. However, the authors instead concluded that the AHR was mediated through a decrease in NOS activity, because L-NAME did not further potentiate the antigen- or cationic protein-induced AHR. Unfortunately, the authors did not attempt to reverse the AHR using L-arginine, nor determine whether the dose of antigen, L-NAME or cationic protein used resulted in a maximum level of hyperresponsiveness,

so such a conclusion cannot be justified. In another study, using a murine model of airway allergy, administration of L-arginine did not reverse the antigen-induced AHR (Takano *et al.*, 1998). In fact, antigen-induced AHR may be independent of NOS, since antigen induces the same degree of hyperresponsiveness in iNOS-deficient mice compared to normal controls (Xiong *et al.*, 1999). There is also no evidence to suggest that cationic proteins act through a NO-dependent pathway (Minnicozzi *et al.*, 1995). Therefore, the observation that PAF-induced AHR is not modulated by either L-NMMA or L-arginine is not unexpected.

Finally, although it would appear that NOS does not modulate antigen-induced AHR, it is interesting to note the blocking iNOS-mediated NO production in the guinea pig and murine model of allergic asthma significantly reduces the late phase response to antigen challenge (Iijima *et al.*, 1998; Xiong *et al.*, 1999). Although NOS inhibitors induce AHR in the normal, non-allergic human nasal airway, it would be interesting to investigate their action on the response to antigen in subjects with allergic rhinitis.

Summary

- Intranasal challenge using histamine, 200 µg, caused a significant decrease in the patency of the human nasal airway.

- The nitric oxide synthase (NOS) inhibitors N^G-nitro-L-arginine methyl ester (L-NAME) or N^G-monomethyl-L-arginine (L-NMMA), 1 µmol, given repeatedly over a six hour period, increased the nasal response to histamine. L-NAME also increased the nasal obstruction induced by bradykinin.

- L-arginine, 30 µmol, given together with L-NAME, abolished the ability of L-NAME to cause a nasal hyperresponsiveness to histamine. Pretreatment with N^G-nitro-D-arginine methyl ester (D-NAME), 1 µmol, did not induce hyperresponsiveness to histamine, implying that inhibition of NOS caused the hyperresponsiveness.

- L-NAME failed to cause a hyperresponsiveness when given at a dose of 0.1 µmol. Administration of 10 µmol of L-NAME did not further increase the nasal response to histamine, but did prolong the duration of the hyperresponsiveness.

- L-NAME, 1 µmol, given repeatedly over six hours, significantly reduced the amount of nitric oxide measured in the nasal cavity.

- L-NAME 1 µmol, given repeatedly over six hours, also caused a small, but significant nasal eosinophilia, six hours after initial administration.

- Neither L-NMMA, 1 µmol, nor L-arginine, 30 µmol, altered the nasal hyperresponsiveness induced by platelet activating factor (PAF), 60 µg. PAF did not alter the production of nitric oxide in the nasal cavity.

- The data suggest that inhibition of nitric oxide synthase can induce nasal hyperresponsiveness in man, via a different mechanism to that involved in PAF-induced hyperresponsiveness.

CHAPTER 8

GENERAL DISCUSSION AND FUTURE AIMS

The aim of this study was to investigate the role of kinins and nitric oxide in the development of airway hyperresponsiveness (AHR) in the human nasal airway. The results indicate that both these mediators are involved in this process, and may, therefore, have a role in the AHR observed in allergic rhinitis.

It was demonstrated that in the human nasal airway, the AHR, induced by PAF in non-atopic subjects, or that following a single nasal challenge with grass pollen antigen in sensitive subjects, is dependent upon the production of kinins, and their subsequent action at bradykinin B_2 receptors. Interestingly, a role for kinins in the recruitment of inflammatory cells was also identified, both *in vivo* and *in vitro.* This effect was, apparently, independent of bradykinin receptor activation, and may have resulted from an interaction between neutrophils, the endothelium/epithelium, kinins and IL-8. In particular, the data from chapter 8 indicates that, following nasal antigen challenge, the release of IL-8 is modulated by kinins, and this could explain the ability of icatibant, a bradykinin B_2 receptor antagonist, to reduce both IL-8 production and the nasal eosinophilia induced by antigen.

Therefore, it would be interesting to investigate the ability of kinins to stimulate IL-8 production, both *in vivo* in the human nasal airway, and *in vitro*, from inflammatory cells such as neutrophils, endothelial and epithelial cells, and also the ability of bradykinin antagonists to alter this process. Since the physiological properties of different endothelial cell populations vary (Liu *et al.*, 1996), the *in vitro* experiments investigating cell transmigration should be repeated, using endothelial and epithelial cells isolated from the nasal mucosa, obtained using explant culture techniques. Furthermore, the ability of an IL-8 antibody to modulate both PAF- and antigen-induced eosinophilia should be studied, in order to clarify the role of IL-8 in the potentiation of cell transmigration by kinins. There are also differences in the migratory properties of eosinophils *in vitro*, depending on the method of isolation used

(Rozell *et al.*, 1996). Therefore, it would be appropriate to repeat the transmigration studies using eosinophils isolated using methods other than magnetic cell separation.

One observation which has been made throughout this study is the ability of icatibant and [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin ([Ad]-BK), also a bradykinin B_2 receptor antagonist, to inhibit kinin generation *in vivo*. Other studies have also identified an anti-kallikrein action of bradykinin receptor antagonists (Spragg *et al.*, 1988; Dear *et al.*, 1996b). This action may contribute significantly to the ability of bradykinin receptor antagonists to reduce allergen-induced responses both in animals (Farmer *et al.*, 1992) and in the human nasal airway (Chapter 5; Dear *et al.*, 1996b). It would, therefore, be useful to study the relative abilities of different bradykinin receptor antagonists to inhibit kinin generation, *in vivo* and *in vitro.*

Pretreatment with icatibant also reduced the increase in ECP levels in nasal lavage fluid, following administration of PAF or antigen. While this could be the result of an inhibition of eosinophil activation by icatibant, it is more likely that the results were a consequence of the fall in eosinophil recruitment. However, it might be helpful to confirm this, by investigating the effect of icatibant on eosinophil activation *in vitro.*

The production of kinins by PAF and antigen, and the kinin-dependent development of AHR, could be independent of eosinophil recruitment and activation. In this study, the AHR induced by PAF, antigen, NK-A and the NOS inhibitor L-NAME was associated with a nasal eosinophilia, although the degree of the eosinophilia was mild following NK-A or L-NAME. While icatibant abolished both the AHR and eosinophilia induced by PAF, [Ad]-BK only prevented the AHR. Thus, it is possible to observe both a nasal eosinophilia, and the release of ECP (a marker of eosinophil activation), in the absence of AHR. Similarly, nasal administration of lyso-PAF also causes an eosinophilia without inducing AHR (Tedeschi *et al.*, 1994a). Both these observations can be explained by the kinin-dependent stage of AHR occurring after eosinophil recruitment and activation. However, Klementsson *et al.* (1991) found that the secretory hyperresponsiveness, induced by allergen in the human nasal airway, occurred prior to eosinophil recruitment. Similar observations have been made in both animal models (Chapman *et al.*, 1991; Spina *et al.*, 1991), and in the lower airways of

man (Crimi *et al.*, 1998). In contrast, a number of other studies have identified a clear association between AHR and eosinophils in the nasal airway (Bascom *et al.*, 1989; Terada *et al.*, 1994; De Graaf-in't Veld *et al.*, 1996), thus confirming the data in the current study. The discrepancies between these reports may be due to the type of hyperresponsiveness under investigation (i.e. secretory or airway obstruction), or differences in the mechanism underlying AHR in the acute and chronic allergic state. Eosinophils may have a greater role in the induction of AHR and allergic inflammation, while the maintenance of this state might depend on other factors, such as airway remodeling or neural dysfunction (Crimi *et al.*, 1998; Oddera *et al.*, 1998). These reports imply a role for eosinophil activation in the development of AHR, though it is likely that other, non-eosinophil-derived mediators are involved. It would be interesting to investigate further the kinetics of the AHR induced by different stimuli in the human nasal airway, particularly with respect to eosinophil recruitment and activation.

The development of both PAF- and antigen-induced AHR appears to be dependent on the activation of bradykinin B_2 receptors. This may, in turn, cause the release of neuropeptides which then induce AHR (Kraneveld *et al.*, 1997a). However, PAF did not cause the release of substance P into the nasal cavity, implying that PAF-induced AHR may be independent of neuropeptide release. Alternatively, the airway responsiveness may be modulated by the non-adrenergic non-cholinergic (NANC) nervous system (Barnes, 1989; Joos, 1989). Bradykinin potentiates excitatory NANC (eNANC) responses both *in vitro* (Miura *et al.*, 1992) and *in vivo* (Miura *et al.*, 1994) in the guinea pig lower airway, and this could result in AHR. In order to determine the role of neuropeptides in the development of AHR, it would be necessary to test the effect of specific neurokinin receptor antagonists on the AHR induced by antigen, PAF and other mediators. One could also investigate whether neurokinin receptors are upregulated in allergic rhinitis, by using *ex vivo* nasal tissue in radioligand binding studies, and by measuring a change in the expression of mRNA for tachykinin receptors.

In chapter 7, it was found that inhibitors of nitric oxide synthase (NOS) induce AHR. However, this was not abolished by pretreatment with icatibant. Similarly, PAF-

induced AHR was not modulated by either L-NMMA or L-arginine, implying that the AHR was not dependent on a change in NOS activity. Therefore, the data imply the existence of at least 2 separate mechanisms by which AHR can be induced in the human nasal airway. However, nitric oxide is a mediator of inhibitory NANC (iNANC) transmission (Li & Rand, 1991). Therefore, AHR could result from an increase in eNANC transmission (for example, caused by kinins), or a decrease in iNANC transmission (achieved by administering a NOS inhibitor). Thus, the responsiveness of the nasal airway could be controlled by a single mechanism, involving NANC transmission, although this could be modulated by a number of different transduction pathways. If PAF does indeed cause AHR by increasing eNANC transmission, one might expect L-arginine to reverse PAF-induced AHR, by increasing iNANC transmission. However, L-arginine is a freely-available amino acid (Moncada *et al.*, 1991), so the administration of L-arginine would not necessarily induce iNANC activity, in the absence of NOS inhibition.

It would be interesting to investigate the role of NOS in antigen-induced AHR in the human nasal airway. Schuiling *et al.* (1998) reported that in the guinea pig, NOS inhibition affects the recovery of the lower airways from AHR, and a similar effect could occur in man. Since inhibition of NOS induces AHR, it is possible that the same mechanism could account for the development of AHR in allergic rhinitis. To test this hypothesis, one could measure the relative expression of mRNA for the different NOS isoforms, in nasal biopsies from subjects with active allergic rhinitis and non-allergic controls. Another area for further study is the novel observation that chronic administration of L-NAME into the human nasal airway induces a mild eosinophilia, in contrast to previous observations in animal studies. The effect of NOS inhibitors on the nasal eosinophilia induced by PAF and antigen challenge could be studied, and this would allow comparisons to made as to the mechanisms of eosinophil recruitment in animal models of airway allergy, and in human subjects.

Whether bradykinin B_2 antagonists could be used as a potential treatment for allergic rhinitis and other allergy airway diseases is unclear. Although icatibant abolished the antigen-induced early phase response in perennial allergic rhinitis (Austin *et al.*, 1994), no similar effect occurred with grass pollen antigen in the study described in

chapter 5, confirming previous findings (Akbary & Bender, 1993; Dear, 1996). Currently, seasonal allergic rhinitis is controlled using histamine H_1 antagonists against the early phase response and corticosteroids to inhibit the chronic phase (Parikh & Scadding, 1997). Corticosteroids have a potent effect in inhibiting the development of AHR in the nasal airway (Andersson *et al.*, 1989; Baroody *et al.*, 1992), and almost totally abolish antigen-induced nasal eosinophilia (Bascom *et al.*, 1989; Wang *et al.*, 1997). Therefore, medication currently used to treat seasonal allergic rhinitis is superior to any effect of icatibant observed. However, bradykinin B_2 receptor antagonists may be useful in the treatment of perennial allergic rhinitis, if their duration of action can be prolonged. To this end, a number of non-peptide bradykinin B_2 receptor antagonists are currently under development (Bertrand & Geppetti, 1996). The experiments described in this thesis could be used to assess the potential of these novel compounds to treat allergic rhinitis.

Although kinins are thought to be involved in the pathology of asthma, a large multicentre, clinical trial found that icatibant had little effect as an acute bronchodilator (although there was a small but significant improvement in expiratory peak flow measurements), possibly because bradykinin does not cause significant bronchoconstriction in man (Akbary *et al.*, 1996). So while antagonists at the bradykinin B_2 receptor may be useful in the treatment of perennial allergic rhinitis, the data currently available suggests a limited potential for this class of drugs in the treatment of other allergic airway diseases in man.

In conclusion, the data presented in this study has added to the current understanding of the development of AHR in the human nasal airway. Furthermore, it was observed that kinins are involved in the recruitment of neutrophils and eosinophils, a process which is an integral part of the inflammatory response. Therefore, these results may be useful in the development of pharmacological compounds which can be used to treat chronic inflammation.

CHAPTER 9

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