AN INVESTIGATION INTO THE ACTIONS OF BRADYKININ RECEPTOR ANTAGONISTS AS INHIBITORS OF KALLIKREIN AND THE GENERATION OF KININS WITHIN THE HUMAN NASAL AIRWAY

A thesis submitted by Gideon Agbanoma for the degree of Doctor of Philosophy in the University of London

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I, Gideon Agbanoma, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Gideon Agbanoma.
Abstract

Intranasal challenge of allergic individuals with allergen causes symptoms of nasal blockage and airway hyper-reactivity (AHR). A group of inflammatory peptides, known as kinins, are synthesised in the nasal airway, in response to challenge with allergen and their production coincides with symptom severity. These effects can be modelled in non-allergic individuals with the inflammatory phospholipid platelet-activating factor (PAF). Antagonism of kinin B$_2$ receptors in vivo reduces the recovery of kinins from the nasal airway of allergic individuals challenged with allergen and non-allergic individuals challenged with PAF, and inhibits AHR in both allergic and non-allergic individuals.

In this thesis, the mechanism by which, icatibant, a kinin B$_2$ receptor antagonist, reduces the recovery of kinins from the nasal airway of non-allergic individuals challenged with PAF has been investigated. In addition, the mechanism by which PAF enhances the congestive responses to intranasal challenge with the kinin, bradykinin, and the mast cell-derived mediator, histamine, has been investigated.

The effect of various kinin receptor antagonists on the recovery of albumin and kinins, by nasal lavage, from individuals challenged with PAF were determined by ELISA and radioimmunoassay respectively.

The effect of various kinin receptor antagonists on the enzyme activity of tissue kallikrein, plasma kallikrein and crude nasal lavage fluid were investigated by spectrophotometry with the chromogenic substrates S-2266 and S-2302.

Nasal blockage was measured objectively using acoustic rhinometry.

The data in this thesis has shown that icatibant potently inhibits the recovery of kinins from PAF challenged non-atopic individuals in vivo. This effect does not appear to be dependant on the inhibition of plasma or tissue kallikrein activity, or the antagonism of kinin B$_2$ receptors which control the influx of prekallikrein and kininogen into the nasal airway. In addition, there is evidence to suggest that nerves within the human nasal airway may play a role in PAF-induced AHR.
# CONTENTS

Title page 1

Declaration 2

Abstract 3

Contents 4

Acknowledgments 13

Abbreviations 14

List of figures 17

List of tables 26

## CHAPTER 1 – INTRODUCTION 27

1.1 Introduction 27

1.2 Anatomy and physiology of the human nasal airway 27
   1.2.1 The nasal cavity 27
   1.2.2 Nasal glands and secretions 29
   1.2.3 The microvasculature 29
   1.2.4 The autonomic and sensory nervous system 31
   1.2.5 The sympathetic nervous system 33

1.3 Allergic rhinitis 35
   1.3.1 Pathophysiology 36
      1.3.1.1 Sensitisation 36
      1.3.1.2 The immediate response 38
         1.3.1.2.1 Mast cell activation 39
         1.3.1.2.2 Change in vascular dynamics 40
         1.3.1.2.3 Activation of sensory-parasympathetic reflex 41
   1.3.1.2.4 Inhibition of sympathetic activity 42
1.4.4 The role of kinins, kallikrein and kininases in the nasal airway

1.4.5 Kinin B2 Receptor Antagonists

1.5 Aims of the project

CHAPTER 2 – MATERIALS AND METHODS

2.1 Techniques which can be used to study the nasal airway
   2.1.1 Estimation of nasal airflow
   2.1.2 Estimation of nasal blood flow
   2.1.3 Estimation of nasal patency
   2.1.4 Measurement of nasal inflammation and mediator production
   2.1.5 Symptoms scores

2.2 Nasal challenge experiments
   2.2.1 Subjects
   2.2.2 Dilution of challenge agents
   2.2.3 Nasal challenge
   2.2.4 Nasal lavage
   2.2.5 Acoustic Rhinometry
   2.2.6 Data analysis
   2.2.7 Statistical analysis

2.3 Endothelial Cells

2.4 Biochemical assays
   2.4.1 Albumin enzyme-linked immunosorbent assay
   2.4.2 slgA enzyme-linked immunosorbent assay
   2.4.3 Bradykinin Radioimmunoassay
   2.4.4 Bradford assay
   2.4.5 Enzyme activity assay

2.5 Enzyme assays
   2.5.1 Crude nasal lavage fluid
   2.5.2 Purified active enzyme
      2.5.2.1 Porcine tissue kallikrein
      2.5.2.2 Human plasma kallikrein
      2.5.2.3 Human pancreatic trypsin
2.5.3 Enzyme activation assays 100
  2.5.3.1 Prekallikrein activation assays 100
  2.5.3.2 Human plasma activation assays 101
2.6 Statistical analysis of biochemical assays 102
2.7 Spectrophotometry 102
2.8 Materials 103

CHAPTER 3 – THE EFFECT OF ICATIBANT ON PAF-INDUCED KININ PRODUCTION 108

3.1 Introduction 108
3.2 Experimental protocol 110
  3.2.1 The effect of PAF on kallikrein-like activity, albumin, slgA and kinin production 110
  3.2.2 The effect of methacholine on kallikrein-like enzyme activity 111
  3.2.3 The effect of icatibant on PAF-induced albumin and kinin production 111
  3.2.4 The effect of icatibant on the binding of bradykinin to rabbit anti-bradykinin serum 112
  3.2.5 The effect of NPC-567 on albumin and kinin production 113
  3.2.5 The effect of aprotinin on albumin and kinin production 113
  3.2.6 The effect of des-arg^{10}-leu^{9}-kallidin on albumin and kinin production 114
  3.2.7 Data analysis 115
3.3 Results 117
  3.3.1 The effect of PAF on kallikrein-like activity, albumin, slgA and kinin production 117
  3.3.2 The effect of methacholine on kallikrein enzyme activity 117
  3.3.3 The effect of icatibant on PAF-induced albumin and kinin production 123
3.3.4 The effect of icatibant on the binding of bradykinin to rabbit anti-bradykinin serum 123
3.3.5 The effect of NPC-567 on albumin and kinin production 123
3.3.6 The effect of aprotinin on albumin and kinin production 128
3.3.7 The effect of des-arg^{10}-leu^{9}-kallidin on albumin and kinin production 128

3.4 Discussion 135
3.5 Summary 140

CHAPTER 4 – THE EFFECT OF KININ RECEPTOR ANTAGONISTS ON KALLIKREIN ACTIVITY 142

4.1 Introduction 142
4.2 Molecular theory 143
4.3 Experimental protocol 144
  4.3.1 Enzyme assay 144
  4.3.2 Data Analysis 144
4.3 Results 145
  4.3.1 The effect of human plasma kallikrein concentration on the cleavage of S-2302 145
  4.3.2 The effect of porcine tissue kallikrein concentration on the cleavage of S-2266 145
  4.3.3 The effect of S-2302 concentration on human plasma kallikrein enzyme activity 145
  4.3.4 The effect of S-2266 concentration on porcine tissue kallikrein enzyme activity 148
  4.3.5 The effect of kinin receptor ligands on human plasma kallikrein enzyme activity 154
  4.3.6 The effect of kinin receptor ligands on porcine tissue kallikrein enzyme activity 155
4.4 Discussion 164
4.5 Summary 169
CHAPTER 5 – CHARACTERISATION OF THE KININ-FORMING ENZYME IN CRUDE NASAL LAVAGE FLUID

5.1 Introduction 170
5.2 Molecular theory 171
5.3 Experimental protocol 171
  5.3.1 Collection of nasal lavage fluid 171
  5.3.2 Enzyme assay 171
  5.3.3 Data analysis 171
5.4 Results 173
  5.4.1 The cleavage of S-2266 by crude nasal lavage fluid and human plasma kallikrein 173
  5.4.2 The cleavage of S-2302 by crude nasal lavage fluid and human plasma kallikrein 176
  5.4.3 The effect of soybean trypsin inhibitor and aprotinin on crude nasal lavage fluid enzyme activity 176
  5.4.4 The effect of soybean trypsin inhibitor and aprotinin on human plasma kallikrein enzyme activity 180
  5.4.5 The effect of soybean trypsin inhibitor and aprotinin on porcine tissue kallikrein enzyme activity 184
  5.4.6 The effect of kinin receptor ligands on the cleavage of S-2266 by crude nasal lavage fluid 184
5.5 Discussion 187
5.6 Summary 191

CHAPTER 6 - THE EFFECT OF ICATIBANT ON PREKALLIKREIN ACTIVATION

6.1 Introduction 192
6.2 Experimental protocol 194
  6.2.1 Collection of human plasma 194
  6.2.2 Human plasma activation and prekallikrein activation assays 194
6.2.2.1 The kinetics of prekallikrein activation in the presence and absence of Zn$^{2+}$ and FXII 195
6.2.2.2 Activation of prekallikrein by Heat Shock Protein 90 195
6.2.2.3 The effect of icatibant on prekallikrein activation in the presence of HSP90 195
6.2.2.4 Activation of prekallikrein by hyaluronic acid binding protein and FXII 196
6.2.2.5 The effect of icatibant on prekallikrein activation in the presence of FXII 196
6.2.2.6 Potentiation of prekallikrein activation by FXII with endothelial cell lysate 196
6.2.2.7 Prekallikrein activation with endothelial cell lysate and without FXII or Zn$^{2+}$ 197
6.2.2.8 The effect of icatibant on prekallikrein activation in the presence of FXII and endothelial cell lysate 197
6.2.2.9 Activation of human plasma by endothelial cell lysate and Zn$^{2+}$ 197
6.2.2.10 The effect of icatibant on the activation of human plasma in the presence of Zn$^{2+}$ and endothelial cell lysate 198
6.2.3 Human pancreatic trypsin enzyme assay 198
6.2.4 Data analysis 198
6.3 Results 200
6.3.1 The kinetics of prekallikrein activation in the presence and absence of Zn$^{2+}$ and FXII 200
6.3.2 Activation of prekallikrein by Heat Shock Protein 90 200
6.3.3 The effect of icatibant on prekallikrein activation in the presence of HSP90 200
6.3.4 Activation of prekallikrein by hyaluronic acid binding protein and FXII 204
6.3.5 The effect of icatibant on prekallikrein activation in the presence of FXII 204
6.3.6 Potentiation of prekallikrein activation by FXII with endothelial cell lysate 204
6.3.7 Prekallikrein activation with endothelial cell lysate and 208
without FXII or Zn$^{2+}$

6.3.8 The effect of icatibant on prekallikrein activation in the presence of FXII and endothelial cell lysate

6.3.9 Activation of human plasma by endothelial cell lysate and Zn$^{2+}$

6.3.10 The effect of icatibant on the activation of human plasma in the presence of Zn$^{2+}$ and endothelial cell lysate

6.3.11 The effect of S-2765 concentration on human pancreatic trypsin enzyme activity

6.3.12 The effect of kinin receptor ligands on human pancreatic trypsin enzyme activity

6.4 Discussion

6.5 Summary

CHAPTER 7 – KININ RECEPTORS AND AIRWAY HYPERREACTIVITY

7.1 Introduction

7.2 Experimental protocol

7.2.1 The effect of bradykinin and captopril on histamine-induced nasal blockage

7.2.2 The duration of PAF-induced airway hyperreactivity

7.2.3 The role of kinin B$_1$ receptors in PAF-induced nasal airway hyperreactivity

7.2.4 The effect of unilateral pre-treatment with PAF on contralateral histamine-induced nasal blockage

7.2.5 The effect of unilateral pre-treatment with PAF on contralateral bradykinin-induced nasal blockage

7.2.6 The effect of bilateral challenge with PAF on unilateral histamine-induced nasal blockage

7.2.7 Data analysis

7.3 Results

7.3.1 The effect of bradykinin and captopril on histamine-induced nasal blockage
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.3.2 The time-course of PAF-induced airway hyperreactivity</td>
<td>231</td>
</tr>
<tr>
<td>7.3.3 The role of kinin B₁ receptors in PAF-induced nasal airway</td>
<td>231</td>
</tr>
<tr>
<td>hyperreactivity</td>
<td></td>
</tr>
<tr>
<td>7.3.4 The effect of unilateral pre-treatment with PAF on contralateral</td>
<td>236</td>
</tr>
<tr>
<td>histamine-induced nasal blockage</td>
<td></td>
</tr>
<tr>
<td>7.3.5 The effect of unilateral pre-treatment with PAF on contralateral</td>
<td>239</td>
</tr>
<tr>
<td>bradykinin-induced nasal blockage</td>
<td></td>
</tr>
<tr>
<td>7.3.6 The effect of bilateral challenge with PAF on unilateral histamine</td>
<td>243</td>
</tr>
<tr>
<td>induced nasal blockage</td>
<td></td>
</tr>
<tr>
<td>7.4 Discussion</td>
<td>248</td>
</tr>
<tr>
<td>7.5 Summary</td>
<td>253</td>
</tr>
</tbody>
</table>

**CHAPTER 8 - GENERAL DISCUSSION AND FUTURE AIMS** 254

**CHAPTER 9 – REFERENCES** 258
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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₁-AT</td>
<td>α₁-antitrypsin</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>[Ad]-BK</td>
<td>[1-adamantaneacetyl-D-Arg⁰, Hyp³-(2-thienyl)-Ala⁵,⁸, D-Phe⁷]-bradykinin.</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyper-reactivity</td>
</tr>
<tr>
<td>ΔAlbumin</td>
<td>Difference in two concentrations of albumin</td>
</tr>
<tr>
<td>Amin.</td>
<td>Minimum cross sectional area</td>
</tr>
<tr>
<td>AMPSF</td>
<td>p-Amidinophenyl-methanesulfonyle fluoride hydrochloride</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>AVA</td>
<td>Arterio-venous anastamoses</td>
</tr>
<tr>
<td>BK</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDX</td>
<td>Cluster of differentiation X</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene related peptide</td>
</tr>
<tr>
<td>CK1</td>
<td>Cytokeratin 1</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclo-oxygenase</td>
</tr>
<tr>
<td>CPN</td>
<td>Carboxypeptidase N</td>
</tr>
<tr>
<td>CystLT</td>
<td>Cysteinyl leukotriene receptor</td>
</tr>
<tr>
<td>DAKD</td>
<td>Des-arg¹⁰-kallidin</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>Der p</td>
<td>Dermatophagoides pteronyssinus</td>
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<tr>
<td>ECP</td>
<td>Eosinophil cationic protein</td>
</tr>
<tr>
<td>EDN</td>
<td>Eosinophil derived neurotoxin</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EFS</td>
<td>Electrical field stimulation</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EPO</td>
<td>Eosinophil peroxidase</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen binding fragment</td>
</tr>
<tr>
<td>Fc</td>
<td>Crystallizable fragment</td>
</tr>
<tr>
<td>FcɛRI</td>
<td>Crystallizable fragment epsilon receptor 1</td>
</tr>
<tr>
<td>FXI</td>
<td>Factor XI</td>
</tr>
<tr>
<td>FXII</td>
<td>Factor XII (unactive)</td>
</tr>
<tr>
<td>FXIIa</td>
<td>Factor XIa (active)</td>
</tr>
<tr>
<td>FXIIf</td>
<td>Factor XIIf (active fragment)</td>
</tr>
<tr>
<td>gC₁qR</td>
<td>Receptor for globular head of complement fragment 1</td>
</tr>
<tr>
<td>HABP</td>
<td>Hyaluronic acid binding protein</td>
</tr>
<tr>
<td>HDM</td>
<td>House dust mite</td>
</tr>
<tr>
<td>HF</td>
<td>Hageman Factor</td>
</tr>
<tr>
<td>Hfα</td>
<td>Hageman Factor (active)</td>
</tr>
<tr>
<td>HMWK</td>
<td>High molecular weight kininogen</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
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<tr>
<td>IC₅⁰</td>
<td>The half maximal inhibitory concentration</td>
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<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
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<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>Immunoglobulin E</td>
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<td>IL-X</td>
<td>Interlukin-X</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>KBP</td>
<td>Kallikrein binding protein</td>
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<td>$k_{cat}$</td>
<td>Catalytic constant</td>
</tr>
<tr>
<td>KD</td>
<td>Kallidin</td>
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<tr>
<td>KD</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Dissociation Inhibition constant</td>
</tr>
<tr>
<td>$\Delta$Kinin</td>
<td>Difference in two concentrations of kinin</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis Menten constant</td>
</tr>
<tr>
<td>LDV</td>
<td>Laser Doppler Velocimetry</td>
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<tr>
<td>LFA-1</td>
<td>Lymphocyte Function Antigen-1</td>
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<tr>
<td>LMWK</td>
<td>Low molecular weigh kininogen</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>HSP90</td>
<td>Heat Shock Protein 90</td>
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<tr>
<td>LPR</td>
<td>Late phase response</td>
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<td>LTB$_4$</td>
<td>Leukotriene B$_4$</td>
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<tr>
<td>LysoPAF</td>
<td>Lyso platelet activating factor</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>Major Basic Protein</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
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<tr>
<td>MHC II</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>MIP-1$\alpha$</td>
<td>Macrophage inflammatory protein 1 alpha</td>
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<td>MIP-1$\beta$</td>
<td>Macrophage inflammatory protein 1 beta</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NANC</td>
<td>Non-adrenergic non-cholinergic</td>
</tr>
<tr>
<td>NEP</td>
<td>Neutral endopeptidase</td>
</tr>
<tr>
<td>nH</td>
<td>Hill coefficient</td>
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<tr>
<td>NK$_1$</td>
<td>Neurokinin 1 receptor</td>
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<td>NK$_2$</td>
<td>Neurokinin 1 receptor</td>
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<td>NK$_3$</td>
<td>Neurokinin receptor 3</td>
</tr>
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<td>NKA</td>
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</tr>
<tr>
<td>NKB</td>
<td>Neurokinin B</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<td>NPC 567</td>
<td>[D-Arg$^5$, Hyp$^3$, D-Phe$^7$]-bradykinin</td>
</tr>
<tr>
<td>NPEF</td>
<td>Nasal peak expiratory flow</td>
</tr>
<tr>
<td>NPIF</td>
<td>Nasal peak inspiratory flow</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NPY Y$_X$</td>
<td>Neuropeptide Y receptor X</td>
</tr>
<tr>
<td>Oic</td>
<td>Octahydroindole-2-carboxylic acid</td>
</tr>
<tr>
<td>$\Delta$mOD</td>
<td>Difference between two milli optical density values</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
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<td>PAR</td>
<td>Protease activated receptor</td>
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<tr>
<td>PGD$_2$</td>
<td>Prostaglandin D$_2$</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>$pK_i$</td>
<td>-$\log$ dissociation Inhibition constant</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation, normal T cell expressed and secreted</td>
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<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
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<tr>
<td>SBTI</td>
<td>Soybean trypsin inhibitor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SCG</td>
<td>Supercervical ganglion</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>Standard error of the mean</td>
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<td>Transforming growth factor beta</td>
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<td>Very Late Antigen-4</td>
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<tr>
<td>Vmax</td>
<td>Maximum velocity</td>
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LIST OF FIGURES

Figure 1.1) The lateral wall of the human nasal cavity.

Figure 1.2) The anatomy of the human nasal mucosa.

Figure 1.3) A schematic diagram of the neuronal arrangement within the nasal mucosa.

Figure 1.4) The amino acid sequence of VIP.

Figure 1.5) The amino acid sequence of NPY.

Figure 1.6) The amino acid sequence of SP, NKA and NKB.

Figure 1.7) The amino acid sequence of α-human CGRP and β-human CGRP.

Figure 1.8) The synthesis and metabolism of PAF.

Figure 1.9) The amino acid sequence of bradykinin and kallidin.

Figure 1.10) The domains within HMWK.

Figure 1.11) The structure of FXIIa and its cleavage sites.

Figure 1.12) The activation of prekallikrein by FXII and FXIIIf on artificial surfaces.

Figure 1.13) A summary of the kallikrein-kinin pathway.

Figure 2.1) Diagram of a syringe, with a quill tube and medical grade silicone attached, used for nasal lavage.

Figure 2.2) General protocol for studying the effect of intranasal challenge on mediator production on in the nasal airway.
Figure 2.3) A map of the cross-sectional area of a nasal cavity prior and post nasal blockage.

Figure 2.4) The time course of bradykinin-induced nasal blockage.

Figure 2.5) The effect of bilateral intranasal challenge with saline or bradykinin on nasal patency.

Figure 2.6) Standard curve for albumin ELISA.

Figure 2.7) Standard curve for secretory IgA ELISA.

Figure 2.8) Standard curve for bradykinin RIA.

Figure 2.9) Standard curve for Bradford assay.

Figure 2.10) Amino acid composition of bradykinin, HMWK(Arg\textsuperscript{362}-Ser\textsuperscript{371}) and S-2302.

Figure 2.11) A graph showing the dose-dependent aprotinin-induced inhibition of S-2266, 400 \(\mu\)M, cleavage by crude nasal lavage fluid.

Figure 2.12) An inhibition curve showing the effect of aprotinin on the cleavage of S-2266 400 \(\mu\)M, by crude nasal lavage fluid.

Figure 3.1) The amino acid sequence of the kinin B\textsubscript{2} receptor agonist, bradykinin, and the kinin B\textsubscript{2} receptor antagonists, NPC 567 and icatibant.

Figure 3.2) Protocol for investigating effect of PAF on kallikrein enzyme activity, albumin production, sIgA levels and kinin content of lavage fluid from the nasal airway.

Figure 3.3) Protocol for investigating effect of methacholine on kallikrein enzyme activity of lavage fluid from the nasal airway.

Figure 3.4) Protocol for investigating the effect of icatibant on PAF-induced albumin and kinin production within the nasal airway.
Figure 3.5) Protocol for investigating the effect of NPC-567 on PAF-induced albumin and kinin production within the nasal airway.

Figure 3.6) Protocol for investigating the effect of aprotinin on PAF-induced mediator production in the nasal cavity.

Figure 3.7) Protocol for investigating the effect of des-arg^{10}-leu^{9}-kallidin on PAF-induced albumin and kinin production within the nasal airway.

Figure 3.8) The effect of intranasal challenge with PAF, 60 µg, or saline on the kinin content of nasal lavage fluid recovered prior to and 20 minutes after challenge.

Figure 3.9) Enzyme activity in nasal lavage fluid recovered 20 minutes after intranasal challenge with PAF, 60 µg, or saline.

Figure 3.10) The effect of intranasal challenge with PAF, 60 µg, or saline on the secretory IgA content of nasal lavage fluid recovered prior to and 20 minutes after challenge.

Figure 3.11) The effect of intranasal challenge with PAF, 60 µg, or saline on the albumin content of nasal lavage fluid recovered prior to and 20 minutes after challenge.

Figure 3.12) The effect of saline or methacholine (1, 3 and 10 mg) on the kallikrein enzyme activity of nasal lavage fluid recovered 10 minutes after intranasal challenge.

Figure 3.13) The effect of intranasal challenge with PAF, 60 µg, or saline on the kinin content of nasal lavage fluid after treatment with saline or icatibant.

Figure 3.14) The effect of intranasal challenge with PAF, 60 µg, or saline challenge on the albumin content of nasal lavage fluid after pre-treatment with saline or icatibant.
Figure 3.15) The effect of icatibant on the binding of rabbit anti-bradykinin serum to bradykinin, 11 µg/100 µl.

Figure 3.16) The effect of intranasal challenge with PAF, 60 µg, or saline on the kinin content of nasal lavage fluid after pre-treatment with saline or NPC-567, 200 µg.

Figure 3.17) The effect of intranasal challenge with PAF, 60 µg, or saline treatment on the albumin content in nasal lavage fluid after treatment with saline or NPC-567, 200 µg.

Figure 3.18) The effect of intranasal challenge with PAF, 60 µg, or saline on the kinin content of nasal lavage fluid after pre-treatment with saline or aprotinin, 100 µg.

Figure 3.19) The effect of intranasal challenge with PAF, 60 µg, or saline treatment on the albumin content of nasal lavage fluid after treatment with saline or aprotinin, 100 µg.

Figure 3.20) The effect of intranasal challenge with PAF, 60 µg, or saline on the kinin content of nasal lavage fluid after pre-treatment with saline or des-arg\(^{10}\)-leu\(^9\)-kallidin, 200 µg.

Figure 3.21) The effect of intranasal challenge with PAF, 60 µg, or saline on the albumin content of nasal lavage fluid after pre-treatment with saline or des-arg\(^{10}\)-leu\(^9\)-kallidin, 200 µg.

Figure 4.1) A graph showing the effect of human plasma kallikrein concentration on the cleavage of S-2302, 100 µM.

Figure 4.2) A graph showing the effect of porcine tissue kallikrein concentration on the cleavage of S-2266, 10 µM.

Figure 4.3) A Michaelis-Menten plot (A) and Hill plot (B) showing the effect of S-2302 concentration on human plasma kallikrein (6.78 nM) enzyme activity.
Figure 4.4) A Lineweaver-Burk plot showing the effect of S-2302 concentration on human plasma kallikrein (6.78 nM) enzyme activity.

Figure 4.5) A Michaelis-Menten plot (A) and Hill plot (B) showing the effect of S-2266 concentration on porcine tissue kallikrein (276 nM) enzyme activity.

Figure 4.6) A Lineweaver-Burk plot showing the effect of S-2266 concentration on porcine tissue kallikrein enzyme activity.

Figure 4.7) Human plasma kallikrein, 6.78 nM, cleavage of S-2302, 100 μM, in the presence of kinin receptor ligands.

Figure 4.8) Porcine tissue kallikrein, 276 nM, cleavage of S-2266, 10 μM, in the presence of kinin receptor ligands.

Figure 4.9) A Hill plot showing the effect of kinin receptor ligands on the cleavage of S-2302, 100 μM, by human plasma kallikrein, 6.78 nM.

Figure 4.10) A Hill plot showing the effect of kinin receptor ligands on the cleavage of S-2266, 10 μM, by porcine tissue kallikrein, 276 nM.

Figure 5.1) A Michaelis-Menten plot (A) and Hill plot (B) showing the effect of S-2266 concentration on nasal lavage fluid and human plasma kallikrein (6.78 nM) enzyme activity.

Figure 5.2) A Lineweaver-Burk plot showing the effect of S-2266 concentration on nasal lavage fluid and human plasma kallikrein (6.78 nM) activity.

Figure 5.3) A Michaelis-Menten plot (A) and Hill plot (B) showing the effect of S-2302 concentration on nasal lavage fluid and human plasma kallikrein (6.78 nM) enzyme activity.
Figure 5.4) A Lineweaver-Burk plot showing the effect of S-2302 concentration on nasal lavage fluid and human plasma kallikrein (6.78 nM) activity.

Figure 5.5) Inhibition curves (A) and Hill plots (B) showing the affect of aprotinin and soybean trypsin inhibitor (SBTI) on the cleavage of S-2302, 400 μM, by crude nasal lavage fluid.

Figure 5.6) Inhibition curves (A) and Hill plots (B) showing the affect of aprotinin and soybean trypsin inhibitor (SBTI) on the cleavage of S-2302, 100 μM, by human plasma kallikrein, 6.78 nM.

Figure 5.7) Inhibition curves showing the affect of aprotinin and soybean trypsin inhibitor (SBTI) on the cleavage of S-2266, 10 μM, by porcine tissue kallikrein, 276 nM.

Figure 5.8) The cleavage of S-2266, 400 μM, by nasal lavage fluid in the presence of kinin receptor ligands. Percentage changes were normalised to the control (mean of 1.00 x10^{-3} OD/min, standard error of the mean 0.36 x10^{-3} OD/min).

Figure 6.1) The hydolysis of S-2302 (0.3 mM) in the presence of plasma prekallikrein (8.75 nM) and HMWK (8.75 nM) and the effect of addition of FXII (8.75 nM) and Zn^{2+} (50 μM).

Figure 6.2) The kinetics (A) and the percentage change in the non-exponential cleavage of S-2302, 0.3 mM (B) in the presence and absence of plasma prekallikrein (35 nM), HSP90 (10 nM), Zn^{2+} (50 μM) and HMWK (35 nM).

Figure 6.3) HSP90-potentiated prekallikrein (35 nM) activation in the presence of icatibant (10 μM), aprotinin (1 μM) and soybean trypsin inhibitor (1 μM).

Figure 6.4) Hydolysis of S-2302 (0.3 mM) in the presence and absence of plasma prekallikrein (8.75 nM), HMWK (8.75 nM), HABP (5.33 μg/ml), FXII (8.75 nM) and Zn^{2+} (50 μM).
**Figure 6.5**) FXII-potentiated prekallikrein (8.75 nM) activation in the presence of icatibant (10 μM), soybean trypsin inhibitor (1 μM) and aprotinin (1 μM).

**Figure 6.6**) The rate of hydrolysis of S-2302, 0.3 mM (A) and Log_{10}[dose]-potentiation curves (B) in the presence of Zn^{2+} (50 μM), prekallikrein (8.75 nM), HMWK (8.75 nM), FXII (8.75 nM) with whole (EA.hy926) cell, supernatant and pellet lysate.

**Figure 6.7**) The rate of S-2302, 0.3 mM, hydrolysis by prekallikrein (8.75 nM), with HMWK (8.75 nM), in the presence and absence of FXII (8.75 nM), Zn^{2+} (50 μM) and 1.2 mg/ml of protein from whole (EA.hy926) cell endothelial lysate.

**Figure 6.8**) The inhibition of prekallikrein activation in the presence of icatibant (10 μM), soybean trypsin inhibitor (1 μM) and aprotinin (1 μM).

**Figure 6.9**) The kinetics (A) and the rate of S-2302 (0.3 mM) hydrolysis (B) by human plasma activated by 3.74 mg/ml of protein from EA.hy926 whole cell lysate in the presence and absence of Zn^{2+} (1 mM).

**Figure 6.10**) The effect of icatibant (10 μM), SBTI (1 μM) and aprotinin (1 μM) on the hydrolysis of S-2302 (0.3 mM) by human plasma activated in the presence of the protein (3.74 mg/ml) from EA.hy926 whole cell lysate and Zn^{2+} (1 mM).

**Figure 7.1**) Protocol for investigating the effect of captopril and bradykinin on histamine-induced nasal blockage.

**Figure 7.2**) Protocol for investigating duration of PAF-induced airway hyper-reactivity.

**Figure 7.3**) Protocol for investigating the effect of PAF pre-treatment on challenge with a kinin B₁ receptor agonist.
Figure 7.4A and 7.4B) Protocol for investigating the effect of unilateral pre-treatment with PAF on contralateral histamine-induced nasal blockage.

Figure 7.5A and 7.5B) Protocol for investigating the effect of unilateral pre-treatment with PAF on contralateral bradykinin-induced nasal blockage.

Figure 7.6A and 7.6B) Protocol for investigating the effect of bilateral pre-treatment with PAF on nasal blockage after unilateral challenge with bradykinin.

Figure 7.7) (A) A graph showing the change in nasal patency after challenges with bradykinin, 250 µg, prior and post receipt of captopril or placebo. (B) Area under "area under A_{min} vs time curve" at time points 1 and 6 hours.

Figure 7.8) A graph showing the change in nasal patency after challenge with histamine, 200 µg, 6.5 hours after receipt of captopril or placebo.

Figure 7.9) (A) A graph showing the change in nasal patency after challenge with histamine, 200 µg, at 0, 4, 6 and 8 hours after treatment with saline or PAF, 60 µg. (B) Area under "area under A_{min} vs time curve" at time points 4, 6 and 8 hours.

Figure 7.10) A graph showing the change in nasal patency after challenge with bradykinin, 100 µg, and des-arg^{10}-kallidin, 200 µg and 400 µg, prior to saline or PAF, 60 µg, treatment.

Figure 7.11) A graph showing the change in nasal patency after challenge with 100 µg of bradykinin and des-arg^{10}-kallidin, 200 µg and 400 µg, 4 hours after saline or PAF, 60 µg, treatment.

Figure 7.12) A graph showing the change in nasal patency after challenge of the left nasal cavity with histamine, 200 µg, 6 hours after contralateral treatment with saline or PAF, 80 µg.
Figure 7.13) A normalised $A_{\text{min.}}$ vs. time graph showing the change in nasal patency of the left nasal cavity after challenge with histamine, 200 $\mu$g and receipt of treatment of PAF, 80 $\mu$g, in the right nostril 6 hours earlier.

Figure 7.14) A graph showing the change in nasal patency after challenge of the right nasal cavity with histamine, 200 $\mu$g, 6 hours after contralateral treatment with saline or PAF, 80 $\mu$g.

Figure 7.15) A graph showing the change in nasal patency after challenge of the left nasal cavity with bradykinin, 100 $\mu$g, 6 hours after contralateral treatment with saline or PAF, 80 $\mu$g.

Figure 7.16) A normalised $A_{\text{min.}}$ vs. time graph showing the change in nasal patency of the left nasal cavity after challenge with bradykinin, 100 $\mu$g, and receipt of treatment of PAF, 80 $\mu$g, in the right nostril 6 hours earlier.

Figure 7.17) A graph showing the change in nasal patency after challenge of the right nasal cavity with bradykinin, 100 $\mu$g, 6 hours after contralateral treatment with saline or PAF, 80 $\mu$g.

Figure 7.18) A graph showing the change in nasal patency after challenge of the right nasal cavity with histamine, 200 $\mu$g, 6 hours after bilateral treatment with saline or PAF, 80 $\mu$g.

Figure 7.19) A graph showing the change in nasal patency after challenge of the left nasal cavity with histamine, 200 $\mu$g, 6 hours after bilateral treatment with saline and PAF, 80 $\mu$g.


**List of tables**

**Table 1.1**  Distribution of PAR receptors, agonists and activating enzymes within the airway.

**Table 2.1**  Kinetic constants and amino acid sequence of chromogemic substrates available for use with plasma kallikrein, tissue kallikrein and trypsin.

**Table 2.7**  The materials used in this study are shown below along with their sources.

**Table 4.1**  A summary of $V_{\text{max}}$, $k_{\text{cat}}$ and $K_M$ generated by the cleavage of S-2302 and S-2266 by plasma kallikrein and tissue kallikrein, respectively.

**Table 4.2**  Inhibition constants ($K_i$) and Hill coefficients (nH) for kinin receptor ligands inhibiting human plasma kallikrein cleavage of S-2302, 100 μM.

**Table 4.3**  Inhibition constants ($K_i$) and Hill coefficients (nH) for kinin receptor ligands inhibiting porcine tissue kallikrein cleavage of S-2266, 10 μM.

**Table 4.4**  A table showing the correlation between the the pK$_s$ for the displacement of $[^{125}\text{I}]$-icatibant from soluble human nasal turbinates and pK$_s$ for the inhibition of tissue and plasma kallikrein by the kinin B$_2$ receptor antagonists, icatibant, [Ad]-Bk, WIN 64338 and NPC 567.

**Table 5.1**  Inhibition constants ($K_i$) and Hill coefficients (nH) for the enzyme activity of crude nasal lavage fluid, human plasma kallikrein and porcine tissue kallikrein in the presence of aprotinin and soybean trypsin inhibitor (SBTI).
CHAPTER 1

INTRODUCTION

1.1 Introduction

The kinins, bradykinin and kallidin, are agonists of kinin B₂ receptors and these receptors are present on the microvasculature of the human nasal airway. With the aid of selective, high affinity antagonists, such as icatibant, bradykinin has been shown to cause nasal blockage and rhinorrhea in non-allergic individuals via the kinin B₂ receptor. However, introduction of icatibant into the nasal airway of individuals with allergic rhinitis has shown that it inhibits allergen-induced kinin production in addition to inhibiting allergen-induced nasal blockage. In this thesis the mechanism by which icatibant inhibits the recovery of kinins from human nasal airways has been determined using non-allergic individuals to model allergic rhinitis by intranasal challenge with the phospholipid platelet activating factor (PAF).

1.2 Anatomy and physiology of the human nasal airway

1.2.1 The nasal cavity

To assist breathing, the nose must heat, humidify and filter air of foreign particles on entry into the airway (Atkinson and Kaliner, 1995; Mygind and Dahl, 1998; Dykewicz, 2003). Filtration begins in the nasal vestibule, the outermost region of the nose. This region contains a copious amount of hairs, which seize and deposit potentially noxious particles (greater than 10 μM in diameter) in the nasal cavity where they enter the mucociliary transport system (figure 1.1) (Naclerio and Solomon, 1997).

The nasal cavity is a framework of bone and cartilage, lined with soft mucosal tissue. Entry into the nasal cavity occurs via two oblong shaped apertures, which form canals that project dorsally into the airway. These canals join to form a region known as the nasopharynx. The anterior ends of both canals are segregated from each other by a wall of cartilage and bone known as the nasal
septum, while the bases of the canals are situated directly above a bone known as the hard palate (figure 1.1).

Protruding from the lateral walls of both nasal cavities are three bones, known as the superior, the middle and the inferior nasal turbinates (or conchae) (figure 1.1) (Mygind and Dahl, 1998). These structures significantly increase the surface area of the nasal airway and therefore improve its ability to heat and humidify air (figure 1.1).

![Figure 1.1](Image) The lateral wall of the human nasal cavity (Gray's Anatomy, 2000).

The respiratory mucosa consists of a layer of ciliated pseudostratified columnar epithelial cells, intermittently penetrated by goblet cells, basal cells and non-ciliated epithelial cells (figure 1.2) (Thaete et al., 1981; Atkinson and Kaliner, 1995; Christodouloupolos et al., 2000; Salib et al., 2003; Dykewicz, 2003). Both ciliated and non-ciliated cells contain 300 to 400 small cytoplasmic extensions on their apical surfaces called microvilli and like cilia, the function of the microvilli is to increase the surface area of the nasal airway to help maintain a constant temperature and humidity.
Chapter 1 Introduction

Situated directly beneath the mucosal lining is the basement membrane, a fibrous layer composed of type I, III and IV collagen (Christodouloupolous et al., 2000). It forms a stable surface for cells of the mucosal lining to grow on, and prevents cellular detachment during a chemical or inflammatory insult. Beneath the basement membrane is a relatively cell free zone, called the submucosa (or lamina propria). The major constituents of this zone are glands, blood vessels, nerves, interstitial cells and inflammatory cells.

1.2.2 Nasal glands and secretions

There are two types of glands found in the nasal mucosa, serous glands and mucous glands. Serous glands predominantly reside near the surface of the submucosa, where they produce non-viscous secretions which contain antimicrobial proteins such as lactoferrin, lysozyme, secretory IgA and secretory component (Mygind and Dahl, 1998; Kaliner, 2005; Thaete et al., 1981). Each nostril is thought to contain approximately 100 to 150 of these glands. Mucous glands are situated further away from the mucosal surface and they are around 10 times more abundant than their serous counterparts. They produce a more viscous secretion (as a result of a higher glycoprotein content) and are thought to be one of the major contributors to nasal secretions during nasal allergy.

Secretions from both of these glands combine with mucous glycoproteins from the goblet cells, condensed water from inspired air and plasma exudate to produce a bilayered blanket which lines the respiratory epithelium (Salib et al., 2003; Kaliner, 1991). The inner layer is serous in nature, while the outer layer has a high mucus content, and acts a barrier responsible for catching microorganisms and particles found within the inspired air. Epithelial cilia pass through the inner layer to penetrate the outer layer of the blanket. Movement of the cilia causes the mucous layer to move posteriorly towards the nasopharynx where it can be swallowed. The ciliated epithelia, mucosal and submucosal glands work in unity to form the first line of defence against foreign particles and microorganisms.

1.2.3 The microvasculature

Arterioles of the respiratory mucosa receive a large amount of blood from the ethmoidal arteries (branches of the ophthalmic artery) and the sphenopalatine artery (a branch of the maxillary artery) (Dawes and Prichard, 1953; Atkinson
The blood leaves the arterioles to enter arterio-venous anastamoses, capillaries and venous sinusoids of the nasal mucosa.

Arterio-venous anastamoses (AVA) are structures that bypass capillary beds and venous sinusoids present within the nasal airway, and directly join arterioles to venules at the surface of the submucosa (figure 1.2). In doing so, it is thought they allow an increase in blood flow, which helps to cool the nasal airway down in hot ambient temperatures, without causing nasal congestion (Widdicombe, 1997).

Postcapillary venules are potentially very porous in nature, this is because their endothelial cells contract (in response to exposure to certain inflammatory mediators) to form gaps in the junctions between endothelial cells (figure 1.2) (Cauna, 1970; Atkinson and Kaliner, 1995; Widdicombe, 1997; Mygind and Dahl, 1998). Consequently, postcapillary venules are deemed a major site of plasma leakage during inflammatory events. It must be noted that the leakage of plasma into the interstitial space may also cause nasal congestion due to submucosal swelling (oedema).

There are three types of capillary beds found in the nasal airway, all of which are distinguished from each other by their position within the lamina propria (subepithelial capillary beds, glandular capillary beds and periosteal capillary beds). They all contain an endothelial lining with fenestrae (figure 1.2) (Cauna, 1970; Atkinson and Kaliner, 1995; Mygind and Dahl, 1998; Grevers and Kastenbauer, 1996). The fenestrations aid the humidification and heating of the surrounding tissues, increase the efficiency of gaseous exchange, and increase the efficiency of nutrient transfer between the capillaries, the epithelium and the glands of the nasal airway.

Venous sinusoids are the capacitance vessels of the microvasculature, they are extremely prominent in the nasal turbinates. They are responsible for generating the symptom of nasal blockage. When engorged with blood, they distend into the nasal airway (because they are encased in bone), and obstruct airflow (figure 1.2). Sphincter muscles, called throttle veins, are situated at the site where the sinusoids adjoin to venules. It is thought that their contraction increases the level of sinusoidal filling (Widdicombe, 1997). Sinusoid filling is also thought to be controlled by the degree of sympathetic tone regulating the
activity of smooth muscle in sinusoidal walls and the degree of venular compression by adjacent dilated arteries.

**Figure 1.2** The anatomy of the human nasal mucosa adapted from Naclerio et al., 1993 and Baraniuk, 1995.

### 1.2.4 The autonomic and sensory nervous system

The human nasal airway has an extensive neuronal network, comprising of the autonomic nervous system (the sympathetic nervous and the parasympathetic nervous system) and the non-adrenergic non-cholinergic nervous (NANC) system.

Parasympathetic innervation of the respiratory mucosa of the nasal cavity is derived from the greater superficial petrosal nerves which synapse in the sphenopalatine ganglion (SPG) (figure 1.3). The entire sympathetic nervous input of the nasal airway originates from the superior cervical ganglion (SCG), which emits three main clusters of nerve fibre; the ethmoid nerves; the deep petrosal nerves and another nerve(s) which are thought to innervate the arterioles of the contralateral and ipsilateral nasal cavities (Wilson and Yates, 1978; Su et al., 1988; Atkinson and Kaliner, 1995; Rinder, 1996).
Figure 1.3) A schematic diagram of the neuronal arrangement within the nasal mucosa adapted from Wilson and Yates, 1978; Su et al., 1988; Atkinson and Kaliner, 1995 and Widdicombe, 1990. A signifies the arterioles, V signifies the postcapillary venules. a = SCG nerve fibres, b = the vidian nerve, c = the greater superficial petrosal nerve, d = the deep petrosal nerve, e = the caudal nasal nerve.

After leaving the SCG, the ethmoidal nerves directly innervate the nasal mucosa. Prior to entry into the SPG, the deep petrosal nerves become closely associated with the greater superficial petrosal nerves as they traverse the
pterygoid canal. Subsequently, they are given the collective name, the vidian nerve (figure 1.3).

As the vidian nerve enters the SPG, the deep petrosal nerve passes through the ganglion but does not form any synapses. The emerging postganglionic parasympathetic fibres remain in close proximity with the deep petrosal nerves, and are given the name, the caudal nasal nerve (figure 1.3).

The caudal nasal nerve, along with sympathetic fibres traversing from the contralateral nasal cavity provide the remainder of the autonomic input of the nasal airway (Wilson and Yates, 1978; Su et al., 1988; Atkinson and Kaliner, 1995).

Sensory neurones of the nasal airway originate from axons of the maxillary division of the trigeminal cranial nerve. It branches off into two divisions, one forms a varicosity with the parasympathetic branch of the vidian/caudal nasal nerve in the SPG, and the other branch directly innervates the mucosa and submucosa of the nasal airway (figure 1.3) (Widdicombe, 1990; Atkinson and Kaliner, 1995).

1.2.5 The sympathetic nervous system

The degranulation of mast cells after allergen challenge results in the production of an array of humoral mediators, most of which influence the dynamics and integrity of the microvasculature within the nose. However, under non-inflammatory conditions the sympathetic nervous system is predominantly responsible for controlling vascular tone. This has been shown by the severance of sympathetic fibres emanating from the SCG. Cervical sympathectomy causes ipsilateral nasal blockage, suggesting that nasal patency is maintained by a continuous stream of impulses from the sympathetic nervous system (Mygind, 1982; Takanashi et al., 2003). The principal neurotransmitter in this stream of impulses is noradrenaline.

Three adrenoceptor subtypes for noradrenaline are present within the nasal airway: $\alpha_{1^{-}}$, $\alpha_{2^{-}}$ and $\beta_{2^{-}}$-adrenoceptors (van Megen et al., 1991a). $\alpha_{1^{-}}$-adrenoceptors are post-synaptic receptors, which when activated cause vasoconstriction including constriction of venous sinusoids. While, on the other hand, $\alpha_{2^{-}}$-adrenoceptors were originally thought to be exclusively pre-synaptic,
Chapter 1 Introduction

and therefore autoinhibitory. This is no longer the case since pharmacological studies on human nasal mucosa and blood vessels have shown that (1) the tissue contracts in response to the application of \( \alpha_2 \)-adrenoceptor agonists, and (2) this response is inhibited by pre-treatment with a selective \( \alpha_2 \)-adrenoceptor antagonist (Ichimura and Chow, 1988).

These findings brought the following question to light, "what is the precise subanatomical location of the \( \alpha_2 \)-adrenoceptor?". Andersson and Bende, (1984) found that intranasal application of \( \alpha_2 \)-agonists into the nasal airway caused a significant reduction in blood flow, while application of clinically efficacious doses of an \( \alpha_1 \)-agonist (for relief of nasal congestion) did not significantly affect nasal blood flow (Andersson and Bende, 1984). It was therefore concluded that \( \alpha_2 \)-adrenoceptors are the predominant \( \alpha \)-adrenoceptor found on resistance vessels. Another group performed studies on pre-capillary (resistance) vessels in vitro and found that the application of phenylephrine (a selective \( \alpha_1 \)-adrenoceptor agonist) to the preparation caused dose-dependent vasoconstriction (which was inhibited by prazosin, a selective \( \alpha_1 \)-adrenoceptor antagonist), while oxymetazoline (a non-selective \( \alpha \)-adrenoceptor agonist) had very little effect on the vascular tone (Johannssen et al., 1997). A possible reason for the discrepancy between these results could be because the study performed in vitro was carried out using only arterioles dissected from the nose (and not AVAs or venous sinusoids), therefore any contribution of the AVAs and capacitance vessels to the rate of blood flow in vivo were overlooked.

Nonetheless, it is unanimously agreed that (1) functional forms of \( \alpha_1 \)- and \( \alpha_2 \)-adrenoceptor are present within the nasal airway, (2) under non-inflammatory conditions it is likely that \( \alpha_1 \)- and \( \alpha_2 \)-adrenoceptors are both responsible for maintenance of nasal patency and, (3) the exogenous application of their agonists to the nasal airway reduces nasal congestion.

Expression of \( \beta_2 \)-adrenoceptors within the human nasal mucosa of allergic individuals is thought to be downregulated in comparison to that of non-allergic individuals (Ishibe et al., 1983; van Megen et al., 1991a). The biological significance of this finding is not known, as their physiological role within the human nasal airway is not understood (Svensson et al., 1980; Andersson and Bende, 1984; van Megen et al., 1991a; Stewart et al., 1993). \( \beta_2 \)-adrenoceptors have been identified on the nasal turbinates of non-allergic individuals. It is
speculated that they cause nasal blockage by their ability to dilate resistance vessels (Widdicombe, 1997). However, intranasal challenge with selective $\beta_2$ adrenoceptor agonists does not produce significant nasal blockage (Svensson et al., 1980; Andersson et al., 1984). It has also been speculated that because these receptors are found on post-capillary venules, they may play a role in controlling the vascular permeability of the nasal vasculature. However, Svensson et al., (1994) found that intravenous administration of $\beta_2$ adrenoceptor agonists does not alter the albumin or plasma-derived protein content of nasal lavages from non-allergic individuals challenged with histamine.

1.3 Allergic rhinitis

Allergic rhinitis is a condition in which the nasal airway mounts an inflammatory response against foreign particles found on the mucosal surface. It currently affects millions of people worldwide and its prevalence is thought to be increasing dramatically, especially in industrialised nations. During 2001, in the UK alone, it was estimated that 22% of the population suffered from clinically diagnosable forms of allergic rhinitis (Bauchau and Durham, 2004).

Allergic individuals usually experience symptoms of pruiritis (nasal itching), excessive sneezing, nasal blockage, rhinorrhea (a runny nose) and airway hyper-reactivity. Symptoms can be extremely debilitating and it is not uncommon for them to spread to other regions of the body such as the eyes (conjunctivitis), the lower airway (allergic asthma) or even the skin (dermatitis or urticaria). In severe cases symptoms can cause insomnia.

Broadly speaking, there are two forms of allergic rhinitis, a perennial form and a seasonal form (Skoner, 2001). The perennial form is prevalent all year around and is triggered by exposure to indoor allergens such as house dust mite, animal dander and mould spores. The seasonal form of allergic rhinitis tends to arise during the spring and summer months as a result of rises in ambient levels of tree, grass and flower pollens.
1.3.1 Pathophysiology

Under normal conditions, in non-allergic individuals, the nasal mucosa functions effortlessly, maintaining homeostasis and preventing the entry of foreign particles into the nasal mucosa. However, in sufferers of allergic rhinitis, this system fails, resulting in an inflammatory response, which manifests itself in the form of the symptoms mentioned above.

Allergic rhinitis is composed of two phases, a sensitisation phase and an effector phase. Sensitisation is the first of the two phases and is responsible for allergen recognition, the production of CD4+ T-helper 2 (T\(_{h2}\)) lymphocytes and immunoglobulin E (IgE) antibodies specific for the invading particle.

1.3.1.1 Sensitisation

Allergen(s) capable of evading the nose's first line of defence against foreign particles are usually encountered by one of three types of antigen presenting cell (APC): monocytes/macrophages, B-lymphocytes and dendritic cells (DCs) (Kalb et al., 1991; van Cauwenberge, 1997). A pre-requisite for being able to act as an APC, is that the cell must be able to express class II major histocompatibility complexes (MHC II). However, of the three leukocytes mentioned above DCs are the dominant cell type responsible for mediating primary immune responses against allergens (Fokkens, 1999; Howarth et al., 2000). The reason for this is not known but large numbers of their undifferentiated predecessors are abundant within the nasal epithelium and mucosa (Jahnsen et al., 2004).

Recognised allergens are endocytosed by local dendritic cells and passed through an intracellular pathway responsible for processing exogenous antigens. This pathway involves cleaving the allergen into 4-7 amino acid long peptides, binding to MHC II molecules and then transportation to the cell membrane for extracellular expression (Fokkens, 1999).

While processing the antigen, the DC down regulates the expression of chemokine receptors that direct it towards the nasal mucosa, and upregulates the expression of receptors such as CCR7 and CCR8 (Qu et al., 2004). These receptors cause the DC to leave the nasal mucosa, via lymphatic system, and enter a local lymphoid tissue organ in which the chemokines CCL21 and CCL1,
ligands for CCR7 and CCR8 respectively, are produced (Fokkens, 1999; Upham and Stumbles, 2003).

Once in the lymph node, the DC binds to T-cell receptors (TCRs) of undifferentiated CD4+ T-cells via their MHC class II receptors, with the peptide still bound. CD4+ cells differentiate into one of two cellular subtypes: a Th1 subtype, which generates a delayed hyper-sensitivity response to invading allergens, or, a Th2 subtype, which is necessary for a type I and type IV hypersensitivity reaction. In order for the immunoincompetent T-cells to differentiate into a CD4+ Th2 lymphocyte a variety of other co-stimulatory signals are generated, such as the binding of CD4 to MHC II (Lydard et al., 2000), the release of Th2-cell promoting cytokines by the DC (Upham and Stumbles, 2003), the release of Th1 impeding cytokines by the DC (Upham and Stumbles, 2003) and the interaction of the two cell types via other surface receptors (Lydard et al., 2000). Unfortunately, the reason why DC cells promote a Th2 subtype as opposed to Th1 in allergic disorders is not known (Eisenbarth et al., 2003).

In addition to DCs, B-lymphocytes found within the nasal mucosa also act as APC. Allergen which enters the nasal mucosa is captured by the Fab region of antibodies bound to the surface of B-lymphocytes via their Fc regions (Fokkens, 1999). Cross-linkage of two immunoglobulin receptors (by allergen) causes allergen-immunoglobulin complexes to be endocytosed. They are passed through a series of endosomes until they reach specialised endosomes known as the MIIC (MHC-class-II-containing endosome compartments) (Jelinek, 2000; Clark et al., 2004). In the MIIC, the allergen is cleaved into short amino acid sequences and binds to the antigen recognition domain of MHC class II molecules. The MHC class II molecule-allergen complexes are then passed to the plasma membrane for extracellular expression.

Like DCs, antigen processing B-cells travel down a chemokine gradient which cause them to leave the nasal mucosa and enter a local lymphoid tissue organ (Clark et al., 2004).

Once in the local lymphoid organ, B-lymphocytes bind to the T-cell receptors of naïve T-lymphocytes, via their MHC class II-allergen complexes (Clark et al., 2004). This interaction promotes the differentiation of naïve T-lymphocytes into Th2 lymphocytes.
Newly formed CD4+ T_{h2} cells release IL-2 to promote further growth and differentiation of naïve T-lymphocytes into T_{h2} lymphocytes (van Cauwenberge, 1997).

Naïve B-cells present within the local lymphoid organ become activated by binding to antigen-receptor complexes found on the surface of follicular dendritic cells, which are also present within the lymphoid organ. This ultimately results in the differentiation of the B-cells into non-immunoglobulin E (non-IgE) secreting plasma cells. However, it is believed that the plethora of T_{h2} cytokines (including IL-3, IL-4, IL-5, IL-6, IL-7, IL-10 and IL-13) and T_{h2} co-stimulatory signals (such as B-cell CD40 binding with CD40L/CD154, and B-cell CD28 binding with CD80/CD86) cause the B-cells to grow and differentiate into IgE-secreting plasma and memory cells (van Cauwenberge, 1997; Fokkens, 1999; Lydard et al., 2000; Jelinek, 2000; Baraniuk, 1997).

IgE produced by the plasma cells leaves the lymph node and enters the circulation. Subsequently, a rise of specific-IgE titre is thought to be indicative of atopy (or allergy) (Prussin and Metcalfe, 2006). Immunohistochemical examination of nasal biopsies from sensitised individuals has shown that approximately 64% of the IgE observed co-localises with mast cells (Rajakulasingam et al., 1997a). This interaction occurs via high affinity cell surface FcεRI receptors, which are found on both mast cells and basophils. IgE binds to these receptors (via its Fc region), leaving its antigen-binding Fab region exposed to the extracellular environment, awaiting further allergen invasion.

1.3.1.2 The immediate response

Sensitised individuals remain asymptomatic until they encounter allergen again, in which case, a variety of different inflammatory mediators are produced. A handful of these mediators induce an immediate response, which results in the manifestation of rhinitic symptoms for a period of approximately 40 minutes. A key event in the development of the immediate response is mast cell degranulation.
1.3.1.2.1 Mast cell activation

Mast cells usually reside in the submucosa of non-allergic individuals. However, in allergic rhinitis, they traverse the lamina propria and mucosa to enter the epithelium, where they proliferate in anticipation of another invasion of allergen (Howarth, 1995; Baraniuk, 1997; Howarth et al., 2000; Skoner, 2001; Salib et al., 2003).

All subsequent IgE-specific allergen that infiltrates the nasal epithelium/mucosa binds to the high affinity FcεRI receptors. IgE binding is translated into an intracellular signal by the activation of two tyrosine kinases, Syk and Fyn (Blank and Rivera, 2004). The combined activity of Fyn (which ultimately activates protein kinase C) and Syk (which simultaneously generates a phospholipase Cγ-dependent rise in intracellular \([Ca^{2+}]\)) is mandatory for mast cell degranulation (Blank and Rivera, 2004).

Degranulation is a term which refers specifically to the exocytosis of storage granules. These granules contain carboxypeptidases, tryptase, chymase (in mast cells found in connective tissue only) and histamine, which is bound to the proteoglycans, chondritin and heparin (Naclerio et al., 1985; Togias et al., 1988; Dykewicz, 2003; Hansen et al., 2004). Receptor cross-linkage also acts as a signal for the release of lipid mediators, but because they are synthesised on demand their release is delayed, and also prolonged by their diffusion out into the extracellular space. Lipid mediators produced include platelet activating factor (PAF), prostaglandin D\(_2\) (PGD\(_2\)), leukotriene B\(_4\) (LTB\(_4\)), leukotriene C\(_4\) (LTC\(_4\)) and leukotriene D\(_4\) (LTD\(_4\)) (Baraniuk, 1997; Christodoulopoulos et al., 2000; Dykewicz, 2003). Cytokines are the third and final type of mediator released as a result of receptor cross-linkage. Examples include tumour necrosis factor-\(\alpha\) (TNF\(\alpha\)), transforming growth factor-\(\beta\) (TGF\(\beta\)), IL-4, IL-5, IL-6 and IL-13 (Howarth, 1995; Baraniuk, 1997; Christodoulopoulos et al., 2000; Salib et al., 2003).

It is thought that mast cell degranulation only occurs in cells in which one allergen molecule simultaneously binds to two surface FcεRI receptors (cross-linking) (Blank and Rivera, 2004). However, it has recently been discovered that intracellular signalling occurs in the absence of receptor cross-linkage and antigen binding (Kalesnikoff et al., 2001). Binding of IgE to FcεRI (without cross-
linkage) activates specific mitogen-activated protein kinase (MAPK) pathways, which cause the cytokine synthesis and secretion (IL-6, IL-13 and TNFα) without mast cell degranulation or lipid mediator synthesis (Kalesnikoff et al., 2001; Prussin and Metcalfe, 2006).

1.3.1.2.2 Change in vascular dynamics

The blood vessels within the nasal airway contain receptors for a variety of different inflammatory mediators produced during, and as a result of mast cell degranulation. It is generally thought that receptors for these mediators are found on endothelial cells, which line the lumen of the vasculature, and production of their agonists causes vasodilation and/or plasma extravasation (Behrendt and Ganz, 2002).

Vasodilation is an increase in the luminal diameter of a blood vessel. It is a receptor-dependent and receptor-independent event brought about by the activation of the Ca²⁺-dependent enzyme, endothelial nitric oxide synthase (eNOS) (Behrendt and Ganz, 2002). Receptor dependent activation of eNOS can be triggered by mediators such as bradykinin, histamine and acetylcholine, while shear stress, on endothelial cells, can cause receptor independent activation of eNOS (Arnal et al., 1999). eNOS is responsible for the conversion of the amino acid, L-arginine, into nitric oxide (NO) and L-citrulline. NO diffuses into adjacent vascular smooth muscle cells and activates another enzyme called guanylate cyclase. Guanylate cyclase converts guanosine triphosphate into cyclic guanosine monophosphate, which in turn goes on to activate a series of other enzymes that cause the relaxation of vascular smooth muscle (Behrendt and Ganz, 2002).

Objectively, vasodilation is detected as an increase in blood flow through the arterioles and AVAs (Birchall et al., 1993). While subjectively, vasodilation is observed as nasal blockage due to engorgement of the venous sinusoids with blood (which occurs when the rate of blood flow through the arterioles and/or AVAs supplying the sinusoids is greater than that through the venules responsible for emptying them) (Birchall et al., 1993).

Under non-inflammatory conditions, postcapillary blood vessels within the nasal airway are slightly porous in nature and therefore allow the fluid to exit from the microvasculature. However, these pores impede the extravasation of large
proteins (such as albumin and $\alpha_2$-macroglobulin) into the lamina propria (Persson et al., 1991; Persson et al., 1996; Persson et al., 1997). Under inflammatory conditions the endothelial cells of these blood vessels contract in response to exposure to mediators such as histamine and PAF. This contraction results in the formation inter-endothelial gaps, which do not impede the transition of plasma proteins out of the microvasculature (Greiff et al., 2002; Greiff et al., 2003). Consequently, exudate accumulates in the lamina propria and then traverses the mucosa to enter the nasal lumen.

Increases in vascular permeability are potentiated by a simultaneous occurrence of vasodilation. This is because an increase in blood flow, also increases the hydrostatic pressure in post-capillary blood vessels, therefore forcing exudate out of the microvasculature at a faster rate (Berg et al., 2003; Greiff et al., 2003). An increase in vascular permeability also contributes to nasal blockage, due to oedema formation (Persson et al., 1991; Persson et al., 1996).

Objective measurements of an increase in vascular permeability are made by detecting increases of extravasation, using markers such as albumin and $\alpha_2$-macroglobulin (Svensson et al., 1989; Svensson et al., 1995; Berg 2003; Greiff et al., 2005).

1.3.1.2.3 Activation of sensory-parasympathetic reflex

While some of the symptoms of allergic rhinitis are caused by changes in vascular tone (nasal blockage), it has been established that the majority are caused by neural activity. Intranasal challenge with mediators such as bradykinin and histamine induces symptoms of rhinorhoea, pruritus and sneezing, all of which may be mediated partly by neural activation. The common starting point in the generation of all these symptoms is the sensory nervous system.

Sensory nerves fibres innervating the nasal mucosa are unmyelinated small diameter C-fibres. Their activation causes the production of an orthodromic signal, which passes, via the trigeminal sensory root, and diverges on entry into the central nervous system (CNS). Impulses are propagated along ascending neural pathways into higher regions of the CNS where sensations of itch and pain are acknowledged. Impulses also travel via interneurones within the CNS,
Chapter 1 Introduction

until they synapse with parasympathetic fibres in the CNS (Baraniuk, 1992; Canning, 2002; Tai and Baraniuk, 2002; Groneberg et al., 2004). Entry of impulses into the CNS is also bypassed by collateral fibres emanating from the maxillary and mandibular branch of the trigeminal nerve (Lundblad et al., 1983; Widdicombe, 1990; Groneberg et al., 2004). These peripheral sensory fibres form a varicosity with the vidian/caudal nasal nerve in the SPG (figure 1.3). Activation of the parasympathetic nerve fibres causes the signal to be passed antidromically towards the effector organs within the nasal airway (i.e. arterioles, venules, glands and venous sinusoids), where it causes glandular secretion, extravasation, vasodilation and possibly even nasal blockage (Baraniuk, 1992; Kaliner, 1992; Canning, 2002). Sneezing is a reflex caused by the activation of motor neurones (as opposed to parasympathetic fibres), which innervate various muscles in the lower airway.

The principle neurotransmitter thought to be responsible for mediating these parasympathetic effects is acetylcholine (ACh). It activates all five subtypes of G-protein coupled muscarinic receptor (m1-m5) and all five are present within the human nasal airway (atopic and non-atopic). However, they all differ in their subantaomical distribution and density (van Megen et al., 1991b; Nakaya et al., 2002).

1.3.1.2.4 Inhibition of sympathetic activity

Under inflammatory conditions, the parasympathetic nervous system induces effects on the microvasculature which oppose the effects of noradrenaline. These opposing actions are mediated by a neural reflex, which is propagated by postganglionic parasympathetic nerve fibres that synapse with postganglionic sympathetic nerve terminals. Depolarisation of these parasympathetic nerve fibres induces the release of acetylcholine and the activation of presynaptic inhibitory muscarinic receptors (found on sympathetic nerve terminals) responsible for inhibiting the release of noradrenaline (Jackson and Steele, 1985).

1.3.1.2.5 Neurogenic inflammation

By definition, the sensory nervous system is only composed of afferent nerve fibres. However, it is capable of propagating a neural reflex which generates an inflammatory response known as neurogenic inflammation. Activation of the
sensory neurones generates an impulse which, as mentioned before, is projected orthodromically towards the CNS, but also passes antidromically through collateral sensory fibres found within the periphery of the nervous system (figure 1.3) (Foreman, 1987). The collateral sensory fibres innervate the parasympathetic nervous system or project antidromically into the nasal mucosa and release neuropeptides such as substance P (SP), calcitonin gene related peptide (CGRP) and neurokinin A (NKA).

1.3.1.2.6 Glandular exocytosis

Activation of glands within the nasal airway is mediated by the parasympathetic nervous system. Sufferers of allergic rhinitis produce excessive amounts of serosal fluid and this is thought to be a major cause of rhinorrhea (Druce et al., 1985; Meltzer et al., 1992; White, 1993). Intranasal challenge with methacholine (a non-selective, longer acting analogue of acetylcholine) causes dose-dependent rhinorrhea (Druce et al., 1985; Raphael et al., 1988; Raphael et al., 1989a; White, 1993). A major constituent of the fluid is serosal in origin, and a rise in the proportion of glandular proteins such as lysozyme and lactoferrin (with respect to total protein content) is indicative of glandular activation (Raphael et al., 1989a).

Allergen challenge of atopic individuals also causes a rise in the ratio of glandular to total protein content of nasal lavage fluid (Raphael et al., 1991). On the basis that glands within the airway are not thought to express receptors for IgE, it is thought that allergen–induced activation is mediated by a neural reflex within the nasal airway. Pre-treatment of allergen challenged atopic individuals with a non-selective muscarinic receptor antagonists significantly reduces the symptoms of rhinorrhea (Meltzer et al., 1992).

Immunohistochemical analysis of muscarinic receptors found on glands within the submucosa of inferior nasal turbinates has revealed that m1, m2, m3 and m5 subtypes are present (the most abundant being m1 and m3) (Nakaya et al., 2002). Unfortunately, determining which subtype is responsible for glandular activation has been hindered by a lack of selective agonists or antagonists for these receptors.
During nasal allergy or infection, glandular secretions combine with exudate, goblet cell secretions, condensed inspired moisture and epithelial secretions to form the fluid produced during rhinorrhea.

1.3.1.2.7 Production of NANC transmitters

As mentioned above, the classical neurotransmitters found in the autonomic nervous system are noradrenaline and acetylcholine. However, peptides also play a role in neurotransmission referred to as non-adrenergic non-cholinergic (NANC) transmission.

1.3.1.2.7.1 Vasoactive Intestinal Peptide

Vasoactive intestinal peptide (VIP) is a neuropeptide, which predominantly (but not exclusively) co-exists with ACh in cholinergic fibres (figure 1.4). These fibres primarily innervate blood vessels and seromucosal glands, and in allergic rhinitic airways their densities are significantly increased compared to non-allergic rhinitics (Uddman et al., 1981; Fang and Shen, 1998; Fang et al., 1998). Biopsies from nasal turbinates have shown that the levels of VIP in allergic individuals are also significantly elevated compared to those of non-allergic individuals (Fang and Shen, 1998; Fang et al., 1998). Expression of VIP receptors (VPAC1 and VPAC2) within the nasal airway strongly correlates with the distribution patterns of VIP-positive fibres (Baraniuk et al., 1990a). Unfortunately, the importance of each receptor subtypes in allergic rhinitis has not been determined.


Figure 1.4) The amino acid sequence of VIP.

Co-localisation of VIP with ACh-positive fibres has implicated it in cholinergic mediated transmission. Nasal challenge with histamine or allergen (in atopics) also causes the release of VIP via the sensory-parasympathetic reflex (Mosimann et al., 1993). Like ACh, intranasal application of VIP into the human nasal airway causes rhinorrhea (Chatelain et al., 1995). Unfortunately, no studies have been performed in vivo to determine whether glandular proteins are produced during this response. However, it is known that treatment of
human nasal biopsies with VIP does cause a rise in the level of lactoferrin (Baraniuk et al., 1990a).

Another symptom which is thought to be mediated by cholinergic transmission is nasal blockage. This hypothesis is supported by the fact that (1) electrical stimulation of feline trigeminal nerves (with lesions in the sympathetic component of the vidian nerve) causes marked nasal vasodilation (Lundblad et al., 1983), (2) electrically-induced vasodilatory responses are partially sensitive to atropine and (3) a high density of muscarinic receptors are expressed on arterioles, veins and cavernous sinusoids within the human nasal airway (van Megen et al., 1991b; Nakaya et al., 2002).

Similarly, intranasal application of VIP to the human nasal airway causes atropine-resistant vasodilation and nasal blockage in non-atopic individuals (Barnes et al., 1991a; Barnes et al., 1991b; Chatelain et al., 1995).

1.3.1.2.7.2 Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino acid peptide, which predominantly (but not exclusively) co-exists with noradrenaline in noradrenergic fibres of the nasal airway (figure 1.5). NPY-positive fibres innervate arteries, arterioles, AVAs and capacitance vessels within the nasal airway. Binding sites for NPY exhibit an almost identical distribution pattern to NPY-positive nerve fibre distribution (Baraniuk, 1990; Fang and Shen, 1998; Fang et al., 1998).

Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu

\[ \text{Ala} \]

Tyr-Arg-Gln-Arg-Thr-Ile-Leu-Asn-Ile-Tyr-His-Arg-Leu-Ala-Ser-Tyr-Tyr-Arg

Figure 1.5) The amino acid sequence of NPY.

Co-localisation of NPY in noradrenergic fibres has implicated it in the regulation of vascular tone within the nasal airway. Intranasal application of NPY to human airways causes a long-lasting vasoconstriction and an increase in nasal patency (Baraniuk et al., 1992; Cervin et al., 1999). Its vasoconstrictive effects also synergise with those of noradrenaline in vitro (Fischer et al., 1993). However, no
studies have been performed with NPY receptor antagonists \textit{in vivo} to
determine the role of NPY in the control of vascular tone and nasal patency.

NPY has 6 receptor subtypes, NPY Y$_1$ - NPY Y$_6$ (Balasubramaniam, 1997).
However, only NPY Y$_1$ and NPY Y$_2$ have been located in human nasal airways.

NPY Y$_1$ is a post junctional receptor whose activation is thought to be
responsible for the vasoconstrictive effects observed within the microvasculature
(Baraniuk et al., 1990b). A selective antagonist for this receptor subtype has
been developed, called BIP3226. Use of BIP3226 has shown that NPY Y$_1$
contributes to electrically-induced vasoconstriction of porcine nasal
microvasculature (Lundberg and Modin, 1995). NPY Y$_2$ is a pre-synaptic
receptor believed to be present on parasympathetic nerve terminals. Activation
of NPY Y$_2$ attenuates the activity of field-stimulated cholinergic nerves in guinea-
pig tracheal tissue and canine nasal airways (Stretton and Barnes, 1988;
Lacroix et al., 1994). This suggests that NPY Y$_2$ is a presynaptic inhibitory
receptor present on cholinergic nerves. These findings concur with studies in
human nasal airways in which topical pre-treatment with the selective NPY Y$_2$
agonist, TASP-V, attenuates histamine-induced nasal blockage (Malis et al.,
1999).

NPY does not have any significant effects on plasma exudation or glandular
excitation (Baraniuk et al., 1992).

1.3.1.2.7.3 Tachykinins

Substance P, neurokinin A (NKA) and neurokinin B (NKB) all belong to a family
of neuropeptides called tachykinins. These peptides are 10/11 amino acids in
length and all have similar C-terminal sequences recognised by one of three
neurokinin receptors, NK$_1$, NK$_2$ and NK$_3$. Binding is selective, but not specific,
with SP preferentially binding to NK$_1$, NKA to NK$_2$ and NKB to NK$_3$ (figure 1.6).

\textbf{Substance P:} Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met
\textbf{Neurokinin A:} His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met
\textbf{Neurokinin B:} Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met

\textbf{Figure 1.6} The amino acid sequence of SP, NKA and NKB.
Most of the effects of tachykinins in the human airways are mediated by NK₁ and NK₂ receptors, as they are the most abundant neurokinin receptors found in the peripheral nervous system while, (Baraniuk, 1990) NK₃ receptors are predominantly found in the central nervous system and autonomic ganglia (Canning, 1997). SP and NKA tend to be co-expressed in sensory fibres which innervate arterioles, venules, sinusoids, seromucosal glands and the epithelium (sparsely) (Uddman et al., 1981; Uddman et al., 1983; Barnes et al., 1991; Konno et al., 1996).

The levels of SP found in the airways of atopic individuals are greater than those in non-atopic individuals (Fang and Shen, 1998; Fang et al., 1998), and intranasal challenge with allergen causes these levels to rise further (Mosimann et al., 1993).

Exogenous application of SP to atopic airways causes dose-dependent nasal blockage (Devillier et al., 1988; Braunstein et al., 1991; Konno et al., 1996). This blockage is thought to be due to a combination of vasodilation (Konno et al., 1996) and an accumulation of exudate within the submucosa (Braunstein et al., 1991). In non-allergic individuals, higher doses of SP are required to induce nasal blockage (Devillier et al., 1988), but unlike in challenge of atopic individuals, nasal blockage is also associated with facial flushing.

SP is also thought to be able to induce rhinorrhea in vivo. Intranasal administration of SP to allergic rhinitics causes an increase in vascular permeability (Braunstein et al., 1991), and application to human nasal explants induces glandular activation (Mullol et al., 1992).

1.3.1.2.7.4 Calcitonin Gene Related Peptide

Calcitonin Gene Related Peptide (CGRP) is a neuropeptide which is 37 amino acids in length. There are two forms of CGRP: CGRP-α and CGRP-β, which differ in structure by 3 amino acids (Baraniuk and Kaliner, 1991). CGRP-β is predominantly found in the enteric nervous system, while CGRP-α is predominantly found in the sensory nervous system, where it co-exists with SP and NKA (figure 1.7) (Fang and Shen, 1998; Fang et al., 1998). CGRP-positive fibres innervate arterial and venous blood vessels but occasionally extend to the epithelium and serosal glands (Baraniuk et al., 1990c; Baraniuk and Kaliner, 1991).
Chapter 1 Introduction

A

Thr- Ala
Asp  Thr
Ala-Cys  s  s  - Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser

Gly
Gly
Phe-Ala-Lys-Ser-Gly-Val-Asn-Thr-Pro-Val-Phe-Asn-**Asn-Lys-Val-Val**

B

Thr- Ala
Asn  Thr
Ala-Cys  s  s  - Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser

Gly
Gly
Phe-Ala-Lys-Ser-Gly-Val-Asn-Thr-Pro-Val-Phe-Asn-**Ser-Lys-Val-Met**

**Figure 1.7** The amino acid sequence of α-human CGRP (A) and β-human CGRP (B)

CGRP is released in response to allergen challenge (Walker et al., 1988; Mosimann et al., 1993), and exogenous application causes vasodilation without nasal blockage, glandular secretion or an increase in vascular permeability (Baraniuk et al., 1990c; Rangi et al., 1990; Guarnaccia et al., 1994). This suggests that the vessels innervated are AVA as opposed to arterioles or venous sinusoids. However, autoradiographical studies have shown that binding sites for CGRP are mainly found on arterioles but they are also present on veins and epithelial cells (Baraniuk et al., 1990c).

1.3.1.2.8 Termination of NANC transmission

Termination of NANC signalling is carried out by a variety of different non-specific proteases found within the nasal airway. A reduction in the expression of these proteases is thought to contribute to the pathophysiology of allergic rhinitis.

Neutral endopeptidase (NEP) [EC 3.4.24.11] is a zinc containing membrane-bound protein, with a molecular weight between 94 and 100 kD, depending on its degree of glycosylation (Baraniuk and Kaliner, 1991; Baraniuk et al., 1993).
NEP is expressed on epithelial cells, submucosal glands, vascular smooth muscle and endothelial cells and is also found in nasal secretions (Baraniuk et al., 1993; Ohkubo et al., 1994). It is responsible for degrading SP, NKA, NKB, VIP, CGRP and NPY (Ohkubo et al., 1994; Chatelain et al., 1995; Koehne et al., 1998). It is not known whether expression of NEP is altered in atopic airways (Nadel, 1990), but it is known that short-term pre-treatment with steroids reduces the symptoms of allergic rhinitis and this is associated with increased expression of NEP in asthmatic lower airways (Sont et al., 1997).

Angiotensin converting enzyme (ACE) [EC 3.4.15.1] is a membrane bound zinc-containing metalloprotease. It is found on vascular endothelial cells, between epithelial cells and in luminal secretions (Ohkubo et al., 1994). ACE is responsible for degrading SP and NKA (Ohkubo et al., 1994). However, functional studies in vivo suggest that its role in neuropeptide degradation is minor in comparison to NEP (Chatelain et al., 1995).

Non-membrane bound enzymes such as mast cell tryptase and mast cell chymase also contribute to the termination of NANC signalling (Caughey, 1989; Tam and Caughey, 1990; Sommerhoff et al., 2000).

1.3.1.2.9 Inflammatory mediator production

1.3.1.2.9.1 Prostanoids

Prostanoids are 20-carbon, fatty acid derivatives of arachidonic acid (5,8,11,14-eicosatetraenoic acid), synthesised by an inducible enzyme called cyclooxygenase-2 (COX-2). COX-2 converts arachidonic acid (liberated from cell membranes by the action of phospholipase C and/or A₂) into prostaglandins and thromboxanes both of which are referred to as prostanoids.

Prostanoids are produced in atopic airways as a result of allergen challenge (Brown et al., 1987; Sugimoto et al., 1994; Naclerio et al., 1996). It is thought that their production may induce congestion, which occurs as part of the immediate response (Doyle et al., 1990; Nantel et al., 2004). However, high doses of COX inhibitors which are known to inhibit prostanoid production do not affect allergen-induced symptoms (Brooks et al., 1984; Brown et al., 1987; Proud et al., 1987).
1.3.1.2.9.2 Leukotrienes

Leukotrienes are also derivatives of arachidonic acid. Synthesis of these mediators is initiated by the activation of an enzyme called 5-lipoxygenase (Haberal and Corey, 2003). The interaction of 5-lipoxygenase with a membrane bound protein called 5-lipoxygenase activating protein results in the production of leukotriene A$_4$ (LTA$_4$). LTA$_4$ is a precursor of LTB$_4$ and another group of cysteinyl-containing leukotrienes (LTC$_4$, LTD$_4$, LTE$_4$ and LTF$_4$) called sulphidopeptide leukotrienes.

Intranasal challenge of atopic individuals with the relevant airborne allergen dose-dependently elevates the levels of leukotrienes B$_4$, C$_4$, D$_4$ and E$_4$ found within their nasal lavages (Creticos et al., 1984; Shaw et al., 1985; Miadonna et al., 1987). Leukotrienes have also been implicated in nasal allergy due to the strong correlation between symptom severity in allergen-challenged individuals and the time course of their elevation in nasal lavage fluid (Miadonna et al., 1987). Allergen challenge acts as a stimulus for basophils and mast cells to synthesise and release leukotrienes, but it must be noted that other cells (such as neutrophils, eosinophils and epithelial cells) require stimuli other than IgE to contribute to leukotriene production (Howarth, 2000).

LTC$_4$ and LTD$_4$ have been introduced into human nasal airways and the only symptom experienced was nasal blockage, accompanied by vasodilation (Bisgaard et al., 1986; Miadonna et al., 1987). These findings coincide with autoradiographic analysis of inferior human nasal turbinates where it was found that binding sites for cysteinyl-leukotrienes are distributed sparsely on arterioles, veins and sinusoids (Tai and Baraniuk, 2002). However, in this study only one of two types of cysteinyl leukotriene receptor (CysLT1) was visualised. CysLT2 receptors are not thought to play a role in the pathophysiology of allergic rhinitis.

Evidence supporting treatment of allergic rhinitis by targeting leukotrienes is equivocal. The CysLT1 receptor antagonist, zafirlukast, and lipoxygenase inhibitors, A78773 and zileuton, significantly reduce allergen induced nasal blockage in allergic rhinitis (Knapp, 1990; Howarth et al., 1995; Jiang, 2006). However, when used in a clinical setting, zafirlukast's (10, 20, 40 and 100 mg) effects did not appear to be dose-dependent. It was only found to be therapeutically useful at doses of 20 and 40 mg (Donnelly et al., 1995). Two weeks pre-treatment with another CysLT1 receptor antagonist, montelukast (10
and 20 mg), also failed to inhibit seasonal increases in rhinitic symptoms (Malmstrom et al., 1998) but has succeeded in other trials (Philip et al., 2002).

1.3.1.2.9.3 Histamine

Histamine is a decarboxylated derivative of the amino acid histidine. It is synthesised and stored in the cytoplasmic granules of mast cells and basophils, and degraded by the enzyme diamine oxidase. In allergic rhinitis, granular exocytosis is an IgE mediated response to allergen, but it may also be induced by an IgE-independent mechanism (Petersen et al., 1997; Foreman, 1987; Austin et al., 1996; Schierhorn et al., 1995).

Intranasal challenge with allergen causes a rise in the histamine content of nasal lavage fluid of allergic individuals (Naclerio et al., 1985; Togias et al., 1988; Walden et al., 1988; Naclerio, 1990; Baroody et al., 1994; Naclerio et al., 1996). Exogenous application of histamine to the nasal airway generates symptoms of allergic rhinitis (Pipkorn, 1982; Doyle et al., 1990; Taylor-Clark et al., 2005). Therefore, histamine has long been a major target for the treatment of allergic rhinitis.

There are four known histamine receptors, H₁, H₂, H₃ and H₄, all of which have been identified in the human nasal airway (Okayama et al., 1992; Nakaya et al., 2004). Histamine H₁ receptors are densely expressed on arterioles, veins, venules, cavernous sinusoids and the nerves of inferior nasal turbinates, with sparse distribution on the epithelium and glands (Nakaya et al., 2004). Activation of histamine H₁ receptors (vascular and sensory) is responsible for inducing symptoms of nasal blockage (vascular), rhinorrhea (sensory and vascular), pruitis (sensory) and sneezing (sensory). This has been confirmed by the use of the histamine H₁ receptor agonist, betahistine, (Shelton and Eiser, 1994) and the reduction of histamine-induced symptoms in human nasal airways with a selective histamine H₁ receptor antagonist, cetirizine (Braunstein et al., 1992; Austin and Foreman, 1994b). The role of histamine H₁ receptors in allergic rhinitis has been established by the use of selective H₁ receptor antagonists. Antagonism of these receptors eradicates allergen-induced sneezing and itching, with little effect on nasal blockage (Kirkegaard et al., 1983; Wagenmann et al., 1994; Wang et al., 1996).
Histamine H2 receptors are thought to play a minor role in the effects of histamine on the human nasal airway. Immunohistochemical analysis has revealed that the highest density of histamine H2 receptors is found on glands, and the epithelium of inferior nasal turbinates, with sparse distribution on arterioles, veins, venules and cavernous sinusoids (Nakaya et al., 2004). Exogenous application of a selective H2 receptor agonist, dimaprit, to human nasal airways induces reversible nasal blockage without symptoms of rhinorrhea, sneezing or pruritis (Shelton and Eiser, 1994; Taylor-Clarke – thesis, 2005). However, use of histamine H2 receptor antagonists in conjunction with histamine H1 receptor antagonists, does not completely reverse allergen or histamine-induced nasal blockage (Holmberg et al., 1989; Taylor-Clark et al., 2005).

Histamine H3 receptors have been identified on epithelial cells, arterioles, veins, venules and cavernous sinusoids. They are most abundant on the nerves of human nasal turbinates (Nakaya et al., 2004). Further analysis has revealed that H3 receptors are situated on presynaptic sympathetic nerve terminals (Varty et al., 2004). Activation of these receptors in vitro, by the H3-receptor agonist, R-α-methyl-histamine, reversibly inhibits the effects of electrical field stimulation (EFS) on human nasal turbinates. EFS induced an α1- and α2-adrenoceptor-dependent contraction of the human nasal tissue. It was therefore concluded that activation of the histamine H3 receptor inhibits EFS-induced contraction by attenuating the release of noradrenaline. These results were corroborated by studies in vivo. Introduction of R-α-methyl-histamine to human nasal airways causes dose-dependent nasal blockage, which is not significantly augmented by the use of the α1-adrenoceptor agonist, corynanthine (Taylor-Clark et al., 2005).

Histamine H4 receptors have only recently been discovered due to their genetic sequence homology with histamine H3 receptors. They are expressed on nerves, basophils, CD8+ T-cells, mast cell, eosinophils and neutrophils (Nakaya et al., 2004; de Esch et al., 2005). Their role in the pathophysiology of allergic rhinitis is not clearly understood but it is known that they regulate leukocytic chemotaxis and cytokine production (Kobayashi et al., 2001; de Esch et al., 2005).
Platelet activating factor (1-o-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) is a biologically active phospholipid. It was originally discovered by Benveniste et al., in 1972, who found that rabbit IgE-dependent activation of rabbit basophils causes the release of a serum-soluble factor, which activates platelets. Its structure was determined 5 years later, and it was given the name platelet-activating factor.

Cellular sources of PAF are predominantly, but not exclusively, leukocytic in nature. These sources include eosinophils, neutrophils, platelets, macrophages, basophils, mast cells and endothelial cells (Barnes et al., 1988; Townley et al., 1989; Atkinson and Kaliner, 1995). A variety of different stimuli are required to promote the synthesis of PAF in these cells, all of which trigger a rise in free intracellular [Ca²⁺].

Calcium is required for the activation of phospholipase A₂. Phospholipase A₂ converts cell membrane phospholipids into arachidonic acid and lyso-PAF (1-o-alkyl-2-lyso-sn-glycero-3-phosphocholine). Lyso-PAF, is then converted into PAF by the cytoplasmic enzyme, acetyl transferase (figure 1.8) (Barnes et al., 1988; Townley et al., 1989). A second pathway for the synthesis of PAF has been proposed, which involves the transfer of phosphorylcholine groups to 1-o-alkyl-2-acetyl-sn-glycerols by the enzyme phosphocholine transferase (figure 1.8). The extent to which these pathways play a role in the synthesis of PAF in vivo is not known.

![Figure 1.8](image-url) The synthesis and metabolism of PAF adapted from Barnes et al., 1988 and Townley et al., 1989.
Chapter 1 Introduction

Being a phospholipid, PAF is not stored by cells; it is synthesised and released on demand. Recovery of PAF, phospholipase A_2 and acetylhydrolase from atopic airways in vivo has been demonstrated after allergen challenge (Touqui et al., 1994). However, in other studies, lyso-PAF (the inactive precursor/metabolite of PAF), and not PAF, have been detected in atopic nasal lavages. The inability to detect PAF in these samples is thought to be due to elevations in acetylhydrolase activity (Touqui et al., 1994).

Immunohistochemical examination of human nasal mucosa has revealed that receptors for PAF are situated on interstitial cells, vascular endothelial cells and epithelial cells: the highest level being expressed by submucosal serous glands (Shirasaki et al., 2005). This distribution pattern correlates with the most prominent effects of exogenous PAF on the nasal airway. Exogenous PAF causes nasal blockage (Leggieri et al., 1991; Maniscalco et al., 2000), an increase in vascular permeability (Maniscalco et al., 2002) and nasal discomfort/pain (Maniscalco et al., 2000; Maniscalco et al., 2002). Nasal discomfort is a sign of C-fibre activation, which would suggest that PAF either activates sensory neurones directly or by the production of other mediators which activate C-fibres.

Studies on PAF's role in allergic rhinitis have been hampered by a lack of promising results with PAF receptor antagonists in the lower airways. Pretreatment of allergic asthmatics with PAF antagonists (WEB2086, UK74-505 and SR27417A) does not offer protection against allergen-induced bronchoconstriction or airway hyperreactivity (Freitag et al., 1993; Kuitert et al., 1993; Evans et al., 1997). This lack of effectiveness is not likely to be due to problems with the antagonists, as (1) a lower dose of WEB2086 inhibits PAF-induced bronchoconstriction in non-allergic airways (Adamus et al., 1990), and (2) platelets removed from the blood of allergic individuals, after oral receipt of UK74-505, exhibit a significantly diminished aggregatory response to treatment with PAF (Kuitert et al., 1993).

1.3.1.2.9.5 Protease Activated Receptors (PARs)

Protease activated receptors are a relatively new family of self-activating, seven transmembrane G-protein coupled receptor. The N-terminal sequences of these receptors undergo specific site-directed cleavage by extracellular proteases, to
form a tethered ligand that binds to, and irreversibly activates the receptor (Lan et al., 2002; Reed and Kita, 2004).

Four subtypes of these receptors have been cloned and characterised using proteases which cause their activation: PAR1, PAR2, PAR3 and PAR4. PARs are also distinguishable pharmacologically by selective synthetic ligands that have been synthesised based on the N-terminal amino acid sequence of their tethered ligands (table 1.1). Receptor activity is terminated by other enzymes or internalisation and lysosomal degradation, as opposed to agonist dissociation (Trejo et al., 1998; Lan et al., 2002; Reed and Kita, 2004).

PARs are ubiquitously expressed throughout the body, but, of the four receptors, PAR2 receptors of the human airway have gained a lot of interest because of their potential to be activated by mast cell tryptase \textit{in vivo} (table 1.1). Another potential pathophysiological activator of PAR2 receptors \textit{in vivo} is house dust mite (Lan et al., 2002). As well as being an allergen, house dust mite also displays cysteine protease activity \textit{in vivo} (Hewitt et al., 1997). This proteolytic activity enables house dust mite to disrupt epithelial intercellular adhesion and increase paracellular permeability, so it can infiltrate the nasal mucosa to encounter mast cells or antigen presenting cells (Winton et al., 1998).

PAR2 receptors have been identified on epithelial and endothelial cells throughout the human airway, and their expression is up-regulated in asthmatics and allergic rhinitics (Cocks and Moffatt, 2001; Knight et al., 2001; Dinh et al., 2006). Unfortunately, to date no investigations looking at the role of PAR2 receptors in the nasal airway have been published. Studies in other tissues using selective agonists for PAR2 have revealed that it induces vasodilation (Robin et al., 2003; Wang et al., 2005), which if extrapolated into the human nasal airway, could cause nasal blockage.
### Chapter 1 Introduction

#### Enzyme activators

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#### Amino acids of tethered ligand sequence

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#### Amino acids of selective synthetic peptide agonist

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#### Cellular distribution

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**Table 1.1** Distribution of PAR receptors, agonists and activating enzymes within the airway (Lan et al., 2002; Reed and Kita, 2004)

#### 1.3.1.3 Leukocyte infiltration

Leukocytic infiltration into the nasal mucosa is a delayed response to inflammatory mediators produced as a result of the immediate response. The significance of the entry of leukocytes into allergic airways is not completely
understood, but it is thought to play a role in inducing the late phase response and airway hyper-reactivity (AHR).

Mast cells, Th2-lymphocytes, epithelial cells and endothelial cells activated during the immediate response produce an array of cytokines and inflammatory mediators which aid the entry of leukocytes into the nasal airway (Christodoulopoulos et al., 2000; Howarth et al., 2000; Skoner, 2001).

Leukocytic infiltration is initiated by inducing endothelial expression of the adhesion molecules, P-selectin and E-selectin. These selectins form brief bonds with the counterligand, L-selectin, present on the surface of inactive leukocytes. The brief interactions formed between these selectins allow the leukocytes to roll across the endothelium towards the site(s) of inflammation (Howarth, 1995). Rolling leukocytes are eventually activated by various cytokines, chemokines and proinflammatory mediators which induce the expression of adhesion molecules such as Very Late Antigen-4 (VLA-4) and Lymphocyte Function Antigen-1 (LFA-1) (Baraniuk, 1997; Bachert et al., 1998). The counterligands (Intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1)), for the newly expressed leukocytic adhesion molecules are up-regulated on endothelial cells in response to cytokines such as IL-1, IL-4, IL-13 and TNFα (Howarth, 1995; Bachert et al., 1998; Howarth et al., 2000). ICAM-1 and VCAM-1 form firm bonds with VLA-4 and LFA-1 that ultimately terminate cellular rolling and allow the leukocytes to traverse the endothelium to enter the nasal submucosa.

The importance of ICAM-1 in eosinophilic migration has been shown in primate models of asthma that display eosinophilia sensitive to pre-treatment with mAb R6.5, a monoclonal antibody raised against ICAM-1 (Wegner et al., 1990). Allergen challenge of allergic individuals also induces eosinophilia associated with an up-regulation of VCAM-1, ICAM-1 and E-selectin (Lee et al., 1994; Braunstahl et al., 2001).

1.3.1.4 Leukocyte activation

Neutrophils are granulocytes which are usually resident in most sites of infection. They are activated by mediators such as PAF, LTB4, IL-4 and IL-8. In response, they produce a host of different mediators including LTB4, myeloperoxidase, reactive oxygen species and reactive nitrogen species.
Neutrophils are also a source of chemokines such as IL-8, MIP-1α and MIP-1β. These chemokines promote additional neutrophil infiltration, and initiate the infiltration of other leukocytes into the nasal airway (Lukacs et al., 1995). Studies in vitro have revealed that neutrophils release cytotoxic proteins that cause the detachment of epithelial cells from their basement membranes (Venaille et al., 1995). The ability of neutrophils to detach epithelial cells from basement membranes was thought to induce airway hyper-reactivity in vivo, but histological analysis of human atopic nasal biopsies has revealed that there is no increase in epithelial damage or loss (Lim et al., 1995). Moreover, glucocorticosteroids abolish AHR in vivo but have no effect on the survival of neutrophils within atopic human airways (Togias et al., 1988; Baroody et al., 1992; Barnes, 1998). Nonetheless, neutrophil numbers are elevated in allergic airways in- and out of season, and in response to allergen challenge (Lim et al., 1995).

Eosinophils are granulocytes commonly known to assist the combat of parasitic infections. However, in rhinitic nasal mucosa their numbers are elevated out of season and these numbers are increased further in response to challenge with allergen (4-6 hour later) out of season (Bentley et al., 1992; Lim et al., 1995). Activation of eosinophils within the nasal mucosa is thought to aid the inflammatory process by releasing pre-stored cytokines such as TNFα, and chemokines such as eotaxin and Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES) (Baraniuk, 1997). Eosinophils are also a major source of LTC₄, which is chemotactic for neutrophils and eosinophils (Howarth, 2000). Allergen challenge of allergic individuals is associated with an increase in eosinophil markers such as eosinophil derived neurotoxin (EDN), eosinophil peroxidase (EPO), major basic protein (MBP) and eosinophil cationic protein (ECP) (Turner et al., 2001). These proteins are thought to be responsible for inducing airway hyper-reactivity (Coyle et al., 1995; Turner and Foreman, 1999) and the development of late phase response symptoms (Howarth et al., 2000).

Basophils are granulocytes, which form more than 1% of the white blood cells within the human body. Like mast cells, basophils are bone marrow-derived CD34+ cells (Howarth, 1995). They mature in the blood (as opposed to the nasal mucosa), with the aid of cytokines such as IL-3, IL-5 and IL-13 and enter the nasal mucosa in response to cytokines such as RANTES, IL-3, IL-5 and IL-8 (Baraniuk, 1997). They also posses high affinity FceRI receptors which allow
them to release histamine and leukotriene C4 in response to challenge with allergen. Unlike mast cells, they synthesise negligible amounts of PGD2 and tryptase (Howarth, 1995) and it is because of this fact that they are thought to play a role in the late phase response (Togias et al., 1988; Wagenmann et al., 1997).

T lymphocytes are present within the nasal mucosa and two thirds are thought to be CD4+ bearing cells. In perennial allergic (but not seasonal allergic) rhinitic nasal airways, the number of active (CD25+ bearing) cells is increased and they are of the Th2 phenotype (Howarth et al., 2000). Th2 lymphocytes release a variety of cytokines and chemokines which promote additional T-lymphocyte chemotaxis and Th2 differentiation as well as neutrophilia, eosinophilia and B-cell differentiation.

1.3.1.5 Late Phase Response

Approximately 4-12 hours after development of the immediate response, 50% of allergic individuals experience a re-emergence of nasal symptoms, called the late phase response (Naclerio et al., 1985; Naclerio et al., 1996). The manifestation of these symptoms coincides with inflammatory mediator production and leukocyte infiltration (Naclerio et al., 1996), but the mechanism behind late phase development has not been elucidated. Mediators produced in the late phase include kinins, histamine and leukotrienes. Kinins may be synthesised by kallikrein or in response to a delayed influx of leukocytes into the nasal airway (as discussed in chapter 1.4.2.3) to cause symptoms of nasal blockage and rhinorrhea (Naclerio et al., 1985; Naclerio et al., 1996; Lauredo et al., 2004). Histamine is recovered in the absence of tryptase and PGD2, which supports the possibility that it is produced by basophils, and causes symptoms of nasal blockage, sneezing and pruritis (Naclerio et al., 1985; Naclerio et al., 1996). Leukotrienes could be produced by a variety of cells and may also play a part in the congestive response (Togias et al., 1988).

1.3.1.6 Airway Hyper-reactivity

Airway hyperreactivity (AHR) is the exacerbation of nasal symptoms in response to intranasal challenge with allergen or mediators such as bradykinin and histamine. Symptoms affected by AHR may include nasal blockage (Turner et al., 2001), sneezing and even rhinorrhea (Klementsson et al., 1991). It is an
extremely common symptom of allergic rhinitis and usually occurs as early as 4 hours after the immediate response and can persist for a further 20 hours.

The mechanism behind the development of AHR in allergic rhinitis has not been completely elucidated but several theories have been proposed.

A dysfunctional epithelium
A variety of mediators detected in allergic rhinitic airways are cytotoxic or detrimental to epithelial cells in vitro and they include PAF (Ganbo et al., 1991), neutrophil derived products (Venaille et al., 1995), eosinophil derived proteins (Tagari et al., 1992) and bradykinin (Proud and Kaplan, 1988). Disruption of the epithelial barrier in vivo would directly expose mucosal receptors to invading pathogens and noxious or irritant particles found in the nasal lumen. Additionally, enzymes such as ACE and NEP, found within the epithelium, would be lost, and inflammatory peptides would accumulate in the airway lumen. Consequently, nasal responses to allergen may be enhanced. However, histological analysis of human atopic nasal biopsies has revealed no increase in epithelial damage or loss (Wilson and Yates, 1978; Lim et al., 1995).

Allergic rhinitics are reported to have an increase in mucosal permeability. An enhanced influx of irritants from the epithelial surface into the submucosa would be a feasible explanation for the development of AHR. However, the capacity of low molecular weight molecules, present within human upper airways, to infiltrate atopic nasal mucosa is significantly reduced during the allergy season. On the other hand, the histamine-induced exudative capacity is increased during the allergy season (Svensson et al., 1998).

Enhanced inflammatory mediator production
PAF is a mediator not found in the nasal airways of non-atopics, but challenge of atopic individuals with allergen causes the recovery of detectable levels of PAF from nasal lavages (Shin et al., 1994). Exogenous application of PAF to non-atopic airways induces AHR to bradykinin and histamine (Austin and Foreman, 1993), and in seasonal allergic individuals it induces AHR in response to challenge with pollen (Andersson and Pipkorn, 1988). While binding sites for PAF in human nasal airways have been established, the mechanism behind PAF’s ability to induce AHR has not been determined.
Chapter 1 Introduction

Receptor modulation
Cholinergic hyperreactivity is a common feature of allergic rhinitis. Intranasal challenge with methacholine induces excessive rhinorhea and nasal blockage in atopic, but not in non-atopic individuals (Druce et al., 1985; White, 1993; Marquez et al., 2000). One possible reason for this exaggerated response may be an increase in muscarinic receptor density within the nasal airway. Autoradiographic analysis of rhinitic nasal airways by Van Megen et al., (1991) revealed no change in muscarinic receptor density, but an increase in ligand binding affinity (van Megen et al., 1991b). Conversely, Ishibe et al., (1983) found an increased muscarinic receptor density with no change in binding affinity. Ultimately, both findings could be sufficient explanations for cholinergic hyperreactivity in vivo.

Altered neuromodulation
The sympathetic nervous system is not only responsible for maintaining nasal patency, it is also responsible for counteracting the effects of the parasympathetic nervous system. Toluene diisocynate-induced airway hyperreactivity in guinea-pigs has revealed that the noradrenaline content of the nasal mucosa is significantly reduced. Consequently, it is speculated that dysfunctional sympathetic activity may be responsible for causing AHR in allergic rhinitis (Kubo and Kumazawa, 1993).

Tachykinins have also been implicated in the development of AHR. In models of allergic asthma in which guinea-pigs are sensitised to ovalbumin, allergen produces an immediate response, a late phase response, eosinophilia and AHR to histamine (Schuiling et al., 1999). NK1 and NK2 receptor antagonists abolish the AHR but have no effect on histamine-induced bronchoconstriction. Whether neurogenic inflammation plays a role in inflamed human nasal airways has not been established, but it is known that challenge with mediators such as bradykinin significantly elevates the levels of SP within the nasal airways in vivo (Baumgarten et al., 1997).

The eosinophil derived protein, MBP, is thought to modify parasympathetic activity in human airways. Radioligand binding studies have revealed that it displaces muscarinic ligands bound to muscarinic m2 receptors (Jacoby et al., 2001). Within the lower and upper airways, parasympathetic nerve terminals possess presynaptic autoinhibitory m2 receptors. MBP is thought to antagonise
these receptors and prevent feedback inhibition, resulting in the release of excessive amounts of acetylcholine and hence AHR (Jacoby et al., 2001).

Additionally, the density of VIP-positive fibres innervating human nasal airways is increased in atopic individuals (Fang et al., 1998). A concomitant enhanced release of VIP would be expected to contribute to symptoms of nasal blockage, rhinorrhea and AHR.

*Reduced inflammatory mediator metabolism*

The enzymes NEP and ACE are responsible for the degradation of inflammatory peptides within the nasal airway. The down-regulation of both of these enzymes in the lower airways of asthmatic individuals is thought to cause an accumulation of inflammatory peptides in human airways. This is likely to cause persistent activation of receptors for non-degraded inflammatory peptides and subsequently lead to AHR (Nadel, 1990; Sont et al., 1997; Roisman et al., 1999).

*Altered intracellular signalling*

NO production is increased in the nasal airways of seasonal and perennial allergic rhinitis (Henriksen et al., 1999). This suggests that activity of one or more of the three isoforms of NOS is increased. It is speculated that increased NO production in the airway may induce AHR by reacting with superoxide to form free radicals which damage surrounding mucosal and epithelial tissue. Ironically, chronic non-selective inhibition of NOS induces AHR to histamine and bradykinin in the nasal airway (Turner et al., 2000a).

1.4 Kinins

Within human airways there are two subtypes of receptor activated by kinins, which are referred to as kinin B$_2$ receptors and kinin B$_1$ receptors. The kinins, bradykinin and kallidin, are agonists of kinin B$_2$ receptors formed by the degradation of kininogen by enzymes called kallikrein. Kinin B$_1$ receptors are activated by des-arg$^9$-bradykinin and des-arg$^{10}$kallidin which are metabolites of bradykinin and kallidin respectively. For the duration of this thesis I shall use the term kinins to refer to agonists of kinin B$_2$ receptors.
1.4.1 Discovery of the kallikrein-kinin system

The nonapeptide, bradykinin (BK), was discovered in 1949 by Rocha e Silva and his co-workers, as a result of investigations looking into the histamine releasing capability of Bothrops jararaca snake venom on animal tissue (Beraldo and Rosenfield, 1949). It was observed that canine blood, treated with Bothrops jararaca venom or trypsin, elicited an atropine- and antihistamine-resistant contraction of guinea-pig ileum. The substance was given the name bradykinin, due to the relatively slow (=brady in Greek), movement (=kinin in Greek), which it induces compared to acetylcholine and histamine. A decade later, bradykinin was purified and its structure determined from plasma treated with B. jararaca (figure 1.8) (Zuber and Jaques, 1960; Boissonnas et al., 1963).

**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

(Figure 1.9) The amino acid sequence of bradykinin and kallidin.

Kallidin (Lys-bradykinin) was discovered in 1937 by E. Werle and his co-workers who observed that human urine contained a substance which induced intestinal smooth muscle contraction *in vitro* and hypotension *in vivo* (Werle et al., 1937). It became apparent that vasodilation and smooth muscle contraction were only elicited if urine was mixed with serum proteins. The active substance responsible for these actions was kallidin (KD), nicknamed “substance DK” on the basis of its original name darmkontrahierender stoff, which is German for intestine-contracting substance (figure 1.9).

Tissue kallikrein was discovered in 1925 by E. K. Frey, who found that intravenous administration of canine urine into another dog induced hypotension and tachycardia (Frey, 1926). Frey went on to work with H. Kraut to isolate tissue kallikrein from human urine, which they called F-substance (Frey substance). F-substance was renamed kallikrein (Greek for pancreas), after the discovery of high concentrations of tissue kallikrein in human pancreas. Kallikrein was later discovered in human salivary glands (Werle and Von Roden, 1936). It was also observed that it is activated by trypsin and inactivated by human serum.
Plasma kallikrein was also discovered by Werle and his co-workers, during an attempt to prevent the binding and subsequent inhibition of urinary kallikrein by serum proteins present in urine (Kraut et al., 1928). Acidification of urine reactivated very small quantities of the inhibited urinary kallikrein. This was shown by its hypotensive inducing effects *in vivo*. However, acidification of serum produced profound hypotensive-inducing effects. It was subsequently discovered that there is another kallikrein-like enzyme present in plasma. The active enzyme was given the name plasma kallikrein, according to its source, purification requirements and trypsin sensitivity.

In 1961, Erdős isolated carboxypeptidase N (kininase I) from human plasma and discovered that it deactivated kinins (amongst other peptides) by the removal of the carboxyl terminal arginine (Erdős, 1961). Kininase II was isolated from human plasma in 1967 by Yang and Erdős (Yang and Erdős, 1967). It inactivates bradykinin by cleaving the Pro7-Phe8 bond. In 1979, kininase II was found to be the same as angiotensin converting enzyme.

The precursors to bradykinin and kallidin, kininogens I and II, were discovered in bovine plasma by Haberman et al., (1963). The human variants of these kininogens were isolated from human plasma soon after and given the names high molecular weight kininogen (HMWK) and low molecular weight kininogen (LMWK).

### 1.4.2 Synthesis of bradykinin and kallidin

Kinins are generated in seasonal allergic rhinitis (Geppetti et al., 1991; Turner et al., 2001), perennial allergic rhinitis (Dear et al., 1996) and non-atopic individuals challenged with PAF (Turner et al., 2000b). Synthesis of kinins *in vivo* occurs via one of three pathways: a tissue kallikrein-dependent pathway, a plasma kallikrein dependent pathway and a cellular protease dependent pathway.

#### 1.4.2.1 Plasma kallikrein-dependent kinin synthesis

Intranasal allergen challenge of allergic individuals induces an increase in plasma kallikrein activity and an elevation in the level of kininogen (Baumgarten et al., 1986a) and kinins found in nasal lavages (Proud et al., 1983; Naclerio et
al., 1985; Turner et al., 2001). The activation of plasma kallikrein in the presence of Hageman factor catalyses the excision of bradykinin from high molecular weight kininogen.

1.4.2.1.1 High molecular weight kininogen

High molecular weight kininogen is a 115 kD glycoprotein, predominantly, but not exclusively, synthesised in the liver (Neth et al., 2001) by alternative splicing of a single gene (Bhoola et al., 1992). When released into the circulation, it binds to either prekallikrein or coagulation factor XI (FXI) in a ratio of 1:1 (Mandle et al., 1976; Thompson et al., 1977; Proud and Kaplan, 1988). In human plasma, its concentration is between 70 and 90 µg/ml and accounts for approximately 1/3 of the total kininogen present in plasma (Neth et al., 2001; Kaplan et al., 2002).

HMWK is a single glycosylated chain of 626 amino acids, with a single disulphide bond between its amino- and carboxy-terminal cysteine residues (figure 1.10). Excision of bradykinin (domain 4), from the chain, disproportionately splits it into two chains linked only by the disulphide bond. The chain closest to the original amino terminal is called the heavy chain and the chain closest the original carboxy terminal is called the light chain. The heavy chain consists of three separate domains, each with distinct functions. Domain 1 comprises residues 1 to 116, domain 2 comprises residues 117 to 238 and domain 3 comprises residues 239-360. Domain 1 contains a low affinity binding site for Ca²⁺ (Farmer, 1997). Domains 2 and 3 are responsible for the inhibition of cysteine protease activity, namely cathepsins B, H and L, papain, ficin and platelet derived calpains I and II (Bhoola et al., 1992; Farmer, 1997). Domain 3 is also responsible for HMWK binding to cell surfaces (Reddigari et al., 1993a; Wachtfogel et al.1994). The light chain of HMWK, amino acids 371 to 626, contains two domains, domain 5 and domain 6. Domain 5, amino acids 91 to 121, is rich in histidine residues and is also responsible for the binding of HMWK to cell surfaces (Wachtfogel et al., 1994; Shariat-Madar and Schmaier, 1999). Domain 6, amino acids 185 to 242, is responsible for binding to and activating FXI or prekallikrein (Thompson et al., 1979; Kaplan et al., 2002).
A combination of radioligand binding experiments and confocal microscopy have revealed that HMWK requires Zn$^{2+}$ to bind with high affinity to the cell membranes of endothelial cells (Reddigari et al., 1993a), vascular smooth muscle cells (Fernando et al., 2005), unstimulated platelets (Meloni et al., 1992), astrocytes (Fernando et al., 2003) and neutrophils (Gustafson et al., 1989; Figueroa et al., 1992). HMWK is also suspected to bind unactivated tissue kallikrein (prokallikrein) (Raab and Kemme, 2000).

1.4.2.1.2 Hageman Factor [3.4.21.38]

Hageman Factor or coagulation factor XII (FXII) is an 80 kD glycosylated proenzyme, predominantly, but not exclusively, synthesised in the liver (Neth et al., 2001). It circulates in the plasma, unbound to other proteins, at concentrations between 30 and 35 μg/ml (Kaplan et al., 2002). Activation, by site-specific cleavage, of this protein catalyses the progression of the complement pathway, the fibrinolytic pathway, the extrinsic coagulation cascade and the kallikrein-kinin pathway (Bhoola et al., 1992).
FXII is a single glycosylated chain of 596 amino acids, with a single disulphide bond between two cysteine residues (Proud and Kaplan, 1988; Kaplan et al., 2002). The first 279 amino acids of the chain are referred to as the heavy chain region of FXII. Amino acids 331 to 596 are referred to as the light chain region, and amino acids 279 to 330 are referred to as the heavy and light chain connecting region of FXII.

Activation of FXII can be brought about by the binding of its heavy chain to negatively charged surfaces such as glass, kaolin, dextran sulfate and sulfatides (Pixley et al., 1987; Proud and Kaplan, 1988; Citarella et al., 2000). FXII activation is augmented and initiated by self-cleavage. It is believed that binding of FXII to artificial surfaces firstly induces a conformational change in the protein that allows it to become a better substrate for itself (autoactivation) and, secondly, generates a large localised concentration of FXII needed for autoactivation (figure 1.12).

The cleavage of the Arg\textsuperscript{353} - Val\textsuperscript{354} bond by plasma kallikrein or FXI\textalpha (active Hageman factor) causes the activation of FXII (figure 1.1). Arg\textsuperscript{353} and Val\textsuperscript{354} are both enclosed within the disulphide bond and therefore, the single chain is converted into two disulphide-linked chains; a heavy chain (and connecting region) and a light chain (Kaplan et al., 2002). The light chain of FXII\textalpha displays serine protease activity and is responsible for the activation of prekallikrein.

FXII\textalpha may be subjected to further cleavage, which convert it into two smaller fragments, referred to as XIIf. XIIf retains its ability to activate prekallikrein, but lacks a heavy chain and the ability to activate FXI (Dunn and Kaplan, 1982). The first fragment is 30 kD in weight and is liberated from its membrane bound heavy chain by a cleavage at Arg\textsuperscript{334} in the connecting region. The second fragment is 28.5 kD in weight, and is formed by reducing the connecting region of the first XIIf to 9 amino acids in length by a cleavage at Arg\textsuperscript{343} (Dunn and Kaplan, 1982; Kaplan et al., 2002) (figure 1.11).

Studies with purified enzyme preparations and human plasma have revealed that FXII activation by plasma kallikrein, and autoactivation are dependent on the presence of HMWK (Meier et al., 1977). Unfortunately, the mechanism behind how HMWK augments FXII activity has not been completely elucidated. However, it is now appreciated that HMWK is not only a substrate required by
plasma kallikrein for bradykinin-synthesis but it is also an essential cofactor in all reactions catalysed by the activation of FXII.

Radioligand binding and biotinylation studies have revealed that FXII, in the presence of Zn$^{2+}$, binds to a similar population of cells as HMWK (Joseph et al., 1996; Fernando et al., 2003; Johne et al., 2006). In fact, it has been observed that on artificial and endothelial cell surfaces that HMWK competes with FXII for the same binding sites, and FXII competes with HMWK for FXII (Shimada et al., 1985; Reddigari et al., 1993a; Reddigari et al., 1993b).

**Figure 1.11** The structure of FXIIa and its cleavage sites (1), (2) and (3) adapted from Kaplan et al., 2002.

1.4.2.1.3 Plasma prekallikrein [EC 3.4.21.34]

Prekallikrein is a proenzyme predominantly, but not exclusively, synthesised in the liver, the kidney and the pancreas (Neth et al., 2001). Prekallikrein is a single glycosylated chain of 619 amino acids with a single disulphide bond. It is synthesised in two forms, an 85 kD form and an 88 kD form (Hojima et al., 1985). Both isoforms are the product of a single 56 kD heavy chain bound to either a 33 kD or 36 kD light chain. Like FXII, activation of prekallikrein is brought about by the cleavage of a bond (Arg$^{371}$-Ile$^{372}$) encompassed by its disulphide bond (Page et al., 1994). The active form of prekallikrein (plasma kallikrein) is therefore a two-chain protein: a heavy chain (371 amino acids) linked to a light chain (248 amino acids) by a disulphide bond. The light chain of plasma kallikrein displays serine protease activity and is responsible for the
excision of bradykinin from HMWK, the activation of FXII and the autoactivation of prekallikrein (Tans et al., 1987). The heavy chain of prekallikrein consists of four homologous 90-91 amino acid disulphide loops referred to as apple domains 1-4 (A1-4). HMWK binds to prekallikrein via A1, A2 and A4 (Page and Colman, 1991; Page et al., 1994; Herwald et al., 1996; Renne et al., 1999).

![Figure 1.12](image-url) The activation of prekallikrein by FXII and FXIIa on artificial surfaces.

1.4.2.1.4 Activation of prekallikrein on cell surfaces

The high affinity binding of FXII and HMWK to human umbilical vein endothelial cells (HUVECs) (Reddigari et al., 1993a), platelets (Meloni et al., 1992) and neutrophils (Gustafson et al., 1989; Figueroa et al., 1992; Henderson et al., 1994) in the presence of Zn²⁺ occurs in a reversible and saturable manner. This discovery prompted the isolation of the cell surface proteins responsible for the binding of FXII and HMWK. The passage of solubilised-HUVEC cell membranes over a HMWK affinity column revealed that the globular heads of the cell surface receptor, gC1qR, bind with high affinity to the light chain of HMWK (Joseph et al., 1996). However, it was observed that the heavy chain of HMWK also bound with high affinity to the plasma membranes of HUVECs (Reddigari et al., 1993a). The protein responsible for the binding of the heavy chain of HMWK to HUVECs was isolated and revealed to be cytokeratin 1 (CK1) (Joseph et al., 1999).
Antibodies raised against gC1qR and CK1 revealed that gC1qR and CK1 are responsible for 72% and 30% of HMWK binding to HUVECs respectively, while the combined antisera inhibited HMWK binding by 85% (Joseph et al., 1999). Consequently, it is believed that a third protein is responsible for the binding of HMWK to HUVECs. Immunostaining and biotinylation studies have revealed that this protein is urokinase plasminogen activator receptor (u-PAR) (Hasan et al., 1998; Mahdi et al., 2001).

Enzyme assays have revealed that activation of FXII is induced by its incubation with gC1qR and CK1, in the presence of HMWK and Zn^{2+} (Joseph et al., 2001). Hence, the activation of prekallikrein is potentiated by FXII in the presence of HMWK, Zn^{2+} and purified CK1 or gC1qR. However, CK1 and gC1qR do not appear to have any effect on the activation of prekallikrein in the absence of FXII or Zn^{2+}. Studies with human plasma, incubated with HUVECs, have also revealed that the plasma becomes activated in the presence of Zn^{2+} and this activity is inhibited by antibodies raised against gC1qR and CK1 (Joseph et al., 2001). This is consistent with the theory that FXII is activated on the surface of cells by binding to CK1 or gC1qR in the presence of Zn^{2+}.

### 1.4.2.2 Tissue kallikrein-dependent kinin synthesis

#### 1.4.2.2.1 Low molecular weight kininogen

Low molecular weight kininogen is a single chain of 409 amino acids. It also has a single disulphide bond between its amino- and carboxy-terminal cysteine residues. Its heavy chain and the first 12 amino acids of the light chain are identical to that of HMWK, but, it lacks domain 6 and therefore does not posses any procoagulant activity or the capacity to bind prekallikrein.

#### 1.4.2.2.2 Tissue prokallikrein [EC 3.4.21.35]

Tissue or glandular kallikrein is a 26-42 kD glycoprotein (depending on the degree of glycosylation) synthesised from one gene in a variety of organs, including the kidney, pancreas, liver, salivary glands and the brain (Lu et al., 1989). In glands of the upper and lower airway, it is stored as an inactive precursor and released in response to allergen challenge in atopics (Baumgarten et al., 1986b; Christiansen et al., 1987; Christiansen et al., 1992;
Once in the nasal mucosa, it converts LMWK into kallidin and a two-chain glycoprotein. However, under conditions where there is little or no LMWK available, tissue kallikrein may also act on HMWK to produce kallidin (Maier et al., 1983a; Baumgarten et al., 1986b).

The definition of a true tissue kallikrein is an enzyme that exhibits kininogenase activity by the excision of a kallidin molecule from LMWK. However, a new family of 14 other enzymes with molecular homology to glandular kallikrein are also referred to as tissue kallikreins (Mahabeer and Bhoola, 2000; Clements et al., 2001; Yousef and Diamandis, 2001; David et al., 2002; Yousef and Diamandis, 2003). These enzymes are also serine proteases but lack kininogenase activity. They are used as markers for the diagnosis of various forms of human cancer. For clarity, during the remainder of this thesis, the term tissue kallikrein will only be used with reference to the enzyme which displays kininogenase activity in conjunction with LMWK.

Prokallikrein, the inactive precursor of tissue kallikrein, is a single chain of 238 amino acids, with an additional 7 N-terminal amino acids (Ala-Pro-Pro-Ile-Gln-Ser-Arg) (Takahashi et al., 1988; Lu et al., 1989). Removal of these 7 N-terminal amino acids results in the activation of prokallikrein. Enzymes capable of activating prokallikrein in vitro include thermolysin (a bacterial enzyme), and trypsin (Takada et al., 1985). However, their contribution to tissue kallikrein activation in the nose is thought to be limited, as thermolysin and trypsin are not thought to be present in the nasal airway. The only other enzyme capable of activating tissue prokallikrein in vitro is plasma kallikrein. However, it has not been determined whether plasma kallikrein is responsible or capable of activating tissue prokallikrein in vivo.

1.4.2.3 Alternative kininogenase pathways

Kinin production in individuals allergic to the house dust mite, Dermatophagoides farinae, is thought to occur via a kallikrein-dependent and kallikrein-independent pathway. D. farinae contains a biochemically active protease (Df protease), which also exhibits kininogenase activity (Maruo et al., 1991). Studies in vivo have revealed that it is capable of kinin synthesis and activating FXII and prekallikrein in vitro.
In addition to glands within the nasal airway, tissue kallikrein is also synthesised and released by vascular endothelial cells, monocytes, macrophages and neutrophils (Figueroa et al., 1989; Kemme et al., 1999; Lauredo et al., 2004; Yayama et al., 2003). The significance of non-glandular tissue kallikrein activity in the synthesis of kinins within the nasal airway is not known. However, it is possible that they may hamper as opposed to augment kinin synthesis within the nasal airway, as exposure of prokallikrein to the neutrophilic enzymes, neutrophil elastase and cathepsin G, impedes tissue kallikrein activity (Podlich et al., 1999). These results are corroborated by the findings of another group, who found that the ability of tissue kallikrein and plasma kallikrein to release kinins from their respective kininogens is impeded by pre-treatment with neutrophil elastase (Dulinski et al., 2003). Yet, in contrast, a mixture of human neutrophil elastase and mast cell tryptase is thought to produce comparable yields of kinins to plasma kallikrein and tissue kallikrein when acting on HMWK and LMWK (Kozik et al., 1998).

IgE-mediated release of an enzyme with kininogenase activity and the ability to activate prekallikrein has been detected in chopped human lung fragments (Meier et al., 1983; Proud and Kaplan, 1988). The enzyme(s) was originally thought to be mast cell tryptase, but this is unlikely as it was found that tryptase requires a non-physiological pH, pH 5.5, for optimum kinin synthesis in vitro (Proud et al., 1988). Moreover, contraction of guinea-pig ileum in response to exposure to HMWK which has been treated with plasma kallikrein is attenuated if the HMWK is also pre-treated with mast cell tryptase (Maier et al., 1983b). Conversely, studies with guinea-pig skin have revealed that products from the degradation of physiological concentrations of HMWK by mast cell tryptase (at pH 7.4) cause a dose-dependent increase in vascular permeability. It was postulated that tryptase causes kinin production in vivo by the activating plasma kallikrein in addition to directly catalysing the synthesis of bradykinin by the degradation of HMWK. Unfortunately, kinin production was not measured and kinin B₂ receptor antagonists were not used to confirm the findings of this study (Imamura et al., 1996).
1.4.3 Termination of the kallikrein-kinin pathway

1.4.3.1 Kininases

Enzymes responsible for the termination of kinin-mediated activity are referred to as kininases. There are two classes of kininases, kininase I and kininase II.

Type I Kininases

Carboxypeptidase N (CPN) [EC 3.4.17.3] comprises a 50 kD and 48 kD active subunit each bound to an 83 kD regulatory subunit (Erdös, 1992). It is synthesised in the liver, released into the circulation and resides on a variety of cells within the nasal airway (Ohkubo et al., 1994). CPN transforms the kinins bradykinin and kallidin into des-arg⁹-bradykinin and des-arg¹⁰-kallidin by removing their C-terminal arginine amino acids (figure 1.13).

Type II Kininases

Angiotensin converting enzyme (ACE) [EC 3.4.15.1] is a single chain 140-170 kD glycoprotein found in most bodily fluids and anchored to the plasma membrane of cells all over the human body (Bhoola et al., 1992). In the nasal airway, it has been identified on vascular endothelial cells, between epithelial cells and in luminal secretions (Ohkubo et al., 1994). ACE has two Zn²⁺-containing active sites: one found in its N-terminal domain and the other in its C-terminal domain. Both domains contribute to kinin degradation, but the N-terminal domain is thought to be responsible for 76% of its activity (Jaspard et al., 1993). ACE terminates kinin activity by the lysis of the Pro⁷-Phe⁸ and Ser⁸-Pro⁷ bonds (figure 1.13) (Bhoola et al., 1992).

Neutral endopeptidase (NEP) [EC 3.4.24.11] is a membrane bound glycoprotein. It has one Zn²⁺-containing active site and is expressed in the human nasal airway (Baraniuk et al., 1993). It terminates kinin activity by the lysis of the Pro⁷-Phe⁸ bond (figure 1.13).
1.4.3.2 Endogenous kallikrein inhibition

Termination of kallikrein-dependent kinin synthesis is mediated by a group of endogenous plasma proteins known as SERPINs (serine proteinase inhibitors). They inhibit serine protease activity by binding to enzyme active sites. The most notorious SERPINs include the complement fragment 1 (C1) inhibitor, $\alpha_2$-macroglobulin, $\alpha_1$-antitrypsin, antithrombin III and $\alpha_2$-antiplasmin.

Hageman Factor enzyme activity is inhibited by a combination of C1 inhibitor, $\alpha_2$-macroglobulin, antithrombin III and $\alpha_2$-antiplasmin. Approximately 91% of its inhibition in human plasma is mediated by C1 inhibitor (Pixley et al., 1985) and over 50% of this inhibition is achieved in 10 minutes (Schousboe, 2003). Plasma kallikrein activity in normal human plasma is inhibited by approximately 89% in 5 minutes. 52% of its inhibition is mediated by C1 inhibitor, 35% by $\alpha_2$-macroglobulin and the remaining 13% by a combination of other plasma proteins (van der Graaf et al., 1983; Olson et al., 1993). Tissue kallikrein is inhibited by $\alpha_1$-antitrypsin ($\alpha_1$-AT) and kallikrein binding protein (KBP) (Geiger et al., 1981; Chen et al., 1990). Half maximal binding of tissue kallikrein to KBP takes approximately 30 minutes and in excess of 6 hours with $\alpha_1$-AT. The slow binding kinetics of tissue kallikrein to plasma proteins suggests that it is relatively resistant to inhibition \textit{in vivo}. 

\textbf{Figure 1.13} A summary of the kallikrein-kinin pathway.
1.4.4 The role of kinins, kallikrein and kininases in the nasal airway

Kinin B₁ receptors are not constitutively expressed in human nasal airways. However, expression of kinin B₁ receptor mRNA is significantly greater in allergic individuals and is increased further by allergen challenge (Christiansen et al., 2002). Unsurprisingly, intranasal challenge of both allergic and non-allergic individuals with the kinin B₁ receptor agonist, des-arg¹⁰-kallidin, produces no observable symptoms (Rajakulasingam et al., 1990; Austin and Foreman, 1994a; Reynolds et al., 1999; Christiansen et al., 2002).

Kinin B₂ receptors are constitutively expressed in both allergic and non-allergic airways. Autoradiographic analysis of human nasal mucosa has revealed that binding sites for bradykinin are present on nerves, arterioles, capillaries, small venules and venous sinusoids, with no specific binding to submucosal glands or goblet cells (Baraniuk, 1990). Intranasal challenge of atopic and non-atopic individuals with bradykinin causes dose-dependent nasal blockage, nasal discomfort and rhinorrhea (extravasation and glandular activation). However, nasal responses to bradykinin are altered in atopic individuals during the allergy season (Riccio and Proud, 1996). Unilateral application of bradykinin to seasonal and perennial allergic individuals causes contralateral glandular secretion, a response not seen in non-allergic individuals. This response was attenuated by pre-treatment of the contralateral nasal cavity with atropine, suggesting that the glandular activation was a cholinergic-mediated reflex response. Additionally, bradykinin also activates sensory neurones, shown by its capacity to produce a significant increase in substance P in atopic nasal lavages (Baumgarten et al., 1997).

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Kinins are also thought to play a role in the development of airway hyperreactivity in the upper and lower airways. Allergic individuals challenged with allergen display an immediate and delayed increase in nasal lavage kinin content (Naclerio et al., 1985; Togias et al., 1988; Turner et al., 2001). Additionally, kinin B₂ receptor antagonists inhibit allergen-induced airway hyper-reactivity in seasonal allergic individuals (Turner et al., 2001). However, these findings are not supported by the fact that intranasal challenge with kinins does not induce airway hyper-reactivity to histamine in non-atopic individuals (Turner et al., 2000b).
The kinin B$_2$ receptor antagonist, icatibant, exhibits different efficacies in the immediate response of individuals with perennial allergic rhinitis and seasonal allergic rhinitis. In perennial allergic rhinitis, icatibant (previously known as HOE 140) abolishes HDM-induced nasal blockage, but, in seasonal allergic rhinitis it has no significant effect on pollen induced nasal blockage (Turner et al., 2001). Coincidently, icatibant also reduces the allergen-induced increases in kinin content of nasal lavage fluid from individuals with perennial allergic rhinitis (Dear et al., 1996).

1.4.5 Kinin B$_2$ Receptor Antagonists

The first kinin receptor antagonist was a D-Phe7 substitute of bradykinin (Stewart et al., 1999). Further substitution of bradykinin amino acids and the addition of an amino-terminal arginine residue lead to the synthesis of the first generation of kinin antagonists, such as NPC 567 ([D-Arg$^9$, Hyp$^3$, D-Phe$^7$]-bradykinin). Unfortunately, the potency of these drugs in vivo was very limited (Pongracic et al., 1991; Austin and Foreman, 1994a). High doses of NPC 567 do not antagonise bradykinin-induced effects in non-atopic nasal airways. This lack of potency was attributed to their relatively low affinity for kinin B$_2$ receptors and their susceptibility to degradation by CPN (Stewart et al., 1999).

Second generation bradykinin antagonists were created by the introduction of two artificial amino acids, octahydroindole-2-carboxylic acid (Oic) and D-tetrahydroisoquinoline-3-carboxylic acid (Tic), into the structure of first generation antagonists. Icatibant was the first successful second generation compound to be recognised for its resistance to CPN degradation, increased affinity for kinin B$_2$ receptors and increased potency in vivo (Hock et al., 1991; Wirth et al., 1991). This has been exemplified by its potency in human nasal airways where icatibant effectively antagonises bradykinin-induced effects at doses of 30 μg (Austin and Foreman, 1994; Proud et al., 1995; Dear et al., 1996).

Substitution of amino acid residues in icatibant for other artificial amino acids has led to the synthesis of third and fourth generation receptor antagonists. These drugs are also resistant to NEP activity, retain their affinity for kinin B$_2$ receptors and have significantly improved affinities for kinin B$_1$ receptors (Burkard et al., 1996; Stewart et al., 1996; Stewart et al., 1999).
1.5 Aims of the project

a) To determine the effect of kinin receptor antagonists on kinin synthesis in the human nasal airway.

b) To determine the effect of kinin receptor antagonists on kallikrein enzyme activity.

c) To determine the effect of kinin receptor antagonists on enzyme activity from human nasal lavage fluid.

d) To determine the effect of kinin receptor antagonists on kallikrein activation.

e) To determine whether kinin B₁ and kinin B₂ receptors play a role in nasal airway hyper-reactivity.

f) To determine if nerves in the nasal airway play a role in PAF-induced airway hyper-reactivity.
CHAPTER 2
MATERIALS & METHODS

2.1 Techniques which can be used to study the nasal airway

Gaining an understanding of the physiology and pathophysiology of diseases of the upper airway is enhanced by being able to manipulate its function in situ. This usually involves pharmacological intervention and/or provocation, in conjunction with a means of assessing the nasal airway. The most common route of pharmacological application is nasally, in the form of a spray or paper disc.

2.1.1 Estimation of nasal airflow

Rhinomanometry is a technique used to determine the level of resistance to airflow within the nasal airway while breathing. More than 80% of the resistance within the nasal airway is generated within the region of the inferior nasal turbinate (Malm, 1997). Resistance is therefore used as a direct measure of nasal congestion. Nasal airway resistance is calculated by the division of pressure within the posterior region of the nares, by the rate of airflow through the nasal airway. Airflow and pressure are determined with aid of an airtight mask, with a 'flow head' and a pressure sensing tube inserted into the mouth or the nostrils (Malm, 1997; Nathan et al., 2005). Calculation of pressure within the nasal airway by insertion of the tube into the mouth is called posterior rhinomanometry, while insertion of the tube into each nostril is called anterior rhinomanometry. Anterior rhinomanometry is favoured over posterior rhinomanometry because it calculates the resistance of each nostril individually and requires less subject compliance, but it is flawed if unilateral nasal obstruction occurs.

Nasal peak flow measures either the maximum inspiratory flow rate (NPIF) in litres per second or the maximum nasal expiratory flow rate (NPEF) in litres per second (Malm, 1997; Nathan et al., 2005). Readings are obtained with the aid of a flow meter attached to a mask. Both methods are quick, non-invasive and require very little subject compliance. As with rhinomanometry, nasal peak flow
readings are influenced by resistance within the region of the inferior nasal turbinate, however, the level of reproducibility is limited by a number of factors including subject technique, vestibular collapse (with NPIF), poor inspiratory reserve, nasal obstruction and poor equipment sterility.

2.1.2 Estimation of nasal blood flow.

Xenon clearance and laser-Doppler velocimetry (LDV) are techniques used to measure changes in blood flow within the nose. Xenon is a radioactive marker, injected into the nasal mucosa using a small-gauge needle. Blood flow is determined by detection of xenon activity, taking into consideration its solubility within the blood and the nasal mucosa (partition coefficient) (Druce, 1993). Unfortunately, the accuracy of this technique is limited by its flaws. Firstly, it is invasive, therefore nasal trauma is likely to produce undesirable effects on the vasculature. Secondly, measurements can only be taken when the patient is supine, however, they must receive allergen while in a seated position, which means changes in posture may affect the results. Thirdly, xenon activates neural receptors and may also interfere with results.

LDV works on the principle of the Doppler effect. The Doppler effect is a change in frequency or wavelength of a wave (electromagnetic or sound) perceived by a moving object relative to its position to the source of the wave. In LDV the wave is light and the objects are red blood cells within the microvasculature. Light is scattered by red blood cells as they pass through the microvasculature. The wavelength and frequency of the scattered light is identical to the wavelength and frequency which is received by the red blood cells. However, this changes relative to their position to the laser. Scattered light is detected by a photomultiplier tube. A reference wave, which is not passed through microvasculature, is also detected by the photomultiplier tube. Both sets of waves, scattered and reference, are transformed into an electrical signal and used to detect a change or "shift" in the light's wavelength. The faster the red blood cells move, the greater the degree of scatter and therefore the greater the change in the frequency (or wavelength) of the scattered light (Druce, 1993). LDV is non-invasive and can be used to measure dynamic changes in blood flow and volume. However, positioning of the probe, which emits and detects the light, is likely to be complicated by the inability to see the nasal turbinate and by subject non-compliance.
2.1.3 Estimation of nasal patency

Magnetic resonance imaging and computed tomography are techniques that are used to visualise the nasal airway (Malm, 1997). They provide three-dimensional images of the nasal airway and can be used to measure changes in nasal congestion. However, the downfalls with these techniques are the large running costs and the high doses of radiation exposure.

Acoustic rhinometry measures the minimum nasal cross sectional area ($A_{\text{min}}$), within a certain depth of the nasal airway. Measurements are taken from the narrowest region of the nasal cavity, which tends to be around the inferior nasal turbinate, to give an objective measurement of nasal blockage.

Acoustic rhinometry uses sound waves to map out the internal anatomy of the nasal cavity. The rhinometer is a long hollow pipe with a pulse generator at the bottom end and a microphone just below the top end. A sterile plastic tube (approximately 6 cm long) is attached to the top of the rhinometer and is placed in the anterior aperture of the nostril. Subjects sit on a stable stool, with a straight back and face forwards. They are then required to take a deep breath through their mouths, then close their mouths and hold still whilst the measurement is taken. The pulse generator discharges a sound wave up the tube, this echoes within the nasal passage and reflected sound waves are recorded by a microphone, then amplified and sent to the computer where a graph of $A_{\text{min}}$ against distance is generated, as shown in figure 2.3. Each recording takes approximately 0.5 seconds to obtain.

While acoustic rhinometry may not directly calculate the nasal airway resistance, changes in $A_{\text{min}}$ correlate strongly with changes in airway resistance calculated by rhinomanometry (Austin and Foreman, 1994b). Acoustic rhinometry provides quick, reproducible and accurate results with very little subject compliance.

Assuming that the nasal airway is a tube in which air flows without turbulence, the minimal cross sectional area of the nasal cavity can be calculated as follows:

$$A_{\text{min}} = \pi r^2$$
Where:

\( r \) = the radius of the nasal airway.

Poiseuille's law states that:

\[
I = \frac{\pi \Delta P \cdot r^4}{8 \eta l}
\]

where:

\( I \) = rate of airflow through nasal airway
\( \Delta P \) = pressure difference across the nasal airway
\( r \) = Radius of airway
\( l \) = length of nasal airway
\( \eta \) = viscosity of air

Ohms law can be used to describe the magnitude of resistance within the nasal airway as follows:

\[
R = \frac{\Delta P}{I}
\]

where:

\( \Delta P \) = pressure difference across the nasal airway
\( I \) = rate of airflow through nasal airway

Therefore Poiseuille's law can be re-written as follows (after using ohms law for substitution of \( I \)):

\[
R = \frac{8 \eta l}{\pi \cdot r^4}
\]

\( R = \Delta P/I \)

\( P \) = pressure diff across nasal cavity
\( I \) = rate of airflow through the nasal cavity
Therefore the resistance to airflow through the tube is inversely proportional to the fourth power of its radius, meaning that a reduction in the diameter of the nasal airway results in a large increase in the resistance to airflow.

The nose is clearly not a smooth tube, it has a number of internal protrusions, which prevent streamlined movement of air through the nasal cavities and increase its surface area. The most prominent protrusions are the nasal turbinates. Thus, Poiseuille's law is an approximation for determining the resistance to airflow in the nasal airway.

2.1.4 Measurement of nasal inflammation and mediator production

Irrigation of the nasal airway is known as nasal lavage. Nasal lavage is a technique used to recover cells, mediators and enzymes from the nasal airway for biochemical and/or histological analysis (Baumgarten et al., 1986a; Svensson et al., 1989; Turner et al., 2001). It cannot be used to determine mediator concentrations on the surface of the nasal airway, it is simply used to monitor changes in mediator concentration. Basal mediator concentrations within the nasal airway may be high, therefore, pre-challenge lavages are usually performed and repeated to dilute mediator concentrations so a baseline mediator concentration, with little mediator accumulation, can be established. Nasal lavages may also be performed in conjunction with nasal biopsy. Tissue samples may be removed surgically, by scraping or by brushing and used for microscopy, cell staining, radioligand binding or a variety of other in vitro techniques (Mullol et al., 1992; Dear et al., 1996; Rajakulasgingam et al., 1997b)

2.1.5 Symptoms scores

Subjective assessments of symptoms severity quantified on a numerical scale. Symptoms usually assessed include nasal blockage, pruitis, sneezing and rhinorrhea.
2.2 Nasal challenge experiments

2.2.1 Subjects

All studies were carried out on non-atopic individuals. Any subjects with ill health or taking medication less than 4 weeks prior to the study were excluded. All subjects gave informed consent and studies were approved by UCL Research Ethics Committee. Experiments were performed in a laboratory at controlled temperature and humidity.

2.2.2 Dilution of challenge agents

Stock solutions of the nasal challenge agents were made by dilution in sterile saline (154 mM NaCl). 1.5 ml aliquots were then placed into 7 ml bijoux and stored at -20°C. The concentration of each stock aliquot was as follows: bradykinin, 1 and 2.5 mg/ml; histamine, 2 mg/ml; [D-Arg⁰, Hyp⁵, D-Phe⁷]-bradykinin, 2 mg/ml; des-arg¹⁰-kallidin, 2 and 4 mg/ml; des-arg¹⁰-leu⁹-kallidin, 2 mg/ml; aprotinin, 1 mg/ml; icatibant, 0.1, 0.3, 1 and 2 mg/ml. Platelet activating factor (PAF) was initially dissolved in ethanol and then diluted in deoxygenated saline, to produce a 0.6 mg/ml or 0.8 mg/ml solution of PAF, in 1% ethanol. 1.5 ml aliquots were placed into 7 ml bijoux and filled with nitrogen gas before being frozen at -20°C. Aliquots of PAF were only stable for 3 to 4 days at -20°C.

2.2.3 Nasal challenge

All nasally administered drugs were delivered via a hand-held pump spray (Perfect-Valois UK Ltd), which delivers 100 μl of solution with 98% accuracy. Drugs were dissolved and diluted in sterile saline. All nasal sprays were warmed to 37°C before administration.

2.2.4 Nasal lavage

A nasal olive (made of medical grade silicone) was attached to the end of a 5 ml syringe, to ensure a watertight seal when applied to each nostril. A kwill tube was also attached to the end of the syringe and used to withdraw 2.5 mls of
sterile saline from vessel heated to 37°C (figure 2.1). The kwill tube was then removed and the olive left attached to the syringe.

![Diagram of a syringe, with a kwill tube and medical grade silicone attached, used for nasal lavage.](image)

**Figure 2.1** Diagram of a syringe, with a kwill tube and medical grade silicone attached, used for nasal lavage.

Subjects were then asked to lean over a sink, at an angle of approximately 50° from vertical, to prevent fluid entering the nasopharynx. The saline was slowly instilled into the nasal cavity and then mixed for 10 seconds. The contents of the nasal cavity were expelled into a sink. This procedure was repeated twice in each nasal cavity. The fluid from the third pre-challenge lavages, of each nostril, was collected into a 15 ml conical tube and kept in ice until the end of the experiment. Approximately five minutes later subjects were challenged (to induce a nasal response) or treated (to inhibit a nasal response) with the appropriate agent. After intranasal challenge, subjects underwent a final lavage without any pre-washes, and the contents were collected in a new 15 ml conical tube. All nasal lavage fluid was then centrifuged at 1000 x g for 10 minutes at 4°C. The supernatant was then placed in polypropylene Eppendorf tubes and stored at -70°C until assay, unless otherwise stated. This protocol is summarised in figure 2.2.
Chapter 2 Materials & Methods

Figure 2.2) General protocol for studying the effect of intranasal challenge on mediator production on in the nasal airway

2.2.5 Acoustic Rhinometry

Nasal patency was determined with an acoustic rhinometer from GM Instruments, Kilwinning, UK. The minimum cross sectional area of both nasal cavities was measured (in triplicate) immediately prior to and then at, 2, 5, 10, and 15 minutes after all nasal challenges. The units of the minimum cross sectional area ($A_{\text{min}}$) are cm$^2$ (figure 2.3).

Figure 2.3) A map of the cross-sectional area of a nasal cavity prior and post nasal blockage. The x-axis corresponds to the distance from the end of the rhinometer which the cross-sectional area is measured. The y-axis corresponds to the cross-sectional area. The deepest troughs found in both the pre and post challenge recordings corresponds to the minimum cross-sectional area and probably represent the distribution caused by the inferior nasal turbinate.
2.2.6 Data analysis

The resting patency of each nasal cavity was determined by calculating the mean, for each nostril, triplicate resting $A_{\text{min}}$ values ($A_{\text{min}}$ values obtained prior to nasal challenge). The overall resting nasal patency was the mean of the resting values for each nostril. These calculations were repeated with each set of triplicate values, for both nasal cavities, at each time point after nasal challenge to determine the average nasal patency after intranasal challenge.

Due to intra- and inter-subject variation in nasal patency, all post challenge values of $A_{\text{min}}$ were normalised to the baseline $A_{\text{min}}$. Figure 2.4 is a graph showing the effect of saline and bradykinin on nasal patency after intranasal challenge.

Figure 2.4) The time course of bradykinin-induced nasal blockage. The effect of bilateral intranasal challenge with saline or bradykinin (100 μg) on nasal patency. $A_{\text{min}}$ was measured in triplicate in each nasal cavity immediately prior, 2, 5, 10 and 15 min after each challenge. Values were normalised by expressing $A_{\min}$ as a percentage decrease in $A_{\min}$ from baseline for each subject. Data are means from 9 subjects, displayed as mean ± standard error of the mean.

To quantify the degree of nasal blockage caused by a particular drug, nasal $A_{\text{min}}$ vs. time graphs (figure 2.4) were integrated (mathematically) to find the area under the curve (AUC). If measurements were taken over a 15-minute period a value of 15 units.min corresponds to no change in nasal patency (i.e. an average normalised value of 1 unit over the 15 minute recording period) and a
value less than 15 units min corresponds to a reduction in nasal patency or an increase in nasal congestion. In this way figure 2.4, can be transformed into figure 2.5.

![Chart](image)

**Figure 2.5** The effect of bilateral intranasal challenge with saline or bradykinin (100 µg) on nasal patency. Amin was measured immediately prior, 2, 5, 10 and 15 min after each challenge. Values were normalised by expressing Amin as a percentage decrease in Amin from baseline for each subject, following which the AUC was determined. Data are means from 9 subjects, displayed as mean ± standard error of the mean.

### 2.2.7 Statistical analysis

All data was analysed statistically using a one-tailed non-parametric analysis, and when appropriate a post hoc Dunn's test. Non-parametric analyses were used because the data did not follow a Gaussian distribution. The null-hypothesis for each test assumed there was no difference between the control and active treatments.

In every experiment, each subject was exposed to both the active and control treatments, and so repeated measure statistical tests were used. As only two treatments were being compared (i.e. the active and inactive group) a Wilcoxon signed-rank tests was used. Probability values (p) less than 0.05 were considered statistically significant.
2.3 Endothelial Cells

EA.hy926 cells were kindly provided by Dr M. Peretti of the William Harvey Institute, London, UK. EA.hy926 are a hybrid of A459 epithelial cells and human umbilical vein endothelial cells (HUVECs). They retain their endothelial characteristics for up to 50 passages and are therefore frequently used as a substitute for HUVECs (Edgell et al., 1983).

**EA.hy926 culture**

EA.hy926 were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with nutrient mixture F12 HAM at 37°C, 5% CO₂, 95% O₂ and 100% humidity. DMEM was also supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), fetal calf serum (10%) and glutamine (2 mM). Once the cells reached 100% confluence, they were passaged, which occurred every 3-4 days.

**EA.hy926 Passage**

EA.hy926 were scraped from the surface of the T75 flasks and then centrifuged at 4°C and 500 x g for 5 minutes. They were then re-suspended in fresh nutrient medium at a ratio of 1:3 (old medium with EA.hy926: new medium) and placed in new T75 flasks to grow further in the conditions mentioned above. After achieving 100% confluence a total of three times, the EA.hy926 were homogenized.

**EA.hy926 homogenization**

The cells were scrapped off the surface of the T75 flasks and centrifuged at 4°C and 1000 x g for 10 minutes. The nutrient medium was aspirated and the cells were re-suspended in 1.5 mls of HEPES buffer (10 mM HEPES, 137 mM NaCl, 4 mM KCl, 11 mM D-glucose, 0.5 mg/ml RIA grade BSA, pH 7.4). They were then lysed by sonication (Ultrasonic sonicator XL, Heat systems). 500 µl of the whole cell homogenate was aliquoted into Eppendorf tubes and stored at -70°C until use. The remaining millilitre of homogenate was centrifuged at 15 000 x g for 8 minutes. The supernatant was aliquoted and stored at -70°C until use. The pellet was re-suspended in 0.5 ml HEPES buffer aliquoted and stored at -70°C until use.
2.4 Biochemical assays

2.4.1 Albumin enzyme-linked immunosorbent assay (ELISA)

The albumin content of nasal lavage fluid was measured by an indirect ELISA. This method is quick, reproducible, sensitive and economical with respect to other techniques, such as radioimmunoassay and radial immunodiffusion (Raphael et al., 1988).

Albumin lining the surface of 96-well microtiter plates competes with soluble albumin (from standards or samples), for a binding site on antihuman serum albumin conjugated to horseradish peroxidase (HRP). HRP cleaves the chromogenic substrate, o-phenylenediamine dihydrochloride, to produce a change in absorbance detectable by spectrophotometry.

Protocol:

(1) Human serum albumin (50 μg/ml) was dissolved in 0.1 M carbonate buffer at pH 9.6. The albumin-buffer was incubated overnight in a 96-well polypropylene microtiter plate at 4°C.

(2) The wells were washed 4 times with PT (PT: 0.05% Tween 80 in phosphate buffered saline at pH 7.4).

(3) The plate was incubated at 23°C for 30 minutes, with 200 μl of 0.1% goat serum diluted in PT.

(4) The wells were washed again, 4 times with PT.

(5) 50 μl of albumin standard (2-200 μg/ml) and diluted samples were added to separate microtiter wells in duplicate. 50 μl of goat antihuman serum albumin-HRP (diluted 1:1000 in PT) was added to all wells containing albumin standard and diluted nasal lavage samples. The plate was then incubated for 90 minutes at 23°C.

(6) The wells were washed again, 4 times with PT.
(7) The chromogenic reaction was initiated by the addition of 200 μl of o-phenylenediamine dihydrochloride (0.4 mg/ml, dissolved in distilled water).

(8) The plate was incubated at room temperature in the dark for 30 minutes before being read in a 96-well plate reader, at 450 nm.

The difference in absorbance of the albumin standards (ODx) and the blank (OD0) were plotted against the logarithmic value of the standard albumin concentration (figure 2.6). GraphPad (Prism 4) software was used to fit a curve to the graph and calculate the concentrations of albumin in the nasal lavage samples.

Figure 2.6) A standard curve showing the change in optical density with soluble albumin content. The optical density is reduced with increasing soluble albumin content, due to an increasing reduction of antihuman serum albumin-HRP binding to albumin coating the wells.

2.4.2 slgA enzyme-linked immunosorbent assay (ELISA)

Secretory immunoglobulin A content of nasal lavages was determined using a commercially available sandwich ELISA (Oxford Biosystems). Specific binding of slgA (unknown and standards) to a rabbit anti-slgA polyclonal antibody, is determined by the simultaneous binding of a signal antibody (peroxidase labelled anti-slgA antibody) and a chromogenic substrate for peroxidase, tetramethylbenzidine (TMB). The change in optical density produced by the addition of TMB is directly proportional to the specific binding of slgA.
Protocol:

(1) A 96-well microtiter plate, pre-coated with excess immobilised polyclonal rabbit slgA, was washed five times with ELISA buffer.

(2) Nasal lavage samples were diluted 1:1000 with the ELISA buffer.

(3) 100 μl of diluted nasal lavage fluid, slgA standards (0, 22.2, 66.6, 200 and 600 ng/ml) and calibrators were incubated and shaken, on a horizontal mixer, at room temperature for 1 hour in the 96-well microtiter plate.

(3) The wells were washed with the ELISA buffer.

(4) 100 μl of peroxidase-labelled slgA antibody was incubated and shaken, on a horizontal mixer, for 1 hour at room temperature in the 96-well microtiter plate.

(5) The wells were washed with the ELISA buffer.

(6) 100 μl of TMB (provided in assay kit, concentration not known) was added to the microtiter plate and incubated for 5-10 minutes at room temperature.

(7) 50 μl of stop solution (as provided in assay kit) was added to the microtiter plate and the absorption was determined immediately afterwards at 450 nm.

The slgA standards were used to generate a standard curve (figure 2.7) of slgA concentration against change in optical density. GraphPad (Prism 4) software was used to fit a curve to the graph and calculate the concentration of slgA in the nasal lavage samples.
2.4.3 Bradykinin Radioimmunoassay (RIA)

The kinin content of nasal lavage fluid was determined by radioimmunoassay. This method is reproducible and more sensitive than an ELISA, but it is less economical and takes 3 days to complete.

The assay is based on a competition between a labelled $^{125}\text{I}-\text{bradykinin}$ and unlabelled bradykinin (standard and unknown) binding to a limited amount of antibody. As the concentration of the standard or unknown peptide increases, the amount of $^{125}\text{I}$-bradykinin bound to the antibody decreases. A standard curve displaying the amount of $^{125}\text{I}$-bradykinin bound as a function of the concentration of unlabeled bradykinin is generated and used to calculate the amount of bradykinin in the lavage samples (figure 2.8).

In order to reduce kinin degradation in nasal lavage fluid, 0.5 ml samples were mixed with EDTA solution (1.5 mls of 53.3 mM) and kept on ice until the end of the experiment (Proud et al., 1983). These samples were then centrifuged at $-4^\circ\text{C}$ and 1000 x g, for 10 minutes. The supernatant was aliquoted into Eppendorf tubes and be frozen at $-70^\circ\text{C}$ until assay.
Figure 2.8) A standard curve displaying the relationship between the concentration of non-labelled bradykinin and the binding of $^{125}\mathrm{I}$-bradykinin to rabbit anti-bradykinin serum.

Protocol:
(1) 100 µl of nasal lavage samples and bradykinin standards were incubated overnight (16-24 hours) at 4°C with rabbit anti-bradykinin serum (100 µl) in polypropylene tubes. These tubes were also supplemented with 100 µl of RIA buffer. Rabbit anti-bradykinin serum was not added to the tubes in which the total count and non-specific binding of $^{125}\mathrm{I}$-bradykinin were to be determined: these tubes only contain RIA buffer (200 µl).

(2) 100 µl of [$^{125}\mathrm{I}$]-bradykinin (10,000-15,000 cpm) was added to all the RIA tubes in the assay. They were then incubated overnight (16-24 hours) at 4°C.

(3) All bradykinin (labelled and non-labelled) bound to the rabbit anti-bradykinin antibodies was precipitated by centrifugation (1700 x g for 30 minutes at 4°C) after the addition of normal rabbit serum (100 µl) and goat anti-rabbit IgG serum (100 µl).

(4) The supernatant was then aspirated from all the tubes in the assay (apart from those used to determine the total activity) and the activity of the pellets determined.
The specific binding of all the samples and the bradykinin standards was determined using the following equation:

\[
\frac{B_O}{B_{\text{max}}} = \frac{X - \text{NSB}}{\text{TB} - \text{NSB}} \times 100
\]

Where:
X = the counts per minute of in the pellets of the standard or unknown samples.
NSB = the counts per minute in the pellets of the non-specific binding tubes.
TB = the counts per minute in the pellets of the total binding tubes.

A graph showing the effect of increasing non-labelled bradykinin concentrations on the binding of [\text{125I}]-bradykinin to rabbit anti-bradykinin serum was generated (figure 2.8). GraphPad (Prism 4) software was then used to fit a curve to the graph and calculate the concentrations bradykinin in the nasal lavage samples.

2.4.4 Bradford assay

The amount of protein found in the homogenized EA.hy926 cells was determined using a Bradford assay. This protein assay is fast and fairly accurate. It works on the principle that the degree of absorbance exhibited by a coloured dye found in Bradford reagent, increases proportionally with its binding to protein (figure 2.9).

Protocol:

1. Human serum albumin standards (20 - 100 µg/ml) were created in Tris buffer (50 mM Tris, 113 mM NaCl, pH 7.5).

2. Protein samples were diluted 1:10 with Tris buffer.

3. 10 µl triplicates of the standards and samples were placed in a polystyrene microtiter 96-well plate.

4. The Bradford reagent was diluted 1:1 with distilled water.
(5) 190 µl of the diluted Bradford reagent was incubated for 30 minutes at room temperature, with all the wells that contained protein samples and standards.

(6) The absorbance was read at 570 nm.

(7) Samples with an absorbance that fell within the range of the standard curve were used to calculate the protein content of the undiluted samples.

![Standard Curve](image)

**Figure 2.9** A standard curve displaying the linear relationship between protein content and absorbance at 470 nm.

2.4.5 Enzyme activity assay

Chromogenic substrates are short synthetic peptides with a chromophore attached to their terminus. When mixed with specific enzymes, chromogenic substrates produce a colour change by separation from their chromophores in solution due to the hydrolysis of the bond connecting the chromophore to the rest of the substrate. This hydrolytic reaction is catalysed by specific enzymes and its rate of cleavage is proportional to the rate of enzyme activity. The amino acids incorporated into the chromogenic substrate enhance enzyme selectivity and are usually similar or identical to those found adjacent to the site of cleavage in the natural substrate (figure 2.10) (Claeson et al., 1978).
Chapter 2 Materials & Methods

**Figure 2.10** The amino acid composition of high molecular weight kininogen (Arg^{362}-Ser^{371}), bradykinin and the chromogenic substrate, S-2302. The arrows correspond to sites of HMWK cleavage by plasma kallikrein (PK) and tissue kallikrein (TK).

Chromogenic substrates selective for enzymes used throughout this thesis are stated in table 2.1.

One of the most commonly used chromophores is para-nitroaniline (pNA), when exposed to electromagnetic radiation, it selectively absorbs light at the wavelength 400 nm (violet). Atomically, absorption is the conversion of light energy (photons) into chemical energy, used by electrons of the chromophore to enter into a higher energy state. Simultaneously, certain wavelengths of light are not absorbed and these give rise to the yellow colour observed with pNA in solution. The intensity of this colour is directly proportional to the degree of violet light absorbed by the chromophore.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Amino acid sequence</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma kallikrein, Human</td>
<td>S-2302</td>
<td>D-Pro-Phe-Arg-pNA</td>
<td>220</td>
<td>150</td>
</tr>
<tr>
<td>(50 mM TRIS, pH 7.8, 16 mM NaCl)</td>
<td>S-2266</td>
<td>D-Val-Leu-Arg-pNA</td>
<td>500</td>
<td>85</td>
</tr>
<tr>
<td>Tissue kallikrein, Human</td>
<td>S-2266</td>
<td>D-Val-Leu-Arg-pNA</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>(50 mM TRIS, pH 9, 16 mM NaCl)</td>
<td>S-2765</td>
<td>D-Arg-Gly-Arg-pNA</td>
<td>10</td>
<td>170</td>
</tr>
<tr>
<td>Trypsin, Bovine</td>
<td>S-2222</td>
<td>Bz-Ile-Glu-Gly-Arg-pNA</td>
<td>20</td>
<td>240</td>
</tr>
<tr>
<td>(50 mM TRIS, pH 9, 245 mM NaCl)</td>
<td>S-2765</td>
<td>D-Arg-Gly-Arg-pNA</td>
<td>10</td>
<td>170</td>
</tr>
</tbody>
</table>

Table 2.1) Kinetic constants and amino acid sequence of chromogenic substrates available for use with plasma kallikrein, tissue kallikrein and trypsin (Diapharma, Chromogenix Booklet).
2.5 Enzyme assays

2.5.1 Crude nasal lavage fluid

Enzyme activity in nasal lavage fluid was determined by its ability to cleave the chromogenic substrate S-2266, a chromogenic substrate for kallikrein (table 2.1). Nasal lavage fluid was centrifuged at 1000 x g and 4°C for 10 minutes. 37.5 µl of the supernatant was incubated with 175 µl of Tris-HCl buffer (pH 7.8, 50 mM Tris, 113 mM NaCl) at 37°C for 15 minutes in a polystyrene 96-well microtiter well plate. After the addition of S-2266 (37.5 µl of 2.66 mM), spectrophotometric readings were taken at 10-minute intervals for one hour. Figure 2.11 is an example of such an experiment.

![Graph](image)

**Figure 2.11** A graph showing the dose-dependent aprotinin-induced inhibition of S-2266, 400 µM, cleavage by crude nasal lavage fluid. Enzyme activity was measured by spectrophotometry. Readings were taken at 10-minute intervals for 1 hour at 37°C in Tris-HCl buffer.

Inhibition curves, in which various doses of enzyme inhibitor were added to crude nasal lavage fluid, were created by determining the initial rate of the reaction at various doses of inhibitor. The activity of each dose of inhibitor was normalised with respect to the negative control (which had no inhibitor present). This transforms figure 2.11 into figure 2.12.
Figure 2.12) An inhibition curve showing the effect of aprotinin on the cleavage of S-2266 400 μM, by crude nasal lavage fluid. Readings were taken at 10-minute intervals for 1 hour at 37°C in Tris-HCl buffer. Where $v_i$ is the initial velocity of the enzyme reaction and $V_{max}$ is the velocity of the reaction without inhibitor present.

2.5.2 Purified active enzyme

2.5.2.1 Porcine tissue kallikrein

Protocol:

(1) 100 μl of Tris-HCl buffer (pH 8.2, 200 mM Tris at 25°C) with or without various enzyme inhibitors and icatibant present, was added to a polystyrene 96-well plate.

(2) 80 μl of porcine tissue kallikrein dissolved in Tris-HCl buffer was added to the 96-well microtiter plate and incubated at 37°C for 15 minutes.

(3) 20 μl of S-2266 were added to the microtiter plate and spectrophotometric readings were taken at 9-second intervals with Soft Max Pro3 (plate reader software). Note: The chromogenic substrate S-2266 was chosen according to its kinetic constants displayed in table 2.1.

(4) The initial rate of each reaction was determined and normalised with respect to the negative control (with no enzyme inhibitors or icatibant present or the maximum dose of substrate).
2.5.2.2 Human plasma kallikrein

Protocol:

(1) 100 µl of Tris-HCl buffer (pH 7.8, 50 mM Tris, 113 mM NaCl at 25°C) with or without various enzyme inhibitors and icatibant present, was added to a polystyrene 96-well plate.

(2) 80 µl of human plasma kallikrein dissolved in Tris-HCl buffer was added to the 96-well microtiter plate and incubated at 37°C for 15 minutes.

(3) 20 µl of S-2302 were added to the microtiter plate and spectrophotometric readings were taken at 9-second intervals with Soft Max Pro3 (plate reader software). Note: The chromogenic substrate S-2302 was chosen according to its kinetic constants displayed in table 2.1.

(4) The initial rate of each reaction was determined and normalised with respect to the negative control (with no enzyme inhibitors or icatibant present or the maximum dose of substrate).

2.5.2.3 Human pancreatic trypsin

Protocol:

(1) 100 µl of Tris-HCl buffer (pH 7.8, 200 mM Tris, 20 mM CaCl₂ at 25°C) with or without various enzyme inhibitors and icatibant present, was added to a polystyrene 96-well plate.

(2) 80 µl of human pancreatic trypsin dissolved in Tris-HCl buffer was added to the 96-well microtiter plate and incubated at 37°C for 15 minutes.

(3) 20 µl of S-2765 were added to the microtiter plate and spectrophotometric readings were taken at 9-second intervals with Soft Max Pro3 (plate reader software). Note: The chromogenic substrate S-2765 was chosen according to its kinetic constants displayed in table 2.1.
(4) The initial rate of each reaction was determined and normalised with respect to the negative control (with no enzyme inhibitors or icatibant present or the maximum dose of substrate).

2.5.3 Enzyme activation assays

2.5.3.1 Prekallikrein activation assays

Stock solutions of proteins used for prekallikrein activation assays were made by dilution in 4 mM sodium acetate buffer (pH 5.5 and 0.15 M NaCl) treated with 0.1 mM (4-aminophenyl)-methanesulfonylfluoride (APMSF) and stored in aliquots at -70°C. The concentration of each stock aliquot was as follows: FXII, 400 μM; heat shock protein 90, 125.5 nM; HMWK, 400 μM; prekallikrein, 400 μM; and hyaluronic acid binding protein, 66.66 μg/ml.

Protocol:

(1) 96-well microtiter polystyrene plates were coated with 250 μl of 1% polyethylene glycol diluted in HEPES buffer (10 mM HEPES, 137 mM NaCl, 4 mM KCl, 11 mM D-glucose, 0.5 mg/ml RIA grade Bovine serum albumin, pH 7.4) for 2 hours at room temperature.

(2) Prior to assay all proteins were mixed with equal volumes of 8 mM sodium acetate buffer (pH 5.5 and 0.15 M NaCl) for 15 minutes.

(3) The proteins were then diluted to the appropriate concentration in HEPES buffer for 45 minutes at room temperature.

(4) 85 μl or 105 μl of HEPES buffer, with or without various enzyme inhibitors and icatibant, was then added to the 96-well microtiter plate.

(5) Assay proteins were then added to the 96-well microtiter plate. The volumes were as follows: prekallikrein, 35 μl; HMWK, 35 μl; FXII, 35 μl; heat shock protein; 20 μl; hyaluronic acid binding protein; 20 μl and endothelial cell lysate; 20 μl.
(6) 20 μl of S-2302 were added to the microtiter plate and incubated at 37°C for 15 minutes. Note: The chromogenic substrate S-2302 was chosen according to its kinetic constants displayed in table 2.1.

(7) 20 μl of freshly made ZnCl2 (diluted in HEPES buffer) were then added to the 96-well microtiter plate and spectrophotometric readings were taken with Soft Max Pro3 (plate reader software).

The final concentration of each protein and reagent used in these assays is stated in chapter 6.2.2. The protocol and doses used were based upon pilot studies and previous publications (Joseph et al., 2001: Joseph et al., 2002).

2.5.3.2 Human plasma activation assays

Collection of blood

Human blood (9 ml) was collected directly into a 15 ml polypropylene conical flask containing 0.1 mol/litre sodium citrate (1 ml) using a 10 ml syringe and 21 gauge needle. The blood was centrifuged at 2000 x g and 25°C and for 15 minutes. The plasma was aliquoted into 1.5 ml Eppendorffs and frozen at -70°C.

Protocol:

(1) 96-well microtiter polystyrene plates were coated with 250 μl of 1% polyethylene glycol diluted in HEPES buffer (10 mM HEPES, 137 mM NaCl, 4 mM KCl, 11 mM D-glucose, 0.5 mg/ml RIA grade Bovine serum albumin, pH 7.4) for 2 hours at room temperature.

(2) 123.5 μl of HEPES buffer, with or without various enzyme inhibitors and icatibant, was then added to the 96-well microtiter plate.

(3) 7.5 μl of human plasma was then added to the microtiter plate.

(4) 20 μl of endothelial cell lysate was then added to the microtiter plate.
(5) 25 µl of S-2302 (2.4 µM) was added to the microtiter plate and incubated at 37°C for 15 minutes. Note: The chromogenic substrate S-2302 was chosen according to its kinetic constants displayed in table 2.1.

(6) 25 µl of freshly made ZnCl₂, 8 mM, (diluted in HEPES buffer) was then added to the 96-well microtiter plate and spectrophotometric readings were taken with Soft Max Pro3 (plate reader software).

The final concentration of each protein and reagent used in these assays is stated in chapter 6.2.2.

2.6 Statistical analysis of biochemical assays

All biochemical assay and enzyme assay data was analysed statistically using a one-tailed non-parametric analysis, and when appropriate a post hoc Dunn's test. Non-parametric analyses were used because the data did not follow the Gaussian distribution. The null-hypothesis for each test assumed there was no difference between the control and active treatments. Probability values (p) less than 0.05 were considered statistically significant.

The suitability of one equation (or model) over another for data was chosen using an F test (global fitting analysis). A p value less than 0.05 was deemed statistically significant.

Statistical differences in Hill co-efficients from unity were determined using an F test. A p value less than 0.05 was deemed statistically significant.

2.7 Spectrophotometry

Spectrophotometry was performed with a 96-well plate reader (Molecular Devices, Thermomax microtiter plate reader) kindly provided by Pfizer, UK.

Spectrophotometry works on the principle that the absorption intensity is directly proportional to the concentration of the chromophore in solution. This is expressed by the Lambert-Beer law:
Absorbance = \text{Log}\left(\frac{I_o}{I}\right) = \varepsilon \cdot c \cdot l

Where:
\varepsilon = \text{extinction coefficient (molar absorptivity)}, \ c = \text{concentration of the solute (M)}
\ l = \text{length/thickness of the solution (cm)}, \ I_o = \text{intensity of the light of incidence}
\ I = \text{intensity of the light after passing through the solution}

Spectrophotometers quantify the intensity of monochromatic light which passes through a sample, and therefore can be used to determine the concentration of the chromophore in solution.

The spectrophotometer was used to measure the change in optical density of solutions associated with ELISAs, enzyme assays and Bradford assays.
2.7 Materials

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

<table>
<thead>
<tr>
<th>MATERIAL</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pharmacological Agents</strong></td>
<td></td>
</tr>
<tr>
<td>Acetyl-β-methylcholine chloride</td>
<td>Sigma, Poole, UK.</td>
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<tr>
<td>[1-adamantaneacetyl-D-Arg&lt;sup&gt;0&lt;/sup&gt;, Hyp&lt;sup&gt;3&lt;/sup&gt;, beta-(2-thienyl)-Ala&lt;sup&gt;5,8&lt;/sup&gt; D-Phe&lt;sup&gt;7&lt;/sup&gt;]-bradykinin</td>
<td>Bachem, St. Helens, UK.</td>
</tr>
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<td>Aprotinin (5500 KIU/mg)</td>
<td>Calbiochem, Nottingham, UK.</td>
</tr>
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<td>Bradykinin</td>
<td>Bachem, St. Helens, UK.</td>
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<td>Captopril, 12.5 mg (tablets)</td>
<td>UCH Pharmacy, London, UK.</td>
</tr>
<tr>
<td>[D-Arg&lt;sup&gt;0&lt;/sup&gt;, Hyp&lt;sup&gt;3&lt;/sup&gt;, D-Phe&lt;sup&gt;7&lt;/sup&gt;]-bradykinin (NPC 567)</td>
<td>Bachem, St. Helens, UK.</td>
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<td>Des-arg&lt;sup&gt;10&lt;/sup&gt;-kallidin</td>
<td>Bachem, St. Helens, UK.</td>
</tr>
<tr>
<td>Des-arg&lt;sup&gt;10&lt;/sup&gt;-leu&lt;sup&gt;9&lt;/sup&gt;-kallikdin</td>
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<td>Dimethyl Sulfoxide (DMSO)</td>
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<td>Histamine</td>
<td>Sigma, Poole, UK.</td>
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<td>Icatibant</td>
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</tr>
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<td>Soybean trypsin inhibitor</td>
<td>Calbiochem, Nottingham, UK.</td>
</tr>
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<td>WIN 64338</td>
<td>Torcris, Bristol, UK.</td>
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## Biochemical and Enzyme assays

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
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<tbody>
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<td>96-well microtiter polystyrene plates</td>
<td>Griener, Gloucestershire, UK.</td>
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<td>96-well microtiter polypropylene plates</td>
<td>Dynatech Labs., Virginia, USA.</td>
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<td>Bovine serum albumin (RIA grade)</td>
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<td>Bradykinin Radioimmunoassay Kit</td>
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<td>D-glucose</td>
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<td>Goat serum</td>
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<td>Hageman factor, human (FXII)</td>
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<td>Heat sock protein-90, human</td>
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<td>HEPES</td>
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<td>High molecular weight kininogen (human, single chain)</td>
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<td>Human serum albumin, Fraction V</td>
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<td>Hyaluronic acid binding protein (bovine nasal cartilage)</td>
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<td>Kaolin</td>
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<td>o-phenylenediamine dihydrochloride</td>
<td>Sigma, Poole, UK.</td>
</tr>
<tr>
<td>p-Amidinophenyl-methanesulfonyl fluoride hydrochloride (p-APMSF)</td>
<td>Calbiochem, Nottingham, UK.</td>
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<td>Material/Reagent</td>
<td>Vendor/Location</td>
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<td>----------------------------------</td>
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<td>Prekallikrein, human</td>
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<td>S-2266</td>
<td>Quadraturech, Surrey, UK.</td>
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<tr>
<td>S-2302</td>
<td>Quadraturech, Surrey, UK.</td>
</tr>
<tr>
<td>S-2675</td>
<td>Quadraturech, Surrey, UK.</td>
</tr>
<tr>
<td>Sheep antihuman serum albumin conjugated to horseradish peroxidase</td>
<td>Europa Bioproducts, Cambridge, UK</td>
</tr>
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<td>Sodium acetate</td>
<td>Sigma, Poole, UK.</td>
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<td>Sodium bicarbonate</td>
<td>Sigma, Poole, UK.</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>BDH, Poole, UK.</td>
</tr>
<tr>
<td>Sodium phosphate (monobasic)</td>
<td>Sigma, Poole, UK.</td>
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<tr>
<td>Sodium phosphate (dibasic)</td>
<td>Sigma, Poole, UK.</td>
</tr>
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<td>Tissue kallikrein, porcine</td>
<td>Calbiochem, Nottingham, UK.</td>
</tr>
<tr>
<td>Tris</td>
<td>Calbiochem, Nottingham, UK.</td>
</tr>
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<td>Trypsin, human pancreas</td>
<td>Calbiochem, Nottingham, UK.</td>
</tr>
<tr>
<td>Tween 80</td>
<td>Sigma, Poole, UK.</td>
</tr>
</tbody>
</table>
### Materials & Methods

**ZnCl₂**  
Sigma, Poole, UK.

**Cell culture**

**DMEM + F12 nutrient**  
Sigma, Poole, UK.

**Fetal calf serum**  
Invitrogen, Paisley, UK.

**L-Glutamine**  
Sigma, Poole, UK.

**Penicillin**  
Sigma, Poole, UK.

**Streptomycin**  
Sigma, Poole, UK.

**EA.hy926 cells**  
William Harvey Institute, London, UK.

**Miscellaneous**

**Acetic acid**  
BDH, Poole, UK.

**EDTA**  
Calbiochem, Nottingham, UK.

**Hydrochloric acid**  
BDH, Poole, UK.

**Medical grade silicone seals**  
Kapitex Healthcare, Wetherbery, UK

**Sodium chloride**  
BDH, Poole, UK.

**Sodium citrate**  
Calbiochem, Nottingham, UK.
CHAPTER 3
THE EFFECT OF ICATIBANT ON PAF-INDUCED KININ PRODUCTION IN THE HUMAN NASAL AIRWAY

3.1 Introduction

Individuals who suffer from perennial allergic rhinitis, which is caused by house dust mite (HDM) allergen, also produce kinins in response to intranasal challenge with house dust mite (Dear et al., 1996). Kinins play a significant role in the nasal blockage of individuals who suffer from perennial allergic rhinitis as intranasal pre-treatment with the kinin B2 receptor antagonist, icatibant, abolishes HDM-induced nasal blockage and attenuates the recovery of kinins from nasal lavage fluid (Dear et al., 1996). Kinin generation stimulated in non-atopic nasal airways, by intranasal challenge with PAF, is also inhibited by pre-treatment with icatibant (Turner et al., 2000b).

Exogenous application of PAF to the upper airway induces kinin production and also mimics symptoms of allergic rhinitis (Miadonna et al., 1996; Turner et al., 2000b; Maniscalco et al., 2000). Therefore, it is possible that PAF generates an increase in tissue or plasma kallikrein activity within the human nasal airway.

In this chapter non-atopic individuals will be challenged intranasally with PAF: (1) to determine whether it induces glandular activation (by measurement of secretory IgA in nasal lavage fluid using an ELISA); (2) to determine whether it induces an increase in vascular permeability (by measurement of the albumin content of nasal lavage fluid using an ELISA) and; (3) to determine the level of kinin production in nasal lavage fluid (measured by radioimmunoassay). The effect of PAF on kallikrein enzyme activity in crude nasal lavages fluid will also be determined by spectrophotometry using the chromogenic substrate S-2266. Non-atopic individuals will also be topically pre-treated with a kallikrein inhibitor, aprotinin, to determine whether it reduces PAF-induced kinin production within the nasal airway. Subjects will also be challenged intranasally with methacholine to determine if it increases...
kalikrein enzyme activity in nasal lavages fluid. Lastly, the effectiveness of the kinin B$_2$ receptor antagonists, icatibant and NPC 567, and the kinin B$_1$ receptor antagonist, des-arg$^{10}$-leu$^9$kallidin, at reducing PAF-induced kinin and albumin production in non-atopic airways will be investigated.

**Bradykinin:** Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg  
**NPC 567:** Arg-Arg-Pro-Hyp-Gly-Phe-Ser-D-Phe-Phe-Arg  
**Icatibant:** Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg

**Figure 3.1** The amino acid sequence of the kinin B$_2$ receptor agonist, bradykinin, and the kinin B$_2$ receptor antagonists, NPC 567 and icatibant.
3.2 Experimental protocol

All experiments were performed on non-atopic individuals. Subjects were made blind to the treatments which they received during the course of the investigation.

3.2.1 The effect of PAF on kallikrein-like activity, albumin, sIgA and kinin production

The aim of this study was to determine the effect of PAF on the kallikrein-like enzyme activity, kinin production, secretory IgA (sIgA) levels and albumin content of crude nasal lavage fluid. Subjects underwent three pre-challenge nasal lavages (the third lavage was used as the baseline while the first two lavages were discarded) followed by a bilateral intranasal challenge with a 100 μl aerosol of saline or PAF, 60 μg. Twenty minutes after intranasal challenge with PAF or saline, subjects underwent a final nasal lavage. All subjects received PAF or saline in a random order, on separate occasions, at least 3 days apart. The dose of PAF used in this study was chosen on the basis of previously published data (Turner et al., 2000b). The protocol of this investigation is summarised in figure 3.2:

![Figure 3.2](image.png)

**Figure 3.2** Protocol for investigating effect of PAF on kallikrein enzyme activity, albumin production, sIgA levels and kinin content of lavage fluid from the nasal airway.
3.2.2 The effect of methacholine on kallikrein-like enzyme activity

The aim of this study was to determine whether challenge with the muscarinic receptor agonist, methacholine, induces the release of tissue kallikrein from serosal glands within the nasal airway. Subjects underwent three pre-challenge nasal lavages (the third lavage was used as the baseline while the first two lavages were discarded) followed by a 100 μl aerosol of saline or methacholine (1 mg, 3 mg or 10 mg) in both nostrils. Ten minutes after intranasal challenge subjects underwent a final nasal lavage. All 6 subjects received all 4 treatments in a random order, on separate occasions, at least 2 days apart. The protocol of this investigation is summarised in figure 3.3:

![Figure 3.3](image)

**Figure 3.3** Protocol for investigating effect of methacholine on kallikrein enzyme activity of lavage fluid from the nasal airway.

3.2.3 The effect of icatibant on PAF-induced albumin and kinin production

The aim of this study was to determine the effect of the high affinity kinin B₂ receptor antagonist, icatibant, on PAF-induced kinin and albumin production in nasal lavage fluid. Subjects underwent three pre-challenge nasal lavages (the third lavage was used as the baseline while the first two lavages were discarded) followed by bilateral pre-treatment with a 100 μl aerosol of saline or icatibant. Two minutes later they were challenged bilaterally with PAF, 60 μg, or saline. Twenty minutes after intranasal challenge with PAF or saline, subjects underwent a final nasal lavage. All 7 subjects received each of the following 7 treatments in a random order, on separate occasions, at least 3 days apart:
Chapter 3  

The effect of icatibant on PAF-induced kinin production

- Saline / saline
- Saline / PAF, 60 µg
- Icatibant, 10 µg / PAF, 60 µg
- Icatibant, 30 µg / PAF, 60 µg
- Icatibant, 100 µg / PAF, 60 µg
- Icatibant, 200 µg / PAF, 60 µg
- Icatibant, 200 µg / saline

The doses of icatibant used in this study are based on previous published studies (Dear et al., 1996). The protocol of this investigation is summarised in figure 3.4:

![Diagram](image.png)

Figure 3.4) Protocol for investigating the effect of icatibant on PAF-induced albumin and kinin production within the nasal airway.

3.2.4 The effect of icatibant on the binding of bradykinin to rabbit anti-bradykinin serum

The aim of this study was to determine whether icatibant interferes with the detection of bradykinin by radioimmunoassay (RIA). RIA rabbit anti-bradykinin serum was diluted in radioimmunoassay buffer then incubated with 11.5 pg/µl bradykinin and icatibant (10 nM, 100 nM, 1 µM and 10 µM). The kinin content of these samples were determined by radioimmunoassay. The concentration of bradykinin used in this investigation was chosen on the basis that it reduces the binding of [³H]-bradykinin to rabbit anti-bradykinin serum by 50%.
3.2.5 The effect of NPC-567 on albumin and kinin production

The aim of this study was to determine the effect of the low affinity kinin B$_2$ receptor antagonist, NPC-567, on PAF-induced kinin and albumin production in nasal lavage fluid. Subjects underwent three pre-challenge nasal lavages (the third lavage was used as the baseline while the first two lavages were discarded) followed by bilateral pre-treatment with a 100 µl aerosol of saline or NPC-567, 200 µg. Two minutes later they were challenged bilaterally with PAF, 60 µg or saline. Twenty minutes after intranasal challenge with PAF or saline, subjects underwent a final nasal lavage. All 8 subjects received each of the following 4 treatments in a random order, on separate occasions, at least 3 days apart:

- Saline / saline
- Saline / PAF, 60 µg
- NPC-567, 200 µg / PAF, 60 µg
- NPC-567, 200 µg / saline

The protocol of this investigation is summarised in figure 3.5:

![Protocol diagram](image)

Figure 3.5) Protocol for investigating the effect of NPC-567 on PAF-induced albumin and kinin production within the nasal airway.

3.2.5 The effect of aprotinin on albumin and kinin production

The aim of this study was to determine the effect of the kallikrein inhibitor, aprotinin, on PAF-induced kinin and albumin production in nasal lavage fluid. Subjects underwent three pre-challenge nasal lavages (the third lavage was used as the
baseline while the first two lavages were discarded) followed by bilateral pre-treatment with a 100 µl aerosol of saline or aprotinin, 100 µg. Two minutes later they were challenged bilaterally with PAF, 60 µg or saline. Twenty minutes after intranasal challenge with PAF or saline, subjects underwent a final nasal lavage. All 8 subjects, received each of the following 4 treatments in a random order, on separate occasions, at least 3 days apart:

- Saline / saline
- Saline / PAF, 60 µg
- Aprotinin, 100 µg / PAF, 60 µg
- Aprotinin, 100 µg / saline

The protocol of this investigation is summarised in figure 3.6:

Figure 3.6) Protocol for investigating the effect of aprotinin on PAF-induced mediator production in the nasal cavity.

3.2.6 The effect of des-arg^{10}-leu^{9}-kallidin on albumin and kinin production

The aim of this study was to determine the effect of the kinin B₁ receptor antagonist, des-arg^{10}-leu^{9}-kallidin, on PAF-induced kinin and albumin production in nasal lavage fluid. Subjects underwent three pre-challenge nasal lavages followed by bilateral pre-treatment with a 100 µl aerosol of saline or des-arg^{10}-leu^{9}-kallidin, 200 µg. Two minutes later they were challenged bilaterally with PAF, 60 µg or saline. Twenty minutes after intranasal challenge with PAF or saline, subjects underwent a
final nasal lavage. All 6 subjects received each of the following 4 treatments in a random order, on separate occasions, at least 3 days apart:

- Saline / saline
- Saline / PAF, 60 μg
- Des-arg^{10}-leu^{9}-kallidin, 200 μg / PAF, 60 μg
- Des-arg^{10}-leu^{9}-kallidin, 200 μg / saline

The protocol is of this investigation is summarised figure 3.7:

![Protocol for investigating the effect of des-arg^{10}-leu^{9}-kallidin on PAF-induced albumin and kinin production within the nasal airway. DAKD is des-arg^{10}-leu^{9}-kallidin.](image)

**Figure 3.7** Protocol for investigating the effect of des-arg^{10}-leu^{9}-kallidin on PAF-induced albumin and kinin production within the nasal airway. DAKD is des-arg^{10}-leu^{9}-kallidin.

### 3.2.7 Data analysis

Three nasal lavages were performed prior to intranasal challenge or pre-treatment with the appropriate agent, the first two lavages were discarded and the third lavage was kept on ice until the end of the experiment. A single lavage was performed after challenge with the appropriate agent and collected for analysis. Nasal lavage fluid was then centrifuged at 1000 x g for 10 minutes and 4°C. Aliquots of the supernatant were stored at -70°C until assay. The albumin and slgA content of nasal lavage fluid were determined by ELISA and the kinin content by radioimmunoassay.

The supernatant of the nasal lavage fluid was incubated with Tris-HCl buffer (200 mM Tris, pH 7.8 at 37°C) for 15 minutes at 37°C. Kallikrein enzyme activity of the lavage fluid was determined by spectrophotometry. The change in absorbance
associated with the cleavage of S-2266, 400 µM, at 405 nm, was measured at 10-minute intervals for a duration of one hour, as mentioned in chapter 2.5.1. Results were displayed as the rate of increase in optical density/minute.

A Wilcoxon sign-rank test (non-parametric, paired test), Friedman test (non-parametric, one-way analysis of variance test) and Dunn's test (non-parametric, post hoc test) were used to determine statistical differences in albumin, slgA, kinin production and kallikrein activity. A p value less than 0.05 (p<0.05) was taken as statistically significant.
3.3 Results

3.3.1 The effect of PAF on kallikrein-like activity, albumin, sIgA and kinin production

Intranasal application of PAF, 60 µg, compared to saline, caused a significant rise in the kinin content of nasal lavage fluid recovered 20 minutes after challenge (p<0.05, Wilcoxon sign-rank test), as shown in figure 3.8.

Intranasal application of PAF, 60 µg, compared to saline, did not elevate kallikrein-like enzyme activity in nasal lavage fluid recovered 20 minutes after challenge, as shown in figure 3.9. Incubation of nasal lavage fluid with aprotinin, 1 µM, and soybean trypsin inhibitor (SBTI), 1 µM, significantly reduced kallikrein-like activity in samples of nasal lavage fluid collected before and after challenge with saline or PAF (p<0.05, Friedman test).

Intranasal application of PAF, 60 µg, compared to saline, did not cause a significant rise in the sIgA content of nasal lavage fluid recovered 20 minutes after challenge, as shown in figure 3.10.

Intranasal application of PAF, 60 µg, compared to saline, caused a significant rise in the albumin content of nasal lavage fluid recovered 20 minutes after challenge (p<0.05, Wilcoxon sign-rank test), as shown in figure 3.11.

3.3.2 The effect of methacholine on kallikrein enzyme activity

Intranasal challenge with the muscarinic receptor agonist, methacholine (1, 3 and 10 mg), did not increase kallikrein enzyme activity in nasal lavage fluid recovered 10 minutes after challenge compared to saline, as shown in figure 3.12. Incubation of nasal lavage fluid with aprotinin, 1 µM, and SBTI, 1 µM, significantly reduced kallikrein-like activity in samples collected before and after challenge with saline and methacholine (p<0.05, Wilcoxon sign-rank test).
Figure 3.8) The effect of intranasal challenge with PAF, 60 µg, or saline on the kinin content of nasal lavage fluid recovered prior to and 20 minutes after challenge. The difference (Δ) in kinin content of the pre-challenge and post-challenge lavages was determined. Data are the means and s.e.m. from 6 subjects. The mean and standard errors of the levels of kinin found in nasal lavage fluid collected prior to challenge with saline and PAF were 29.7 ± 12.23 pg/ml and 11.65 ± 3.02 pg/ml respectively. *Significant increase in the kinin content of nasal lavage fluid after challenge with PAF, compared to nasal lavage fluid collected after challenge with saline (p<0.05, Wilcoxon sign-rank test).
Figure 3.9) Enzyme activity in nasal lavage fluid recovered 20 minutes after intranasal challenge with PAF, 60 µg, or saline. Samples were incubated with aprotinin, 1 µM, or soybean trypsin inhibitor (SBTI), 1 µM. Data are the means and s.e.m. from 6 subjects. Basal levels of enzyme activity in nasal lavage fluid were $0.70 \pm 0.25 \times 10^{-3}$ OD/min prior to challenge with saline and $0.72 \pm 0.18 \times 10^{-3}$ OD/min prior to challenge with PAF. *Significant reduction in the enzyme activity of the nasal lavage fluid compared to the activity of nasal lavages fluid collected after challenge with saline or PAF with no inhibitors present in the assay ($p<0.05$, Friedman test).
Figure 3.10) The effect of intranasal challenge with PAF, 60 μg, or saline on the secretory IgA content of nasal lavage fluid recovered prior to and 20 minutes after challenge. The difference (Δ) in slgA content of the pre-challenge and post-challenge lavages was determined. Data are the means and s.e.m. from 8 subjects. The mean and standard errors of the basal levels of slgA found in nasal lavage fluid collected prior to challenge with saline and PAF were 19.95 ± 2.56 μg/ml and 22.04 ± 3.26 μg/ml respectively.
The effect of intranasal challenge with PAF, 60 µg, or saline on the albumin content of nasal lavage fluid recovered prior to and 20 minutes after challenge. The difference (Δ) in albumin content of the pre-challenge and post-challenge lavages was determined. Data are the means and s.e.m. from 9 subjects. The mean and standard errors of the basal levels of albumin found in nasal lavage fluid collected prior to challenge with saline and PAF were 4.02 ± 0.69 µg/ml and 3.15 ± 0.5 µg/ml respectively. *Significant increase in the albumin content of nasal lavage fluid after challenge with PAF, compared to nasal lavage fluid collected after challenge with saline (p<0.05, Wilcoxon sign-rank test).
Chapter 3 The effect of icatibant on PAF-induced kinin production

Figure 3.12) The effect of intranasal challenge with saline or methacholine (1, 3 and 10 mg) on the kallikrein enzyme activity of nasal lavage fluid recovered 10 minutes after intranasal challenge. Samples were incubated with aprotinin, 10 μM, and soybean trypsin inhibitor (SBTI), 10 μM. The mean levels of enzyme activity found in nasal lavage fluid collected prior to challenge ranged from 0.67 OD x10⁻³/min to 0.78 OD x10⁻³/min. Data are means and standard errors from 6 subjects.
3.3.3 The effect of icatibant on PAF-induced albumin and kinin production

Intranasal pre-treatment with the kinin B2 receptor antagonist icatibant (10, 30, 100 and 200 µg) failed to dose-dependently reduce the rise in the kinin content of nasal lavage fluid recovered 20 minutes after challenge with PAF, 60 µg, as shown in figure 3.13 (p>0.05, Friedman's test). The increase in kinin production caused by challenge with PAF, 60 µg, was significantly reduced after pre-treatment with icatibant, 200 µg (p<0.05, Dunn's test). Intranasal pre-treatment with saline did not reduce the elevation in the kinin content of nasal lavage fluid recovered 20 minutes after challenge with PAF, 60 µg. Icatibant, 200 µg, did not affect the kinin content of nasal lavage fluid recovered after challenge with saline.

Intranasal pre-treatment with icatibant (10, 30, 100 and 200 µg) failed to reduce the significant increase in albumin content of nasal lavage fluid recovered 20 minutes after challenge with PAF, 60 µg, as shown in figure 3.14 (p>0.05, Friedman's test). Intranasal pre-treatment with saline did not reduce the elevation in albumin content of nasal lavage fluid recovered 20 minutes after challenge with PAF, 60 µg. Icatibant, 200 µg, did not affect the albumin content of nasal lavage fluid recovered after challenge with saline.

3.3.4 The effect of icatibant on the binding of bradykinin to rabbit anti-bradykinin serum

The aim of this study was to determine whether icatibant interferes with the detection of bradykinin by the radioimmunoassay rabbit anti-bradykinin serum. Icatibant, 10 nM to 10 µM, did not reduce the binding of rabbit anti-bradykinin serum to bradykinin, 11.5 pg/μL, as shown in figure 3.15.

3.3.5 The effect of NPC-567 on albumin and kinin production

Intranasal pre-treatment with the kinin B2 receptor antagonist NPC-567, 200 µg, did not reduce the rise in the kinin content of nasal lavage fluid recovered 20 minutes after challenge with PAF, 60 µg, as shown in figure 3.16. NPC-567,
Figure 3.13) The effect of intranasal challenge with PAF, 60 µg, or saline on the kinin content of nasal lavage fluid after treatment with saline or icatibant. Nasal lavage fluid was recovered prior to intranasal pre-treatment and 20 minutes after intranasal challenge. The difference (Δ) in kinin content of the pre-challenge and post-challenge lavages was determined. The mean levels of kinin found in nasal lavage fluid collected prior to challenge ranged from 10 to 19.1 pg/ml. Data are the means and standard errors from 7 subjects.

*Significant reduction in the kinin content of nasal lavage fluid compared to nasal lavage fluid collected after challenge with PAF and pre-treatment with saline (p<0.05, Dunn's test).
Chapter 3 The effect of icatibant on PAF-induced kinin production

Figure 3.14) The effect of intranasal challenge with PAF, 60 μg, or saline challenge on the albumin content of nasal lavage fluid after pre-treatment with saline or icatibant. Nasal lavage fluid was recovered prior to intranasal pre-treatment and 20 minutes after intranasal challenge. The difference (Δ) in albumin content of the pre-challenge and post-challenge lavages was determined. The mean levels of albumin found in nasal lavage fluid collected prior to challenge ranged from 3.3 to 4.9 μg/ml. Data are the means and standard errors from 7 subjects. *Significant increase in the albumin content of nasal lavage fluid compared to nasal lavage fluid collected after challenge and pre-treatment with saline (p<0.05, Dunn's test).
Figure 3.15) The effect of icatibant on the binding of rabbit anti-bradykinin serum to bradykinin, 11 pg/100 µl. Data are the means and standard errors from 3 experiments.
Figure 3.16) The effect of intranasal challenge with PAF, 60 μg, or saline on the kinin content of nasal lavage fluid after pre-treatment with saline or NPC-567, 200 μg. Nasal lavage fluid was recovered prior to intranasal pre-treatment and 20 minutes after intranasal challenge. The difference (Δ) in kinin content of the pre-challenge and post-challenge lavages was determined. The mean levels of kinin found in nasal lavage fluid collected prior to challenge ranged from 11.6 to 12.1 pg/ml. Data are the mean and standard errors from 8 subjects. NS, insignificant difference in the kinin content of nasal lavage fluid compared to nasal lavage fluid collected after challenge with saline and pre-treatment with NPC 567 (p>0.05, Wilcoxon sign-rank test).
Chapter 3 The effect of icatibant on PAF-induced kinin production

200 μg, did not affect the kinin content of nasal lavage fluid recovered after challenge with saline.

Intranasal pre-treatment with NPC-567, 200 μg, did not reduce the rise in the albumin content of nasal lavage fluid recovered 20 minutes after challenge with PAF, 60 μg, as shown in figure 3.17. Intranasal pre-treatment with saline did not affect the elevation in albumin content of nasal lavage fluid recovered 20 minutes after challenge with PAF, 60 μg. NPC-567, 200 μg, did not affect the albumin content of nasal lavage fluid recovered after challenge with saline.

3.3.6 The effect of aprotinin on albumin and kinin production

Intranasal pre-treatment with the kallikrein inhibitor aprotinin, 100 μg, significantly reduced the rise in the kinin content of nasal lavage fluid recovered 20 minutes after challenge with PAF, 60 μg (p<0.05, Wilcoxon sign-rank test), but did not abolish it, as shown in figure 3.18. Aprotinin, 100 μg, did not affect the kinin content of nasal lavage fluid recovered after challenge with saline.

Aprotinin, 100 μg, failed to reduce the rise in the albumin content of nasal lavage fluid recovered 20 minutes after challenge with PAF, 60 μg, as shown in figure 3.19. Aprotinin, 100 μg, did not affect the albumin content of nasal lavage fluid recovered after challenge with saline.

3.3.7 The effect of des-arg^{10}-leu^{9}-kallidin on albumin and kinin production

Intranasal pre-treatment with the kinin B1 receptor antagonist, des-arg^{10}-leu^{9}-kallidin, 200 μg, did not reduce the rise in the kinin content of nasal lavage fluid recovered 20 minutes after challenge with PAF, 60 μg, as shown in figure 3.20. Des-arg^{10}-leu^{9}-kallidin, 200 μg, did not affect the kinin content of nasal lavage fluid recovered after challenge with saline.

Intranasal pre-treatment with des-arg^{10}-leu^{9}-kallidin, 200 μg, did not reduce the rise in the albumin content of nasal lavage fluid recovered 20 minutes after challenge with PAF, 60 μg, as shown in figure 3.21. Intranasal pre-treatment with saline did
not affect the elevation in albumin content of nasal lavage fluid recovered 20 minutes after challenge with PAF, 60 μg. Des-arg\textsuperscript{10}-leu\textsuperscript{9}-kallidin, 200 μg, did not affect the albumin content of nasal lavage fluid recovered after challenge with saline.
Chapter 3 The effect of icatibant on PAF-induced kinin production

Figure 3.17) The effect of intranasal challenge with PAF, 60 μg, or saline on the albumin content in nasal lavage fluid after treatment with saline or NPC-567, 200 μg. Nasal lavage fluid was recovered prior to intranasal pre-treatment and 20 minutes after intranasal challenge. The difference (Δ) in albumin content of the pre-challenge and post-challenge lavages was determined. The mean levels of albumin found in nasal lavage fluid collected prior to challenge ranged from 2.9 to 3.9 μg/ml. Data are means and standard errors from 8 subjects. NS, insignificant difference in the albumin content of nasal lavage fluid compared to nasal lavage fluid collected after challenge with saline and pre-treatment with NPC 567 (p>0.05, Wilcoxon sign-rank test).
Figure 3.18) The effect of intranasal challenge with PAF, 60 µg, or saline on the kinin content of nasal lavage fluid after pre-treatment with saline or aprotinin, 100 µg. Nasal lavage fluid was recovered prior to intranasal pre-treatment and 20 minutes after intranasal challenge. The difference (Δ) in kinin content of the pre-challenge and post-challenge lavages was determined. The mean levels of kinin found in nasal lavage fluid collected prior to challenge ranged from 11.1 to 39.4 pg/ml. Data are the mean and standard errors from 8 subjects. * Significant reduction in the kinin content of nasal lavage fluid compared to nasal lavages fluid collected after pre-treatment with saline and challenge with PAF (p<0.05, Wilcoxon sign-rank test).
Figure 3.19) The effect of intranasal challenge with PAF, 60 μg, or treatment on the albumin content of nasal lavage fluid after treatment with saline or aprotinin, 100 μg. Nasal lavage fluid was recovered prior to intranasal pre-treatment and 20 minutes after intranasal challenge. The difference (△) in albumin content of the pre-challenge and post-challenge lavages was determined. The mean levels of albumin found in nasal lavage fluid collected prior to challenge ranged from 3.6 to 3.9 μg/ml. Data are means and standard errors from 8 subjects. NS, insignificant difference in the albumin content of nasal lavage fluid compared to nasal lavage fluid collected after challenge with saline and pre-treatment with aprotinin (p>0.05, Wilcoxon sign-rank test).
Figure 3.20) The effect of intranasal challenge with PAF, 60 μg, or saline on the kinin content of nasal lavage fluid after pre-treatment with saline or des-arg₁₀-leu⁹-kallidin, 200 μg. Nasal lavage fluid was recovered prior to intranasal pre-treatment and 20 minutes after intranasal challenge. The difference (Δ) in kinin content of the pre-challenge and post-challenge lavages was determined. The mean levels of kinin found in nasal lavage fluid collected prior to challenge ranged from 10.6 to 29.7 pg/ml. Data are means and standard errors from 6 subjects. NS, insignificant difference in the kinin content of nasal lavage fluid compared to nasal lavage fluid collected after challenge with saline and pre-treatment with des-arg₁₀-leu⁹-kallidin (p>0.05, Wilcoxon sign-rank test).
Figure 3.21 The effect of intranasal challenge with PAF, 60 μg, or saline on the albumin content of nasal lavage fluid after pre-treatment with saline or des-arg\(^{10}\)-leu\(^{9}\)-kallidin, 200 μg. Nasal lavage fluid was recovered prior to intranasal pre-treatment and 20 minutes after intranasal challenge. The difference (Δ) in albumin content of the pre-challenge and post-challenge lavages was determined. The mean levels of albumin found in nasal lavage fluid collected prior to challenge ranged from 2.5 to 3.9 μg/ml. Data are means and standard errors from 6 subjects. NS, insignificant difference in the albumin content of nasal lavage fluid compared to nasal lavage fluid collected after challenge with saline and pre-treatment with des-arg\(^{10}\)-leu\(^{9}\)-kallidin (p>0.05, Wilcoxon sign-rank test).
3.4 Discussion

Intranasal challenge with PAF, 60 μg, caused a significant rise in the kinin content of nasal lavage fluid (figure 3.8). However, there was not a concomitant increase in kallikrein enzyme activity of the nasal lavage fluid (figure 3.9). The inability to detect an increase in enzyme activity after challenge with PAF implies one of four things: (1) that the enzyme recovered by nasal lavage is not responsible for kinin synthesis, (2) the enzyme responsible for PAF-induced kinin synthesis is not recoverable by nasal lavage, (3) the enzyme responsible for PAF-induced kinin synthesis is recoverable by nasal lavage but not detectable by spectrophotometry with S-2266 or (4) there is no increase in activity of the enzyme responsible for kinin synthesis.

The latter theory could be explained by a lack of sensitivity of the assay. The kallikrein-kinin pathway is a cascade reaction that works in concert with various other proteolytic pathways in vivo (Heimark et al., 1980), consequently, only a small amount of prekallikrein (or prokallikrein) is required to produce an immense amount of kinin. Therefore, it is possible that the active form of the PAF-activated kininogenase is present in solution, but not detectable by spectrophotometry. However, it has been reported that the cleavage of S-2266 by crude bronchial lavage fluid of atopic asthmatics correlates well with kinin production induced by challenge with allergen (Christiansen et al., 1992). Kallikrein-like activity in crude nasal lavage fluid has also been detected by measuring the degradation of $[^3H]$-TAME ($[^3H]$-N-α-tosyl-L-arginine methyl ester) (Baumgarten et al., 1986b; Baumgarten et al., 1986c). Unfortunately, TAME is a non-specific substrate which is degraded by tissue kallikrein, plasma kallikrein (bound and unbound to $\alpha_2$-macroglobulin), mast cell tryptase, trypsin, plasmin and thrombin. Therefore, to conclusively identify tissue and plasma kallikrein in nasal lavage fluid, samples had to be concentrated, purified, probed by antibody affinity techniques and kininogenase activity measured by radioimmunoassay.

If PAF-induced kinin synthesis is not catalysed by tissue or plasma kallikrein, it could be mediated by mast cell tryptase (Imamura et al., 1996). However, there is very little evidence to show that PAF induces mast cell degranulation in vivo (Petersen et al., 1997) and up to 50% of kinin synthesis induced by PAF, 60 μg, within the nasal airway is inhibited by intranasal pre-treatment with the serine
protease inhibitor, aprotinin, 100 μg, which does not inhibit mast cell tryptase (figure 3.18) (Tanaka et al., 1983).

An inability to recover the enzyme responsible for PAF-induced kinin synthesis by nasal lavage could also be explained by the fact that enzyme precursors, such as prekallikrein, are not very stable in solution. They are also extremely dependant on the presence of cofactors and cell membrane receptors for their activation, interactions with both of these are disrupted by nasal lavage.

PAF, 60 μg, generated a significant increase in the albumin content of nasal lavage fluid recovered 20 minutes after intranasal challenge (figure 3.11). This is likely to have been caused by the activation of PAF receptors found on postcapillary vessels (Shirasaki et al., 2005).

Albumin leakage into the nasal cavity may be indicative of an increase in vascular permeability (Baumgarten et al., 1986) which is also associated with the exudation of prekallikrein and prekallikrein activating proteins, such as HMWK and FXII. This could explain the rise in kinin content of nasal recovered from subjects challenged with PAF.

Intranasal challenge with PAF, 60 μg, did not cause a rise in the slgA content of nasal lavage fluid recovered 20 minutes after challenge (figure 3.10). Secretory IgA, as well as tissue kallikrein, is stored within serosal glands of the nasal airway and is used as a marker of glandular activation (Poblete et al., 1993; Proud and Vio, 1993). Therefore, the inability to detect glandular activation suggests that tissue kallikrein is not released into the nasal cavity in response to challenge with PAF, 60 μg. However, receptors for PAF have been identified on submucosal glands within the human nasal airway (Shirasaki et al., 2005).

A second approach to investigate whether activation of glands within the nasal airway causes an increase in kallikrein activity of nasal lavage fluid, measurable by spectrophotometry with the chromogenic substrate S-2266, was to challenge subjects with the muscarinic receptor agonist methacholine. Intranasal challenge with methacholine (1 - 10 mg) failed to cause a detectable increase in kallikrein activity of the nasal lavage fluid (figure 3.12). However, methacholine, 10 mg, did
cause facial flushing and symptoms of rhinorrhea, both of which suggest that challenge with methacholine did cause glandular activation. However, this may have been mucosal, as opposed to serosal, in origin. It must be noted that previous studies have found that methacholine induces an increase in slgA, but only with an intranasal dose of 25 mg (Raphael et al., 1988).

Considering that icatibant is a structural analogue of bradykinin which binds to kinin B<sub>2</sub> receptors with high affinity, it is not unlikely that it may also bind to antibodies specific for bradykinin or kallidin. Topical administration of icatibant to non-atopic nasal airways is capable of inhibiting bradykinin-induced symptoms if administered up to 2 hours prior to intranasal challenge (Proud et al., 1994). Therefore residual icatibant is expected to be present in nasal lavage fluid collected 22 minutes after its introduction into the nasal airway. The aim of experiment 3.3.4 was to determine whether icatibant interferes with the binding of bradykinin to the rabbit anti-bradykinin serum of the radioimmunoassay which was used to detect kinin content of nasal lavage fluid. Unfortunately, the effective dose of icatibant required to inhibit kinin synthesis within the human nasal airway is not known, however, figure 3.15 clearly shows that icatibant does not effect the binding of bradykinin to rabbit anti-bradykinin serum at doses likely to exceed those used in experiment 3.3.3 (figure 3.13 and figure 3.14).

Pre-treatment with increasing doses of icatibant, 10 to 200 µg, failed to significantly increase the reduction in kinin content of nasal lavage fluid recovered after intranasal challenge with PAF, 60 µg (figure 3.13) (p>0.05, Friedman’s test). However, pre-treatment with 200 µg of icatibant significantly reduced PAF-induced kinin production (p<0.05, Dunn’s test) and generates an IC<sub>50</sub> between 100 and 200 µg. It must be noted that the dose at which kinin production was significantly attenuated by icatibant (200 µg) is also equal to the dose required to completely reverse bradykinin-induced nasal blockage and albumin production in human nasal airways, 200 µg (Austin et al., 1994c). A similar finding was also observed by Turner et al., (2000b), with the high affinity kinin B<sub>2</sub> receptor antagonist, [Ad]-Bk ([1-adamantaneacetyl-D-Arg<sup>6</sup>, Hyp<sup>3</sup>-(2-thienyl)-Ala<sup>5,8</sup>, D-Phe<sup>7</sup>]-bradykinin). However, the low affinity kinin B<sub>2</sub> receptor antagonist, NPC-567, failed to inhibit the recovery of kinins from PAF-challenged subjects (figure 3.16). This suggests that the ability of kinin B<sub>2</sub> receptor antagonists to inhibit the recovery of kinins from nasal lavage
fluid may be related to their ability to antagonise kinin $B_2$ receptors activated by bradykinin or kallidin. Antagonism of postcapillary kinin $B_2$ receptors in vivo by icatibant could reduce the recovery of kinins from PAF-challenged non-atopic individuals by impeding the influx of kininogen or prekallikrein into the nasal airway. However, the results of experiments 3.3.3 (figure 3.14) and 3.3.6 (figure 3.19) are contradictory to this theory. Aprotinin, 100 $\mu$g ($p>0.05$, Wilcoxon sign-rank test) and icatibant, 10 to 200 $\mu$g ($p>0.05$, Friedman’s test), failed to inhibit PAF-induced albumin production. This suggests that: (1) it is unlikely that the influx of prekallikrein (or its co-factors) into the nasal airway is induced by bradykinin or kallidin when challenged with PAF and (2) the influx of prekallikrein (or its co-factors) into the nasal airway is not impeded by pre-treatment with icatibant after challenge with PAF. This result concurs with the conclusions of Dear et al., (1996) who deduced that icatibant’s ability to abolish HDM-induced kinin production is independent of the influx of prekallikrein into the nasal airway as an earlier study, Austin and Foreman (1994), found that intranasal challenge of atopic individuals with HDM does not cause an increase in albumin leakage into nasal lavage fluid. If true, this would also imply that there is no leakage of kininogen into the airway, as plasma exudate is the only local source of HMWK (120 kD), LMWK (64 kD) and albumin (66 kD). However, other investigations on individuals with perennial rhinitis airways have revealed that intranasal challenge with house dust mite does cause an increase in vascular permeability (de Graaf-in’t Veld et al., 1997).

Although icatibant is principally a kinin $B_2$ receptor antagonist, it also possesses a low affinity for kinin $B_1$ receptors in vitro (Stewart et al., 1996). Functional kinin $B_1$ receptors are not present within non-inflamed non-atopic nasal airways (Christiansen et al., 2002) but the possibility that they modulate the recovery of kinins from nasal lavage fluid cannot be ruled out as kinin $B_1$ receptors are inducible and usually expressed under inflammatory conditions (Fernandes et al., 2003; Sugahara et al., 2003). In figure 3.20 and figure 3.21, subjects were pre-treated with 200 $\mu$g of the high affinity kinin $B_1$ receptor antagonist, des-arg$^{10}$-leu$^9$-kallidin, and challenged with PAF, 60 $\mu$g. Des-arg$^{10}$-leu$^9$-kallidin failed to modulate the PAF-induced kinin or albumin production. This implies that kinin $B_1$ receptors do not play a role in modulating the recovery of kinins from nasal lavage fluid.
Chapter 3  The effect of icatibant on PAF-induced kinin production

The ability of icatibant to antagonise kinin B\textsubscript{2} receptors with high affinity is unquestionable. Dear et al., (1996), showed that unlabelled bradykinin and icatibant abrogate the binding of \textsuperscript{[125]I}-icatibant to a single high affinity site on human nasal turbinates, $K_i = 0.99$ and $0.48$ nM, respectively. Therefore, if kinin B\textsubscript{2} receptors do not play a role in controlling the recovery of kinins from nasal lavage fluid it is assumed that there is another high affinity binding site for icatibant that is overlooked by the surgical removal of turbinates from the human nasal airways.

An alternative binding site for icatibant \textit{in vivo} is tissue or plasma kallikrein. Structural analogues of kinin B\textsubscript{2} receptor antagonists inhibit tissue kallikrein activity \textit{in vitro}. KKI-8 (Adamantaneacetyl-Phe-Arg-Ser-Val-Gln-NH\textsubscript{2}), an analogue of [Ad]-Bk, inhibits human tissue kallikrein with a $K_i$ of 4.2 $\mu$M (Okunishi et al., 1987; Okunishi et al., 1989). Inhibition of kallikrein activity \textit{in vivo} would explain icatibant’s ability to impede the recovery of kinins from nasal lavage fluid following intranasal challenge. This theory relies on the possibility that PAF-induced kinin production is mediated by tissue or plasma kallikrein, however, as mentioned in chapter 1.4.2.3, this may not necessarily be true. Intranasal challenge with PAF is also associated with a delayed influx of neutrophils and eosinophils, both of which have the capacity to induce kinin synthesis within sites of inflammation. Neutrophils synthesise and release active tissue kallikrein in response to zymosan and phorbol ester (Lauredo et al., 2004) and activated eosinophils release ECP, which enhances the formation of plasma kallikrein in glass-activated human plasma (Venge et al., 1979). However, the PAF-induced influx of these leukocytes into non-atopic airways does occur until at least after 1 hour challenge and therefore does not coincide with kinin synthesis seen in any of the experiments performed in this chapter (Tedeschi et al., 1994; Miadonna et al., 1996; Turner et al., 2000b).

In conclusion, icatibant reduces the recovery of kinins from PAF challenged non-atopic individuals. The mechanism by which it does this is not known but it is likely to be of significance on the basis that the IC\textsubscript{50} for inhibition of bradykinin-induced nasal blockage (Dear et al., 1996) and IC\textsubscript{50} for the inhibition of PAF-induced kinin production \textit{in vivo} are similar.
3.5 Summary

1. Intranasal challenge with PAF, 60 μg, causes a significant rise in the kinin content of nasal lavage fluid recovered 20 minutes after challenge.

2. Nasal lavage fluid recovered 20 minutes after intranasal challenge with PAF, 60 μg, does not exhibit an increase in kallikrein-like enzyme activity. The enzyme responsible for the cleavage of S-2266 is sensitive to the serine protease inhibitors, aprotinin and soybean trypsin inhibitor.

3. Intranasal challenge with PAF, 60 μg, causes a significant rise in the albumin content of nasal lavage fluid recovered 20 minutes after challenge.

4. Intranasal challenge with PAF, 60 μg, failed to cause a significant rise in the secretory IgA content of nasal lavage fluid recovered 20 minutes after challenge.

5. Intranasal challenge with methacholine, 1 to 10 mg, failed to cause an increase in kallikrein-like activity of nasal lavage fluid recovered 10 minutes after challenge.

6. Intranasal pre-treatment with icatibant, 10 to 200 μg, failed to dose-dependently inhibit the increase in kinin content of nasal lavage fluid from individuals challenged with PAF, 60 μg. Icatibant, 200 μg, significantly attenuates kinin production caused by PAF, 60 μg.

7. Intranasal pre-treatment with icatibant, 10 to 200 μg, does not affect the leakage of albumin into the nasal lavage fluid caused by challenge with PAF, 60 μg.

8. Icatibant, 10 nM to 10 μM, does not interfere with the binding of rabbit anti-bradykinin serum to bradykinin, 11.5 pg/μl.

9. Neither NPC-567, 200 μg or des-arg^{10}-leu^{9}-kallidin, 200μg, inhibit the recovery of kinins from nasal lavage fluid of individuals challenged with PAF, 60 μg.
10. Neither NPC-567, 200 μg or des-arg$^{10}$-leu$^{9}$-kallidin, 200μg, inhibit the recovery of albumin from nasal lavage fluid of individuals challenged with PAF, 60 μg.

11. Intranasal pre-treatment with aprotinin, 100 μg, attenuates the recovery of kinins from the nasal lavage fluid of individuals challenged with PAF, 60 μg.

12. Intranasal pre-treatment with aprotinin, 100 μg, does not inhibit the recovery of albumin from the nasal lavage fluid of individuals challenged PAF, 60 μg.
CHAPTER 4

THE EFFECT OF KININ RECEPTOR ANTAGONISTS ON KALLIKREIN ACTIVITY IN VITRO

4.1 Introduction

Tissue and plasma kallikrein are found in the upper and lower human airway (Poblete et al., 1993; Proud and Vio, 1993). Their enzyme activity, in vitro, is attenuated by the serine protease inhibitor, aprotinin. Coincidently, the enzyme responsible for PAF-induced kinin synthesis in non-atopic nasal airways is also susceptible to inhibition by aprotinin, as shown in chapter 3. Thus, it is possible that kininogenase activated by intranasal challenge with PAF may be tissue or plasma kallikrein.

Pre-treatment of non-atopic individuals with the kinin B2 receptor antagonist, icatibant, reduces PAF-induced elevations in the kinin content of nasal lavage fluid, as shown in chapter 3. The underlying mechanism behind this effect has not been determined, but it is thought to be independent of PAF-induced increases in vascular permeability.

An alternative binding site for icatibant may be tissue or plasma kallikrein. Analogues of kinin B2 receptor antagonists have been reported to inhibit tissue kallikrein activity with nanomolar affinity in vitro (Okunishi et al., 1987; Okunishi et al., 1989). Consequently, it is possible that icatibant inhibits PAF-induced kinin production in vivo by inhibiting tissue or plasma kallikrein enzyme activity.

In this chapter the ability of kinin receptor ligands to modulate kallikrein activity in vitro will be determined. The inhibition constants (K_i) obtained from the inhibition of tissue and plasma kallikrein activity will be compared to their inhibition constants obtained for the displacement of [125I]-icatibant from human nasal kinin B2 receptors (Dear et al., 1996) to clarify whether the ability of icatibant to inhibit symptoms of
nasal allergy might result from the inhibition of kallikrein as well as the antagonism of vascular kinin B₂ receptors.

### 4.2 Molecular theory

Enzyme reactions were carried out at equilibrium and assumed to obey equation 4.1 in the presence of substrate concentrations which exceeded the concentration of the enzyme.

\[
[E] + [S] \overset{k_{s1}}{\underset{k_{s-1}}{\rightleftharpoons}} [ES] \overset{k_{s2}}{\rightarrow} [E] + [P]
\]

Where:

- \([E]\) is the concentration of free enzyme.
- \([S]\) is the concentration of free substrate.
- \([ES]\) is the enzyme-substrate complex concentration.
- \([P]\) is the concentration of product formed.

However, if it is assumed that there is negligible degradation of the product, this assumption reduces equation 4.1 to equation 4.2.

\[
[E] + [S] \overset{k_{s1}}{\underset{k_{s-1}}{\rightleftharpoons}} [ES] \rightarrow [E] + [P]
\]

Assuming that the law of mass action applies, equation 4.2 can be re-arranged to form the Michaelis-Menten equation (equation 4.3):

\[
\frac{v_i}{V_{\text{max}}} = \frac{[S]}{[S] + K_M}
\]

Where:

\[
K_M = \frac{k_{-1} + k_{s2}}{k_{s1}} \quad \text{and} \quad V_{\text{max}} = k_{\text{cat}}[E_0]
\]

\(V_{\text{max}}\) is the maximum initial velocity of the enzyme at saturating substrate concentrations. \(v_i\) is the initial velocity of the enzyme. \(k_{\text{cat}} (= k_{s2})\) is the turnover number of the enzyme. \([E_0]\) is the total enzyme concentration. \(K_M\) is the Michaelis-Menten constant.
4.3 Experimental protocol

4.3.1 Enzyme assay

Kallikrein was incubated in a 96-well plate with the appropriate Tris-HCl buffer (see chapter 2.5.2), with or without an antagonist or inhibitor present, for 15 minutes at 37°C. Reactions were initiated by the addition of the appropriate chromogenic substrate (S-2266 for tissue kallikrein and S-2302 for plasma kallikrein, chosen according to kinetics constants shown in table 2.1) and the change in optical density was measured with a spectrophotometer at 9-second intervals and at a wavelength of 405 nm. All reactions were performed in duplicate and were repeated at least three times.

4.3.2 Data Analysis

The rate of each reaction was calculated by determining the gradient of the change in optical density versus time graphs. SoftMax Pro3 (96-well plate reader software) was used to calculate the initial velocity of each assay by linear regression. Log_{10}[dose]-response curves were created from these values and then fitted to the appropriate equation using GraphPad-Prism 4 software.

The suitability of one equation (or model) over another for data was chosen using an F test (global fitting analysis) found in Graphpad-Prism 4. A p value less than 0.05 was deemed statistically significant.

Statistical differences in Hill co-efficients from unity were determined using an F test found in Graphpad-Prism 4. A p value less than 0.05 was deemed statistically significant.
4.3 Results

4.3.1 The effect of human plasma kallikrein concentration on the cleavage of S-2302

Plasma kallikrein cleaved the chromogenic substrate S-2302, 100 µM, to cause a change in optical density. A concentration of 100 µM S-2302 was chosen on the basis of the predicted $K_m$ for S-2302 with human plasma kallikrein, 220 µM (diaPharma, Chromogenix booklet). The change in optical density caused by the release of the chromophore para-nitroaniline (pNA) was directly proportional to the concentration of plasma kallikrein present in each well (figure 4.1).

4.3.2 The effect of porcine tissue kallikrein concentration on the cleavage of S-2266

Porcine tissue kallikrein cleaved the chromogenic substrate S-2266, 10 µM, to cause a change in optical density. A concentration of 10 µM S-2266 was chosen on the basis of the predicted $K_m$ for S-2266 with tissue kallikrein, 30 µM (diaPharma, Chromogenix booklet). The change in optical density caused by the release of pNA was directly proportional to the concentration of porcine tissue kallikrein present in each well (figure 4.2).

4.3.3 The effect of S-2302 concentration on human plasma kallikrein enzyme activity

Plasma kallikrein, 6.78 nM, displayed Michaelis-Menten kinetics in the presence of increasing concentrations of S-2302 (figure 4.3A). The concentration of plasma kallikrein used in this assay was chosen on the basis that it was the lowest concentration of plasma kallikrein which produced a clear increase in enzyme activity with a substrate concentration close to its $K_m$ (figure 4.1). Enzyme activity was saturable, with a single value for $V_{max}$ and $K_m$. 

145
Figure 4.1) A graph showing the effect of human plasma kallikrein concentration on the cleavage of S-2302, 100 μM. The data are results from a single experiment with duplicate samples.
Figure 4.2) A graph showing the effect of porcine tissue kallikrein concentration on the cleavage of S-2266, 10 μM. The data are results from a single experiment with duplicate samples.
Chapter 4  The effect of kinin receptor antagonists on kallikrein activity

Data was fitted to the Michaelis-Menten equation:

\[ v_i = \frac{V_{\text{max}} \cdot [S]}{[S] + K_M} \]

\( V_{\text{max}} \), \( K_M \) and \( k_{\text{cat}} \) were calculated to be 55.68 AOD x10\(^{-3}\)/min, 171.3 \( \mu \)M and 20.25 s\(^{-1}\) respectively (table 4.1).

Equation 4.3 was rearranged to produce equation 4.4.

**Equation 4.4:** \[ \log_{10}\left(\frac{v_i}{V_{\text{max}} - v_i}\right) = nH \cdot \log_{10}[S] - \log_{10}K_M \]

Linearisation of figure 4.3A using equation 4.4 generated a Hill-coefficient of 1.43 ± 0.14 and \( K_M \) of 76.74 \( \mu \)M (figure 4.3B). The Hill co-efficient was significantly greater than unity (\( p<0.05 \), F test).

Equation 4.3 was also re-arranged to form the Lineweaver-Burk equation (equation 4.5).

**Equation 4.5:** \[ \frac{1}{v_i} = \frac{1}{V_{\text{max}}} + \frac{K_M}{V_{\text{max}} \cdot [S]} \]

Linearisation of figure 4.3A using equation 4.5 generated a \( V_{\text{max}} \), \( K_M \) and \( k_{\text{cat}} \) of 55.59 AOD x10\(^{-3}\)/min, 203 \( \mu \)M and 20.23 s\(^{-1}\) respectively (figure 4.4; table 4.1).

### 4.3.4 The effect of S-2266 concentration on porcine tissue kallikrein enzyme activity

Tissue kallikrein, 276 nM, displayed Michaelis-Menten kinetics in the presence of increasing concentrations of S-2266 (Figure 4.5A). The concentration of porcine tissue kallikrein used in this assay was chosen on the basis that it was the lowest concentration of tissue kallikrein which produced a clear
### Table 4.1

A summary of $V_{\text{max}}$, $k_{\text{cat}}$, and $K_{M}$ generated by the cleavage of S-2302 and S-2266 by plasma kallikrein and tissue kallikrein, respectively. Kallikrein activity was measured by spectrophotometry at 405 nm and 37° C. The data points are arithmetic averages and standard errors of 3 different experiments with duplicate samples.

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<th>Curve-fitting</th>
<th>Linweaver-Burk Plot</th>
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<tr>
<td></td>
<td>$K_{M}$ (µM)</td>
<td>$k_{\text{cat}}$ (s(^{-1}))</td>
</tr>
<tr>
<td>Kininogenase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Kallikrein (S-2302)</td>
<td>171.3 ± 4.79</td>
<td>20.25</td>
</tr>
<tr>
<td>Tissue Kallikrein (S-2266)</td>
<td>17.35 ± 0.78</td>
<td>0.35</td>
</tr>
</tbody>
</table>
Figure 4.3) A Michaelis-Menten plot (A) and Hill plot (B) showing the effect of S-2302 concentration on human plasma kallikrein (6.78 nM) enzyme activity. The data points are arithmetic averages and standard errors of 3 different experiments with duplicate samples.
Figure 4.4) A Lineweaver-Burk plot showing the effect of S-2302 concentration on human plasma kallikrein (6.78 nM) enzyme activity. The data points are arithmetic averages from 3 different experiments with duplicate samples.
Figure 4.5) A Michaelis-Menten plot (A) and Hill plot (B) showing the effect of S-2266 concentration on porcine tissue kallikrein (276 nM) enzyme activity. The data points are arithmetic averages and standard errors of 4 different experiments with duplicate samples.
Figure 4.6) A Lineweaver-Burk plot showing the effect of S-2266 concentration on porcine tissue kallikrein enzyme activity. The data points are arithmetic averages from 4 different experiments with duplicate samples.
increase in enzyme activity with a substrate concentration close to its $K_M$ (figure 4.2). Enzyme activity was saturable, with a single value for $V_{\text{max}}$ and $K_M$. Data was fitted to the Michaelis-Menten equation and $V_{\text{max}}$, $K_M$ and $k_{\text{cat}}$ were calculated to be $37.72 \Delta \text{OD} \times 10^{-3}/\text{min}$, $17.35 \mu\text{M}$ and $0.35 \text{s}^{-1}$ respectively (table 4.1).

According to the Hill Plot, the Hill co-efficient and $K_M$ are $1.07 \pm 0.03$ and $14.13 \mu\text{M}$, respectively (figure 4.5B). The Hill co-efficient was not significantly greater than unity (p>0.05, F test)

Linearisation of figure 4.5A using equation 4.5 generated a $V_{\text{max}}$, $K_M$ and $k_{\text{cat}}$ of $52.91 \Delta \text{OD} \times 10^{-3}/\text{min}$, $23.5 \mu\text{M}$ and $0.48 \text{s}^{-1}$ respectively (figure 4.6; table 4.1).

4.3.5 The effect of kinin receptor ligands on human plasma kallikrein enzyme activity

The cleavage of S-2302, 100 $\mu\text{M}$, by human plasma kallikrein was determined in the presence of the following kinin receptor ligands: icatibant, [Ad]-Bk, NPC 567 and WIN 64338 (which are kinin B$_2$ receptor antagonists); bradykinin (which is a kinin B$_2$ receptor agonist); des-arg$^{10}$kallidin (which is a kinin B$_1$ receptor agonist) and; des-arg$^{10}$-leu$^9$kallidin (which is a kinin B$_1$ receptor antagonist). Inhibition curves (figure 4.7) were fitted to equation 4.6.

$$v_i = v_{\text{max}} \cdot \frac{[IC_{50}]^{nH}}{[IC_{50}]^{nH} + [I]^{nH}}$$

$[I]$ is the concentration of the kinin receptor ligand. $V_{\text{max}}$ is the initial velocity in the absence of any kinin receptor ligands. $v_i$ is the initial velocity of the enzyme. $[IC_{50}]$ is the concentration of the kinin receptor ligand needed to reduce the initial velocity by 50%. $nH$ is the hill co-efficient.

The inhibition constants ($K_i$) of each ligand were determined using the Cheng-Prusoff equation (equation 4.7):
Chapter 4 The effect of kinin receptor antagonists on kallikrein activity

Equation 4.7: \[ K_i = \frac{[IC_{50}]}{1 + \frac{[S]}{K_M}} \]

Inhibition constants and Hill coefficients for each ligand are summarised in table 4.2.

Equation 4.6 was re-arranged to generate equation 4.8.

Equation 4.8: \[ \log_{10}\left[ \frac{v_i}{V_{\text{max}} - v_i} \right] = \log_{10}[IC_{50}] - \log_{10}[I] \]

[I] is the concentration of the kinin receptor ligand. \( V_{\text{max}} \) is the initial velocity in the absence of any kinin receptor ligands. \( v_i \) is the initial velocity of the enzyme. \([IC_{50}]\) is the concentration of the kinin receptor ligand needed to reduce the initial velocity by 50%. \( nH \) is the hill coefficient.

Linearisation of figure 4.7 using equation 4.8 (figure 4.9) produced Hill-coefficients and values for \( IC_{50} \), which were converted into inhibition constants using equation 4.7. These values are summarized in table 4.2.

4.3.6 The effect of kinin receptor ligands on porcine tissue kallikrein enzyme activity

The cleavage of S-2266, 10 \( \mu \)M, by porcine tissue kallikrein in the presence of the following kinin receptor ligands (figure 4.8): icatibant, [Ad]-Bk, NPC 567 and WIN 64338 (which are kinin B\textsubscript{2} receptor antagonists); bradykinin (which is a kinin B\textsubscript{2} receptor agonist); des-arg\textsuperscript{10}kallidin (which is a kinin B\textsubscript{1} receptor agonist) and; des-arg\textsuperscript{10}-leu\textsuperscript{9}kallidin (which is a kinin B\textsubscript{1} receptor antagonist). Ligands which produced inhibition curves that could not be fitted to equation 4.6 were fitted to equation 4.9 which assumes there are two non-interacting binding sites on the enzyme.
Equation 4.9  

\[ v_i = V_{\text{max}1} \cdot \frac{[IC_{50}]^{nH1}}{[IC_{50}]^{nH1} + [I]^{nH1}} + V_{\text{max}2} \cdot \frac{[IC_{50}]^{nH2}}{[IC_{50}]^{nH2} + [I]^{nH2}} \]

\( v_i \) is the initial velocity of the enzyme. \([I]\) is the concentration of the kinin receptor ligand.  
\( V_{\text{max}1} \) is the maximum initial velocity at the first binding site of the enzyme.  
\( V_{\text{max}2} \) is the maximum initial velocity at the second binding site of the enzyme.  
\([IC_{50}]\) is the concentration of the kinin receptor ligand needed to reduce the initial velocity by 50%.  
\( nH1 \) is the hill coefficient of binding site 1.  
\( nH2 \) is the hill coefficient of binding site 2.

Data from figure 4.8 was also fitted to equation 4.9 (figure 4.10).

The inhibition constants \((K_i)\) of each ligand were calculated using the Cheng-Prusoff equation (equation 4.7). Inhibition constants and Hill coefficients for each ligand are summarised in table 4.3. WIN 64338 and [Ad]-Bk were fitted with equation 4.9 (which assumes porcine tissue kallikrein bares two binding sites for [Ad]-bk and WIN 64338) as equation 4.9 is a better fit than equation 4.6 to the data points \((p<0.05, F\) test).
Figure 4.7) The cleavage of S-2302, 100 μM, by human plasma kallikrein, 6.78 nM, in the presence of kinin receptor ligands. Percentage changes were normalised to the control (mean of 60.6 OD x10⁻³/min, standard error of the mean 10.2 OD x10⁻³/min. Data represent the means from 3 experiments with duplicate samples.
Figure 4.8) Cleavage of S-2266, 10 μM, by porcine tissue kallikrein, 276 nM, in the presence of kinin receptor ligands. Percentage changes were normalised to the control (mean of 11.7 OD x10^{-3}/min, standard error of the mean 1.6 OD x10^{-3}/min. Data represent the means and standard errors of the mean from 3 experiments with duplicate samples.
Figure 4.9) A Hill plot showing the effect of kinin receptor ligands on the cleavage of S-2302, 100 µM, by human plasma kallikrein, 6.78 nM. Data represent the means from 3 experiments with duplicate samples.
Figure 4.10) A Hill plot showing the effect of kinin receptor ligands on the cleavage of S-2266, 10 μM, by porcine tissue kallikrein, 276 nM. Data represent the means from 3 experiments with duplicate samples.
### Table 4.2

Inhibition constants ($K_i$) and Hill coefficients ($n_H$) for kinin receptor ligands inhibiting human plasma kallikrein cleavage of S-2302, 100 μM. Data represent the means from 3 experiments with duplicate samples. [Ad]-BK is [1-adamantaneacetyl-D-Arg$^9$, Hyp$^3$-(2-thienyl)-Ala$^{5,8}$, D-Phe$^3$]-bradykinin.

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<th>Compound</th>
<th>Curve-fitting</th>
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<td>$K_i$ (μM)</td>
<td>$n_H$</td>
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<td><strong>Kinin B$_2$ receptor antagonists</strong></td>
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<tr>
<td>WIN 64338</td>
<td>33.00 ± 5.70</td>
<td>0.92 ± 0.09</td>
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<tr>
<td>[Ad]-Bk</td>
<td>223.14 ± 27.1</td>
<td>1.33 ± 0.16</td>
</tr>
<tr>
<td>NPC 567</td>
<td>&gt; 500</td>
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</tr>
<tr>
<td>Icatibant</td>
<td>&gt; 500</td>
<td>-</td>
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<td><strong>KininB$_2$ receptor Agonist</strong></td>
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<td>Bradykinin</td>
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<td>1.00 ± 0.04</td>
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<td>Des-arg$^{10}$-kallidin</td>
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<td><strong>Kinin B$_2$ receptor antagonist</strong></td>
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### Chapter 4: The effect of kinin receptor antagonists on kallikrein activity

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<td>$n_H$</td>
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<tr>
<td>WIN 64338*</td>
<td>0.70 ± 0.01</td>
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<td></td>
<td>6.62 ± 27.92</td>
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<td>[Ad]-Bk*</td>
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<td>Icatibant</td>
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<td>0.86 ± 0.08</td>
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<td><strong>Kinin B&lt;sub&gt;2&lt;/sub&gt; receptor agonist</strong></td>
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<td>Bradykinin</td>
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<td><strong>Kinin B&lt;sub&gt;1&lt;/sub&gt; receptor agonist</strong></td>
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Table 4.3: Inhibition constants ($K_i$) and Hill coefficients ($n_H$) for kinin receptor ligands inhibiting porcine tissue kallikrein cleavage of S-2266, 10 µM. Data represent the means from 3 experiments with duplicate samples. [Ad]-BK is [1-adamantaneacetyl-d-Arg<sup>5</sup>,Hyp<sup>7</sup>-(2-thienyl)-Ala<sup>5,8</sup>,d-Phe<sup>7</sup>]-bradykinin. * WIN 64338 and [Ad]-Bk have two inhibition constants and two corresponding Hill-coefficients with porcine tissue kallikrein as their inhibition curves were fitted with equation 4.9.
Chapter 4

The effect of kinin receptor antagonists on kallikrein activity

Table 4.4) A table showing the correlation between the pK\textsubscript{i}s for the displacement of [\textsuperscript{125}I]-icatibant from soluble human nasal turbulines and pK\textsubscript{i}s for the inhibition of tissue and plasma kallikrein by the kinin B\textsubscript{2} receptor antagonists, icatibant, [Ad]-Bk, WIN 64338 and NPC 567. The pK\textsubscript{i} [\textsuperscript{125}I]-icatibant displacement values were obtained from Dear et al., (1996). * WIN 64338 and [Ad]-Bk have two inhibition constants with porcine tissue kallikrein as their inhibition curves were fitted with equation 4.9.

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<tr>
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<th>pK\textsubscript{i}, [\textsuperscript{125}I]-icatibant displacement</th>
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<th>pK\textsubscript{i}, Plasma kallikrein inhibition</th>
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<td>&lt; 3.3</td>
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<td>[Ad]-Bk*</td>
<td>8.921</td>
<td>6.128</td>
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<td>WIN 64338*</td>
<td>7.385</td>
<td>6.156</td>
<td>5.179</td>
</tr>
<tr>
<td>NPC 567</td>
<td>7.043</td>
<td>4.631</td>
<td>&lt; 3.3</td>
</tr>
</tbody>
</table>
4.4 Discussion

Plasma kallikrein enzyme activity measured by the cleavage of S-2302, 100 μM, was detectable at concentrations of plasma kallikrein as low as 1.7 nM (figure 4.1). The rate of S-2302 cleavage was directly proportional to the concentration of plasma kallikrein. This implies that the reaction was first order with respect to plasma kallikrein and that there is only one active site per enzyme molecule. Plasma kallikrein also displayed a saturable increase in enzyme activity with increasing concentrations of S-2302 (figure 4.3A). A single value for the Michaelis-Menten constant ($K_M$) was obtained by curve fitting (171.3 μM) and Lineweaver-Burk transformation (203 μM). However, analysis of the data using a Hill plot produced an outlying value compared with the Michaelis-Menten constant, $K_M = 76.74$ μM (figure 4.3B). This could be explained by the fact that the Hill plot is curved and has a Hill coefficient significantly greater than unity, $1.43 \pm 0.14$ (p<0.05, F test). This implies that human plasma kallikrein may exhibit positive cooperativity with the plasma kallikrein specific chromogenic substrate S-2302 and therefore may have multiple binding sites. Nonetheless, the former two values for $K_M$ are of similar magnitude to previous studies, 300 μM, in which $K_M$ was also determined by Lineweaver-Burk analysis (Friberger et al., 1986). Conversely, $k_{cat}$, also known as the turnover number (20.25 s$^{-1}$: curve fitting) is approximately 7-fold less than other literature values, 275 s$^{-1}$ (van der Graaf et al., 1982) and 180 s$^{-1}$ (Friberger et al., 1986). This discrepancy in catalytic activity may be accounted for by the difference in assay conditions as kallikrein is extremely sensitive to changes in pH and ionic strength (Friberger et al., 1978; Chao et al., 1983).

Porcine tissue kallikrein enzyme activity measured by the cleavage of S-2266, 10 μM, was detectable at concentrations of enzyme as low as 12 nM (figure 4.2). The rate of S-2266 cleavage was also directly proportional to the concentration of tissue kallikrein, implying that it only has one active site per enzyme molecule. Its $k_{cat}$, 0.35 s$^{-1}$ (curve fitting), is consistent with literature values of tissue kallikrein’s $k_{cat}$ with S-2266, 1 s$^{-1}$ (diaPharma, Chromogenix booklet). Porcine tissue kallikrein also exhibited a saturable increase in enzyme activity with increasing concentrations of S-2266 (figure 4.5A). A single value for $K_M$ was obtained by curve fitting (17.35 μM), Lineweaver-Burk transformation (23.5 μM) and Hill plot analysis (14.13 μM). This is in agreement with literature values of the Michaelis-Menten constant, 30 μM.
(diaPharma, Chromogenix booklet) and implies that tissue kallikrein only has one active site per enzyme molecule.

Initial studies on the kininogenase activity (the excision of kallidin from LMWK) of tissue kallikrein concluded that tissue kallikrein has two active sites per enzyme molecule. This was suggested because cleavage of the Met$^{361}$-Lys$^{362}$ bond of LMWK by was deemed a kinetically unfavourable reaction in comparison to the cleavage of the Arg$^{371}$-Ser$^{372}$ bond (Fiedler and Hinz, 1992). It was postulated that one active site cleaved the Arg$^{371}$-Ser$^{372}$ bond and the other active site cleaved the Met$^{361}$-Lys$^{362}$. However, X-ray analysis of crystal structures of porcine tissue kallikrein suggests that there is only one active site per molecule of porcine tissue kallikrein (Bode et al., 1983). It is now believed that tissue kallikrein cleaves both bonds simultaneously in a single enzyme-substrate complex (Fiedler and Hinz, 1992).

High (millimolar) doses of the kinin receptor antagonist, des-arg$^{10}$-leu$^{9}$-kallidin, failed to inhibit porcine tissue kallikrein activity (figure 4.8). This result concurs with its inability to produce any discernable inhibition of PAF-induced kinin production within non-atopic airways, as shown in chapter 3. However, the kinin B$_1$ receptor agonist, des-arg$^{10}$-kallidin, weakly inhibited porcine tissue kallikrein. This may be due to pseudo product-inhibition, as kallidin is different in structure to des-arg$^{10}$-kallidin by one amino acid. Similar results were obtained with assays of des-arg$^{10}$-kallidin and des-arg$^{10}$-leu$^{9}$-kallidin against human plasma kallikrein (figure 4.7).

Human plasma kallikrein enzyme activity was weakly inhibited by kinin B$_2$ receptor antagonists in vitro. The inhibition constants for icatibant and NPC-567 are in excess of 500 μM, while [Ad]-Bk has an inhibition constant of 223.14 μM (figure 4.7 and table 4.2). [Ad]-Bk's inhibition constant is in accordance with the K$_i$ obtained for KKI-8 (a drug of similar structure to [Ad]-Bk) obtained with human plasma kallikrein, 358 μM (Okunishi et al., 1989). WIN 64338 was the only antagonist with inhibitory activity within the low micromolar range (K$_i$ = 33 μM: curve fitting). However, its inhibition constant is not dissimilar to that of bradykinin (K$_i$ = 128.49 μM: curve fitting). This means it is very unlikely that icatibant inhibits the recovery of kinins from PAF-challenged individuals in vivo by inhibiting human plasma kallikrein as the
The inhibition of porcine tissue kallikrein by the kinin B2 receptor antagonists, WIN 64338 and [Ad]-Bk, produced non-sigmoidal log_{10}[dose]-inhibition curves which could not be fitted to a single binding site model for enzyme inhibition, equation 4.6 (figure 4.8). Consequently, it was considered that porcine tissue kallikrein may possess multiple binding sites for these antagonists and their inhibition curves were re-fitted to a two-binding site model for tissue kallikrein inhibition (equation 4.9). A two-binding site model was considered a better fit for both antagonists \((p<0.05, F\) test). The high and low affinity binding site inhibition constants of [Ad]-Bk were 0.74 \(\mu\)M and 90.71 \(\mu\)M respectively. The high-affinity binding site has a Hill-coefficient of 1.96 \((p<0.05, F\) test), implying that positive cooperativity is occurring at this site and it is responsible for the inhibition of approximately 40\% porcine tissue kallikreins activity. The low-affinity binding site has an inhibition constant of 90.71 \(\mu\)M (curve fitting) which concurs with the ability of KKI-8 (a drug of similar structure to [Ad]-Bk) to inhibit porcine tissue kallikrein \((K_i = 72.7 \, \mu\)M). In addition, the Hill-coefficient of [Ad]-Bk at this site is not significantly different from unity \((nH= 1.3 \pm 0.35: curve\) fitting) implying that it binds to the low affinity binding site of tissue kallikrein in a 1:1 ratio to produce approximately 60\% inhibition of tissue kallikreins enzyme activity. WIN 64338 binds, with similar affinity, to the high-affinity binding site of porcine tissue kallikrein, 0.74 \(\mu\)M (curve fitting) and also has a Hill-coefficient significantly greater unity \((nH= 4.33 \pm 0.25: curve\) fitting) implying that positive cooperativity is occurring at this site. However, the low-affinity binding site of WIN 64338 has a Hill-coefficient of 1.17 \pm 0.03 (curve fitting), which is close to unity. This may imply that WIN 64338 binds to the low affinity site of tissue kallikrein in a 1:1 ratio to produce approximately 50\% inhibition of tissue kallikreins enzyme activity. As only one active site has been identified in tissue kallikrein, as mentioned earlier, it is possible that there are multiple subsites, within its active site, in which multiple molecules of WIN 64338 and [Ad]-Bk can bind (Katz et al., 1998; Power et al., 1984), while only one molecule of S-2266 can bind to the entire active site at once, as suggested by its Hill-coefficient of 1.07 \pm 0.03 (figure 4.5B).
Chapter 4  The effect of kinin receptor antagonists on kallikrein activity

not be accurately fitted to a one- or two-binding site model for the inhibition of tissue kallikrein (figure 4.8). This suggests that tissue kallikrein possesses several binding sites for NPC-567 with inhibition constants of approximately 1 \( \mu \text{M} \) and above. For simplicity, the inhibition curve of tissue kallikrein with NPC-567 was fitted to a one-binding site model. In addition, statistically, a one-binding site model (equation 4.6) was deemed a better fit than a two-binding site model (equation 4.9) for the NPC 567 data of figure 4.8 (p>0.05, F test).

The kinin B\(_2\) receptor antagonist, icatibant, dose-dependently inhibited the enzyme activity of porcine tissue kallikrein (figure 4.8). Its Hill-coefficient obtained by curve fitting, 0.86 ± 0.08, is significantly less than unity and suggests that icatibant binds to porcine tissue kallikrein with negative cooperativity. However, these interactions were of very low affinity, its inhibition constant was 82 \( \mu \text{M} \) (curve fitting). This result contradicts the theory that icatibant-induced inhibition of kinin recovery from the nasal airway of PAF-challenged individuals is mediated by the inhibition of tissue kallikrein. First, because NPC-567 is more potent than icatibant at inhibiting porcine tissue kallikrein \textit{in vitro}, whereas icatibant is more potent than NPC-567 at inhibiting the recovery of kinins from PAF-challenged non-atopic individuals \textit{in vivo}, as shown in chapter 3. Secondly, icatibant has a \( K_i \) for the inhibition of tissue kallikrein which is of similar magnitude to that of bradykinin, a potential product of its kininogenase activity \textit{in vivo} (table 4.3 and 4.4; Dear et al., 1996).

Strong steric interactions between the active site of tissue (and plasma) kallikrein with peptide inhibitors and substrates are usually dependent on the presence of a charged arginine amino acid being adjacent to and at the N-terminal end of the bond to be cleaved. Tissue kallikrein inhibitor selectivity is also aided by the presence of bulky hydrophobic amino acids being an amino acid away and at the N-terminus of the bond to be cleaved. Inhibitors with hydrophobic amino acids in this position tend to have a high affinity for the active sites of tissue and plasma kallikrein (Power et al., 1984; Katz et al., 1998). This could explain the relatively small inhibition constants obtained with NPC 567, WIN 64338 and [Ad]-Bk, as they contain a mixture of hydrophobic and hydrophilic sites, unlike icatibant which is primarily composed of charged amino acids.
Tissue and plasma kallikrein enzyme activity were inhibited by the kinin B<sub>2</sub> receptor antagonists icatibant, [Ad]-Bk, NPC 567 and WIN 64338 between concentrations of 100 nM and 500 μM. This suggests that if high enough concentrations of these antagonists were introduced into the nasal airway it is possible that they could inhibit the recovery of kinins from PAF-challenged individuals by inhibiting the enzyme activity of tissue kallikrein or plasma kallikrein. However, when their pK<sub>i</sub>'s for the inhibition of tissue and plasma kallikrein enzyme activity are compared to their pK<sub>i</sub>'s for the displacement of [¹²⁵I]-icatibant from human nasal kinin B<sub>2</sub> receptors the kallikrein inhibition values are at least an order of magnitude smaller (table 4.4). This implies that the kinin B<sub>2</sub> receptor antagonists used in this study are significantly better at antagonising kinin B<sub>2</sub> receptors than they are at inhibiting tissue kallikrein or plasma kallikrein enzyme activity. In particular, icatibant has a pK<sub>i</sub> for the displacement of [¹²⁵I]-icatibant from human nasal kinin B<sub>2</sub> receptors which is 5 orders of magnitude greater than its pK<sub>i</sub> for the inhibition of tissue kallikrein and plasma kallikrein. This suggests icatibant's ability to inhibit HDM-induced nasal blockage in unchallenged sufferers of perennial allergic rhinitis is probably not as a result of the inhibition of kinin synthesis but due to the antagonism of kinin B<sub>2</sub> receptors in vivo.

Unfortunately, without knowledge of the effective concentration of icatibant needed to significantly inhibit PAF-induced kinin synthesis within the human nasal airway it is not possible to determine whether the concentrations of icatibant used in chapter 3.3.3 correlates well with the concentrations of icatibant needed to inhibit kallikrein activity, as shown in figure 4.7 and figure 4.8.
4.5 Summary

(1) Human plasma kallikrein amidolytic activity (measured by the cleavage of 100 μM S-2302 at pH 7.8, 37°C and 405 nm) is directly proportional to enzyme concentration.

(2) Porcine tissue kallikrein amidolytic activity (measured by the cleavage of 10 μM S-2266 at pH 8.2, 37°C and 405 nm) is directly proportional to enzyme concentration.

(3) Human plasma kallikrein displayed a saturable increase in amidolytic activity with increasing concentrations of S-2302. The Michaelis-Menten constant (determined by curve fitting) at pH 7.8, 37°C and 405 nm was 171.3 ± 4.79 μM.

(4) Porcine tissue kallikrein displayed a saturable increase in amidolytic activity with increasing concentrations of S-2266. The Michaelis-Menten constant (determined by curve fitting) at pH 8.2, 37°C and 405 nm was 17.35 ± 0.78 μM.

(5) The kinin B₂ receptor antagonist, WIN 64338, and the kinin B₂ receptor agonist, bradykinin, inhibited human plasma kallikrein with Kᵢ values in the micromolar range. The kinin B₂ receptor antagonists, NPC-567, icatibant and [1-adamantaneacetyl-D-Arg⁰, Hyp³-(2-thienyl)-Ala⁵,⁸, D-Phe⁷]-bradykinin produced inhibition constants in excess of 500 μM with human plasma kallikrein.

(6) The kinin B₂ receptor antagonists, WIN 64338, NPC-567, icatibant and [1-adamantaneacetyl-D-Arg⁰, Hyp³-(2-thienyl)-Ala⁵,⁸, D-Phe⁷]-bradykinin inhibited porcine tissue kallikrein with Kᵢ values in the micromolar range. Des-arg¹⁰-leu⁸-kallidin and des-arg¹⁰-kallidin produced inhibition constants in excess of 500 μM with porcine tissue kallikrein.

(7) The ability of icatibant ability to inhibit tissue and plasma kallikrein activity in vitro, does not correlate with its ability to inhibit the recovery of PAF-induced kinin production in non-atopic nasal airways as the concentration of kinin B₂ receptor antagonist needed to inhibit agonist binding in vitro are much smaller than the concentration of antagonist required for enzyme inhibition.
CHAPTER 5

CHARACTERISATION OF THE KININ-FORMING ENZYME IN CRUDE NASAL LAVAGE FLUID IN VITRO

5.1 Introduction

Pre-treatment of non-atopic individuals with the kinin B\textsubscript{2} receptor antagonist, icatibant, reduces PAF-induced elevations in the kinin content of nasal lavage fluid, as shown in chapter 3. The underlying mechanism behind this effect has not been determined, but it is not thought to be dependant on kinin-induced increases in vascular permeability.

Reports have shown that low affinity kinin B\textsubscript{2} receptor antagonists are capable of inhibiting tissue and plasma kallikrein enzyme activity in vitro (Okunishi et al., 1987; Okunishi et al., 1989). Therefore, it is suspected that icatibant may reduce the recovery of kinins from the nasal lavage fluid by inhibiting the kininogenase responsible for PAF-induced kinin synthesis. This enzyme has not been identified.

Intranasal challenge of non-atopic individuals with PAF does not induce any signs of glandular activation, which could result in the release of tissue kallikrein, but it does generate an increase in vascular permeability (as shown in chapter 3), which is associated with the exudation of plasma proteins such as albumin and prekallikrein (Baumgarten et al., 1986a). Therefore it is possible that plasma kallikrein could be the kininogenase responsible for PAF-induced kinin production in non-atopic individuals.

In this chapter, the ability of crude nasal lavage fluid, from un-challenged non-atopic individuals, to cleave the chromogenic substrates S-2302 and S-2266 will be characterised to determine whether it displays any similarity to that of tissue or plasma kallikrein.
5.2 Molecular theory

As mentioned in chapter 4.2.

5.3 Experimental protocol

5.3.1 Collection of nasal lavage fluid

Nasal lavage fluid was collected without any pre-washes and centrifuged at 4°C and 1000 x g for 10 minutes. The supernatant was aspirated and used for enzyme assay.

5.3.2 Enzyme assay

Tissue kallikrein, plasma kallikrein and the supernatant of crude lavage fluid were incubated in 96-well plates with the appropriate Tris-HCl buffer (as mentioned in chapter 2.5.2), with or without an antagonist or inhibitor present, for 15 minutes at 37°C. Reactions were initiated by the addition of a chromogenic substrate (S-2266 with tissue kallikrein and crude nasal lavage fluid, S-2302 with plasma kallikrein and crude nasal lavage fluid) and read by spectrophotometry at a wavelength of 405 nm. Chromogenic substrates were chosen according to the kinetic constants and substrate selectivity of tissue and plasma kallikrein shown in table 2.1. Spectrophotometric readings with crude nasal lavage fluid were taken at 10-minute intervals, and 9-second intervals were used with plasma and tissue kallikrein.

5.3.3 Data Analysis

The rate of each reaction was calculated by determining the gradient of the optical density versus time graph. SoftMax Pro3 (96-well plate reader software) was used to calculate the initial velocity of each kallikrein assay by linear regression. The gradient of reactions performed with crude nasal lavage fluid were calculated using GraphPad-Prism 4 software, as mentioned in chapter 2.5.1. Log_{10}[dose]-response curves were created from these values and then fitted to the appropriate equation using GraphPad-Prism 4.
Statistical differences in Hill coefficients from unity were determined using an F test found in GraphPad- Prism 4. A p value less than 0.05 was deemed statistically significant.

Statistical differences in Michealis-Menten constants were determined using a Mann Whitney (non-parametric, unpaired) test. A p value less than 0.05 (p<0.05) was taken as statistically significant.

Statistical differences in dose-response curve kinetics were determined using an F test (global fitting analysis) found in GraphPad- Prism 4. A p value less than 0.05 (p<0.05) was taken as statistically significant.
5.4 Results

5.4.1 The cleavage of S-2266 by crude nasal lavage fluid and human plasma kallikrein

The cleavage of S-2266 by crude nasal lavage fluid and human plasma kallikrein, displayed Michaelis-Menten kinetics (figure 5.1A). Enzyme activity was saturable, with a single value for $V_{\text{max}}$ and $K_M$. Data was fit to the Michaelis-Menten equation:

$$v_i = V_{\text{max}} \cdot \frac{[S]}{[S] + K_M}$$

The $K_M$ of crude nasal lavage fluid and human plasma kallikrein were calculated to be $525.9 \pm 35.62$ µM and $694.5 \pm 76.19$ µM respectively (figure 5.1A). Note: the difference in $K_M$ for human plasma kallikrein (observed in this chapter) to the $K_M$ for human plasma kallikrein obtained in the previous chapter is accounted for by the use of a different chromogenic substrate.

Equation 5.3 was rearranged to produce equation 5.4.

Equation 5.4: $\log_{10} \left( \frac{v_i}{V_{\text{max}} - v_i} \right) = nH\log_{10}[S] - \log_{10}K_M$

Linearisation of figure 5.1A using equation 5.4 generated a Hill-coefficient of $1.25 \pm 0.05$ and $1.17 \pm 0.16$ for crude nasal lavage fluid and human plasma kallikrein respectively (figure 5.1B). Equation 5.3 was also re-arranged to form the Lineweaver-Burk equation (equation 5.5). Using this linearisation plot, the Michaelis-Menten constant of crude nasal lavage fluid and human plasma kallikrein were calculated as $496.78$ µM and $727.27$ µM respectively (figure 5.2).
Figure 5.1) A Michaelis-Menten plot (A) and Hill plot (B) showing the effect of S-2266 concentration on nasal lavage fluid and human plasma kallikrein (6.78 nM) enzyme activity. Percentage changes were normalised to the control (mean of 1.72 x10⁻³ OD/min, standard error of the mean 0.49 x10⁻³ OD/min with crude nasal lavage fluid and mean of 357.9 x10⁻³ OD/min, standard error of the mean 24.7 x10⁻³ OD/min with human plasma kallikrein). The data points are arithmetic averages and standard errors of 3 different experiments with duplicate samples. *Significant difference in the K_M's (p<0.05, Mann Whitney test).
Figure 5.2) A Lineweaver-Burk plot showing the effect of S-2266 concentration on nasal lavage fluid and human plasma kallikrein (6.78 nM) enzyme activity. Kallikrein activity was measured by spectrophotometry at 405 nm and 37° C. The data points are arithmetic averages from 3 different experiments with duplicate samples.
Equation 5.5: \[ \frac{1}{v_i} = \frac{1}{V_{\text{max}}} + \frac{K_M}{V_{\text{max}}M_{\text{max}}} \cdot \frac{1}{S} \]

5.4.2 The cleavage of S-2302 by crude nasal lavage fluid and human plasma kallikrein

The cleavage of S-2302 by crude nasal lavage fluid and human plasma kallikrein, displayed Michaelis-Menten kinetics (figure 5.3A). Enzyme activity was saturable, with a single value for \( V_{\text{max}} \) and \( K_M \). Data was fitted to the Michaelis-Menten equation. The \( K_M \) of crude nasal lavage fluid and human plasma kallikrein were calculated to be 693.7 ± 38.6 \( \mu \text{M} \) and 171.3 ± 4.49 \( \mu \text{M} \) respectively.

Linearization of figure 5.3A using equation 5.4 generated Hill-coefficients of 1.44 ± 0.09 and 1.42 ± 0.07 for crude nasal lavage fluid and human plasma kallikrein respectively (figure 5.3B). Equation 5.3 was also re-arranged to form the Lineweaver-Burk equation (equation 5.5). Using this linearization plot, the Michaelis-Menten constant of crude nasal lavage fluid and human plasma kallikrein were calculated as 509.68 \( \mu \text{M} \) and 203.00 \( \mu \text{M} \) respectively (figure 5.4).

5.4.3 The effect of soybean trypsin inhibitor and aprotinin on crude nasal lavage fluid enzyme activity

The cleavage of S-2302, 400 \( \mu \text{M} \), by crude nasal lavage fluid was dose-dependently inhibited by aprotinin and SBTI (figure 5.5A). The inhibition curves were fit to equation 5.6.

Equation 5.6: \[ v_i = V_{\text{max}} \cdot \frac{[IC_{50}]^{nH}}{[IC_{50}]^{nH} + [I]^{nH}} \]
Figure 5.3) A Michaelis-Menten plot (A) and Hill plot (B) showing the effect of S-2302 concentration on nasal lavage fluid and human plasma kallikrein (6.78 nM) enzyme activity. Percentage changes were normalised to the control (mean of $1.69 \times 10^{-3}$ OD/min, standard error of the mean $0.66 \times 10^{-3}$ OD/min with crude nasal lavage fluid and mean of $44.1 \times 10^{-3}$ OD/min, standard error of the mean $4.4 \times 10^{-3}$ OD/min with human plasma kallikrein). The data points are arithmetic averages and standard errors of 3 different experiments with duplicate samples where the standard errors are contained within the data point.
Figure 5.4) A Lineweaver-Burk plot showing the effect of S-2302 concentration on nasal lavage fluid and human plasma kallikrein (6.78 nM) enzyme activity. The data points are arithmetic averages from 3 different experiments with duplicate samples.
Figure 5.5) Inhibition curves (A) and Hill plots (B) showing the affect of aprotinin and soybean trypsin inhibitor (SBTI) on the cleavage of S-2302, 400 μM, by crude nasal lavage fluid. Percentage changes were normalised to the control (mean of $0.633 \times 10^{-3}$ OD/min, standard error of the mean $0.21 \times 10^{-3}$ OD/min). The data points are arithmetic averages and standard errors of 4 different experiments with duplicate samples.
Inhibition constants were calculated using each inhibitor's IC₅₀ and the Cheng-Prusoff equation (equation 5.7). SBTI produced an inhibition constant of 423.3 ± 65.73 nM with a Hill-coefficient of 0.75 ± 0.05. Aprotinin produced an inhibition constant of 45.06 ± 14.41 nM with a Hill-coefficient of 0.74 ± 0.07.

\[ K_i = \frac{[IC_{50}]}{1 + \frac{[S]}{K_M}} \]

Equation 5.7:

Figure 5.5A was linearised using equation 5.8 to produce figure 5.5B. The Hill-coefficient and inhibition constant of aprotinin with nasal lavage fluid were determined to be 0.69 ± 0.03 and 49.34 nM respectively. The Hill-coefficient and inhibition constant of SBTI with nasal lavage fluid were determined as 0.60 ± 0.05 and 431.94 nM respectively.

\[ \log_{10}\left(\frac{v_i}{V_{max} - v_i}\right) = \log_{10}[IC_{50}] - \log_{10}[I] \]

Equation 5.8:

5.4.4 The effect of soybean trypsin inhibitor and aprotinin on human plasma kallikrein enzyme activity

The cleavage of S-2302, 100 µM, by human plasma kallikrein was dose-dependently inhibited by aprotinin and SBTI (figure 5.6A). The inhibition curves were fitted to equation 5.8. SBTI produced an inhibition constant of 68.4 ± 5.19 nM with a Hill-coefficient of 2.32 ± 0.23. Aprotinin produced an inhibition constant of 104.6 ± 6.32 nM with a Hill-coefficient of 1.78 ± 0.1.

According to the Hill plot, figure 5.6B, the Hill-coefficient and inhibition constant of aprotinin with human plasma kallikrein were 1.34 ± 0.05 and 98.31 nM respectively. According to the Hill plot, figure 5.6B, the Hill-coefficient and inhibition constant of SBTI with human plasma kallikrein were 1.64 ± 0.11 and 92.6 nM respectively.
Figure 5.6) Inhibition curves (A) and Hill plots (B) showing the affect of aprotinin and soybean trypsin inhibitor (SBTI) on the cleavage of S-2302, 100 µM, by human plasma kallikrein, 6.78 nM. Percentage changes were normalised to the control (mean of $64.6 \times 10^{-3}$ OD/min, standard error of the mean $2.57 \times 10^{-3}$ OD/min). The data points are arithmetic averages and standard errors of 4 different experiments with duplicate samples.
Chapter 5  Characterisation of the kinin forming enzyme in crude nasal lavage fluid

Figure 5.7) Inhibition curves showing the affect of aprotinin and soybean trypsin inhibitor (SBTI) on the cleavage of S-2266, 10 μM, by porcine tissue kallikrein, 276 nM. Percentage changes were normalised to the control (mean of $13.7 \times 10^{-3}$ OD/min, standard error of the mean $0.15 \times 10^{-3}$ OD/min). The data points are arithmetic averages and standard errors of 3 different experiments with duplicate samples.
### Table 5.1

Inhibition constants ($K_i$) and Hill coefficients ($n_H$) for the enzyme activity of crude nasal lavage fluid, human plasma kallikrein and porcine tissue kallikrein in the presence of aprotinin and soybean trypsin inhibitor (SBTI). nd means not determined. Values represent the mean and standard errors.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_i$ (nM)</th>
<th>$n_H$</th>
<th>$K_i$ (nM)</th>
<th>$n_H$</th>
<th>$K_i$ (nM)</th>
<th>$n_H$</th>
<th>$K_i$ (nM)</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude lavage fluid</td>
<td>45.06 ± 14.41</td>
<td>0.74</td>
<td>423.3 ± 65.73</td>
<td>0.75</td>
<td>49.34 ± 0.05</td>
<td>0.69 ± 0.03</td>
<td>431.94 ± 0.05</td>
<td>0.60 ± 0.05</td>
</tr>
<tr>
<td>Plasma kallikrein</td>
<td>104.60 ± 6.00</td>
<td>1.78</td>
<td>68.4 ± 6.00</td>
<td>2.32</td>
<td>110.27 ± 0.23</td>
<td>1.41 ± 0.06</td>
<td>92.57 ± 0.11</td>
<td>1.64 ± 0.11</td>
</tr>
<tr>
<td>Tissue kallikrein</td>
<td>46.6 ± 14.57</td>
<td>2.62</td>
<td>&gt; 10000 nd</td>
<td>Poor fit</td>
<td>&gt; 10000 nd</td>
<td>nd</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Chapter 5**

Characterisation of the kinin forming enzyme in crude nasal lavage fluid
5.4.5 The effect of soybean trypsin inhibitor and aprotinin on porcine tissue kallikrein enzyme activity

The cleavage of S-2266, 10 μM, by porcine tissue kallikrein was dose-dependently inhibited by aprotinin, and not SBTI (figure 5.7). However, inhibition of porcine tissue kallikrein by aprotinin does not appear to fit equation 5.6. Nonetheless, its inhibition constant and Hill-coefficient were estimated to be 46.6 nM and 2.62 respectively.

5.4.6 The effect of kinin receptor ligands on the cleavage of S-2266 by crude nasal lavage fluid

Incubation of the kinin B2 receptor antagonists WIN 64338, NPC 567, [Ad]-Bk and icatibant with crude nasal lavage fluid failed to produce any discernable inhibition of the amidolytic activity with doses up to and including 10 μM. Above 10 μM S-2266 cleavage was inhibited dose-dependently. Icatibant, 0.5 mM; WIN 64338, 0.5 mM; [Ad]-Bk, 0.5 mM and NPC 567, 0.5 mM, inhibited the amidolytic activity by 13.74%, 16.88 %, 21.49% and 30.47% respectively.
Figure 5.8) The cleavage of S-2266, 400 μM, by nasal lavage fluid in the presence of kinin receptor ligands. Percentage changes were normalised to the control (mean of 1.00 x10^-3 OD/min, standard error of the mean 0.36 x10^-3 OD/min). Data represent the means and standard errors of the mean from 4 experiments with duplicate samples.
5.4 Discussion

Human plasma kallikrein and crude nasal lavage fluid both display Michaelis-Menten kinetics with the kallikrein selective chromogenic substrate S-2266 (figure 5.1A). The Hill-coefficient of the S-2266 [substrate]-response curve with human plasma kallikrein was not significantly different from unity, $1.17 \pm 0.16$ (p>0.05, F test), implying that it binds to each enzyme molecule in a ratio of 1:1 (figure 5.1B). However, the Hill-coefficient of the S-2266 [substrate]-response curve with crude nasal lavage fluid was significantly greater than unity, $1.25 \pm 0.05$ (p<0.05, F test), implying that positive cooperativity may be occurring (figure 5.1B).

The Michaelis-Menten constant of S-2266 with human plasma kallikrein is 694.5 μM (curve fitting). This value is of similar magnitude to the $K_m$'s of human plasma kallikrein with S-2266 obtained in previous reports, 500 μM (Diapharma, Chromogenix Booklet). The Michaelis-Menten constant of crude nasal lavage fluid with S-2266, 525.9 μM (curve fitting), is significantly smaller (p<0.05, Mann Whitney test) than that of human plasma kallikrein's, 694.5 μM (curve fitting), however, global fitting analysis of both data sets suggests the data from both curves are likely to have come from the same population (p>0.05, F test)(figure 5.1A). This implies that the kinetics of crude nasal lavage fluid with S-2266 are similar to those of human plasma kallikrein with S-2266. It must be noted that the only other kininogenase capable of cleaving S-2266 with Michaelis-Menten kinetics is tissue kallikrein, however, it has a Michaelis-Menten constant more than 40-times smaller than that of human plasma kallikrein with S-2266, as shown in chapter 4.3.4. This suggests that the enzyme present in crude nasal lavage fluid which is responsible for the cleavage of S-2266 is similar to human plasma kallikrein.

Characterisation of the kinin-forming enzyme in crude nasal lavage fluid with the substrate S-2302 produced intriguingly different results from those obtained with S-2266, even though the enzyme present in crude nasal lavage fluid responsible for the cleavage of S-2266 is likely to be the same enzyme responsible for the cleavage of S-2302. Human plasma kallikrein and crude nasal lavage fluid also both display Michaelis-Menten kinetics with the kallikrein selective chromogenic substrate S-2302 (figure 5.3A). The Michaelis-Menten constant of crude nasal lavage fluid, 693.7 μM, with S-2302, is approximately 4-times greater than that of
human plasma kallikreins, 171.3 μM (figure 5.3A). The Michaelis-Menten constant of human plasma kallikrein is corroborated by previous reports with S-2302 (Diapharma Chromogenix Booklet, 220 μM). However, the Hill plot of crude nasal lavage fluid amidolytic activity with S-2302 is parallel but displaced to the right of that of human plasma kallikrein with S-2302 (figure 5.3B). This parallel shift in its Hill plot could be explained by one of two possibilities, firstly, that the enzyme present in crude nasal lavage fluid which is responsible for the cleavage of S-2302 is not human plasma kallikrein and has a distinctly different affinity for S-2302, or secondly, the enzyme present in crude nasal lavage fluid is human plasma kallikrein but is subject to inhibition by a physiological inhibitor also recovered by nasal lavage. The former theory is unlikely to be true because the only other enzyme, apart from plasma kallikrein, capable of cleaving S-2302 with Michaelis-Menten kinetics is FXIIa. However, FXIIa has a $K_M$ of 12 μM with S-2302 (Friberger et al., 1986), it does not cleave S-2266 with a $K_M$ of 525.9 μM and it is weakly inhibited by SBTI ($K_I > 18 \mu M$) (Ratnoff and Saito, 1982), unlike crude nasal lavage fluid and human plasma kallikrein.

The possibility that the enzyme present within crude nasal lavage fluid is: (1), human plasma kallikrein and (2) inhibited by a physiological inhibitor, is a more plausible explanation for the rightward shift of crude nasal lavages Hill-plot (figure 5.3B). The Hill-coefficients of crude nasal lavage fluid and human plasma kallikrein with S-2302, 1.42 ± 0.07 and 1.44 ± 0.09 respectively, imply that there may be multiple binding sites for S-2302 (exhibiting positive cooperatively) within the active sites of both enzymes. The reason for the larger difference in $K_M$ of human plasma kallikrein and crude nasal lavage fluid (in comparison to difference in $K_M$s with S-2266) may be explained by the possibility that the physiological inhibitor partially interacts with a subsite which S-2302 also binds to, while S-2266 only interacts with a subsite which the physiological inhibitor does not interact with.

The possibility that the enzyme present within crude nasal lavage fluid is human plasma kallikrein is also supported by its susceptibility to inhibition by aprotinin and SBTI (figure 5.5; figure 5.6). However, unlike human plasma kallikrein, crude nasal lavage's Hill-coefficients (obtained by curve fitting and Hill plot) were significantly less than unity, as opposed to being closer to a value of 2 ($p<0.05$, F test) (figure 5.5; table 5.1). This suggests that human plasma kallikrein exhibits positive
cooperativity with SBTI and aprotinin, while crude nasal lavage fluid exhibits negative cooperativity with SBTI and aprotinin. In addition, the inhibition constant of SBTI with crude nasal lavage fluid, 423.4 nM (curve fitting), is approximately 6-times greater than the K_i of SBTI with human plasma kallikrein, 68.4 nM (curve fitting) (figure 5.6A; table 5.1). This difference in magnitude of Hill coefficients and inhibition constants could also be explained by the presence of a physiological inhibitor which is bound to a subsite within the active site of human plasma kallikrein. It is possible that the inhibitor: (1) physically obstructs the binding of SBTI to an enzyme subsite to reduce the affinity of SBTI for the enzyme and (2) prevents a conformational change in the enzyme which gives rise to the positive cooperativity and therefore gives rise to negative cooperativity. Conversely, the affinity of aprotinin for the active site of the enzyme in crude nasal lavage fluid is smaller than that of aprotinin with human plasma kallikrein (figure 5.5A; figure 5.6A; table 5.1). This could be explained by the fact that in comparison to SBTI (M_r=22000), aprotinin (M_r=6514) is a low molecular weight inhibitor of kallikrein which may not necessarily interact with the same subsites as SBTI and the physiological inhibitor.

Porcine tissue kallikrein enzyme activity with S-2266, 10 μM, was dose-dependently inhibited by the serine protease inhibitor aprotinin but not SBTI (figure 5.7). This concurs with other reports on the inhibition of tissue kallikrein by aprotinin and SBTI (Baumgarten et al., 1986b). However, the inhibition curve did not appear to fit equation 5.6. This suggests that tissue kallikrein is not inhibited by aprotinin in a simple competitive manner. Reports of tissue kallikrein inhibition by aprotinin suggest that two inhibitor molecules competitively bind to the enzyme active site in a positive cooperative manner and cause a parabolic, as opposed to a sigmoidal, inhibition of its enzyme activity (Miranda et al., 1995). Tissue kallikrein’s lack of susceptibility to inhibition by SBTI make it very unlikely that it is responsible for the enzyme activity present in crude nasal lavage fluid.

The inhibition constants of kinin B_2 receptor antagonists with purified human plasma kallikrein are in the micromolar range, as shown in chapter 4. However, millimolar concentrations of kinin B_2 receptor antagonist failed to significantly inhibit the amidolytic activity of crude nasal lavage fluid. Assuming that the enzyme responsible for the cleavage of S-2266 in nasal lavage fluid is also the enzyme responsible for PAF-induced kinin synthesis in non-atopic airways, it is very unlikely
that icatibant impedes the recovery of kinins from nasal lavage fluid by directly inhibiting this enzyme \textit{in vivo}. Additionally, the inability of the icatibant to inhibit the enzyme activity of crude nasal lavage fluid, while being able to inhibit the recovery of kinins from crude nasal lavage fluid of individuals challenged with PAF may suggest that enzyme responsible for PAF-induced kinin synthesis is different from the enzyme recovered by nasal lavage (from unchallenged individuals).

Intranasal challenge with PAF causes a significant rise in the kinin content of nasal lavage fluid recovered from non-atopic individuals, however, there is no concomitant increase in the kallikrein-like amidolytic activity in nasal lavage fluid, as shown in chapter 3. An increase in amidolytic activity would be expected if the limiting factor in PAF-induced kinin synthesis were the availability of active kininogenase. However, if the basal kininogenase activity of unchallenged non-atopic individuals is sufficient enough to cause a significant rise in the kinin content of the nasal airway after 20 minutes, the limiting factor would be kininogen availability as opposed to kininogenase activity (Scuri et al., 2002). Assuming that the enzyme present in crude nasal lavage is the same as that responsible for PAF-induced kinin synthesis, the enzyme responsible for PAF-induced kinin synthesis could be human plasma kallikrein, according to the enzyme kinetics and inhibition profile of its amidolytic activity in crude nasal lavage fluid (figure 5.1 and figure 5.5).
5.5 Summary

(1) Human plasma kallikrein and crude nasal lavage fluid displayed a saturable increase in amidolytic activity with increasing concentrations of S-2266. The Michaelis-Menten constants (determined by curve fitting) at pH 7.8, 37°C and 405 nm were 694.5 ± 76.19 μM and 525.9 ± 35.62 μM respectively.

(2) Human plasma kallikrein and crude nasal lavage fluid displayed a saturable increase in amidolytic activity with increasing concentrations of S-2302. The Michaelis-Menten constants (determined by curve fitting) at pH 7.8, 37°C and 405 nm were 171.3 ± 4.49 μM and 693.7 ± 38.6 μM respectively.

(3) The cleavage of S-2302, 100 μM, by crude nasal lavage fluid was inhibited by the serine protease inhibitors, aprotinin and SBTI, with inhibition constants of 45.06 ± 14.41 nM and 423.3 ± 65.74 nM respectively.

(4) The cleavage of S-2302, 400 μM, by human plasma kallikrein was inhibited by the serine protease inhibitors, aprotinin and SBTI, with inhibition constants of 104.6 ± 6 nM and 68.4 ± 6 nM respectively.

(5) The cleavage of S-2266, 10 μM, by porcine tissue kallikrein was only inhibited by the serine protease inhibitors, aprotinin and not SBTI, with an inhibition constant of approximately 46.6 ± 14.57 nM respectively.

(6) The cleavage of S-2266, 400 μM, by crude nasal lavage fluid was not inhibited by kinin B2 receptor antagonists WIN 64338, NPC 567, [Ad]-Bk and icatibant. Their inhibition constants were in excess of 500 μM.
CHAPTER 6
THE EFFECT OF ICATIBANT ON THE ACTIVATION OF PREKALLIKREIN

6.1 Introduction

Intranasal challenge with PAF causes a rise in the kinin content of nasal lavage fluid recovered from non-atopic individuals, without a concomitant increase in kallikrein enzyme activity, as shown in chapter 3. This may be due to limited kininogen availability, rather than insufficient kininogenase activity, being a limiting factor of PAF-induced kinin synthesis in vivo. The enzyme kinetics and inhibition profile of the enzyme present within crude nasal lavage fluid of unchallenged individuals is very similar to that of human plasma kallikrein, as shown in chapter 5. Therefore, assuming that: (1) the enzyme responsible for the cleavage of the chromogenic substrates S-2266 and S-2302 in crude nasal lavage fluid of unchallenged individuals is same the enzyme responsible for PAF-induced kinin synthesis in vivo; and (2) that limited kininogen availability is a limiting factor of kinin synthesis in vivo, the kininogenase responsible for PAF-induced kinin synthesis in non-atopic nasal airways could be human plasma kallikrein.

Icatibant’s ability to inhibit the recovery of kinins from PAF-challenged non-atopic individuals in vivo is not dependent on the influx of prekallikrein or HMWK into the nasal airway, as shown in chapter 3. Therefore, it is likely that icatibant either inhibits the synthesis of kinins in vivo or potentiates their degradation. The inhibition constant of icatibant with human plasma kallikrein in vitro is in excess of 500 μM, as shown in chapter 4. This does not correspond with its potency to inhibit the recovery of kinins in vivo, however, amidolytic investigations do not take into consideration the possibility that kinin B₂ receptor antagonists may interfere with activation of plasma prekallikrein in vivo.

The enzyme or cofactors responsible for the activation of tissue prokallikrein within the human nasal airway have not been conclusively identified. However, the
enzymes, trypsin and human plasma kallikrein, are capable of activating tissue prokallikrein \textit{in vitro} (Takada et al., 1985).

In this chapter the ability of icatibant, 10 \(\mu\text{M}\), to inhibit the activation of purified prekallikrein and human plasma will be determined by spectrophotometry with the plasma kallikrein-selective chromogenic substrate S-2302 (table 2.1). In addition, the ability of various kinin receptors ligands to inhibit the enzyme activity of human pancreatic trypsin will be determined using the trypsin-selective chromogenic substrate S-2765 (table 2.1).
6.2 Experimental protocol

6.2.1 Collection of human plasma

Human blood (9 ml) was collected directly into a 15 ml polypropylene conical flask containing 0.1 mol/litre sodium citrate (1 ml) using a 10 ml syringe and 21 gauge needle. The blood was centrifuged at 2000 x g for 15 minutes at 25°C. The plasma was aliquoted into 1.5 ml Eppendorfs tubes and frozen at -70°C until assay.

6.2.2 Human plasma activation and prekallikrein activation assays

Human plasma and prekallikrein activation assays were performed in polystyrene 96-well plates with Hepes buffer (10 mM Hepes, 137 mM NaCl, 4 mM KCl, 11 mM D-glucose, 0.5 mg/ml RIA grade Bovine serum albumin, pH 7.4). Two hours prior to initiation of each assay, the 96-well plates were coated with 1% polyethylene glycol. All proteins to be used in an assay were exposed to 0.1 mM of the serine protease inhibitor (4-amidinophenyl)-methanesulfonylfluoride (AMPSF) in 4 mM sodium acetate buffer (pH 5.5) for 15 minutes, then diluted in Hepes buffer and allowed to equilibrate for 45 minutes, as stated in chapter 2.5.3.

Enzyme activity was measured by spectrophotometry at a wavelength of 405 nm with S-2302, 0.3 mM. All reactions were performed in duplicate and were repeated at least three times. The concentration of icatibant used in these studies were chosen on the basis of previously published data (Dear et al., 1996).

High molecular weight kininogen (HMWK), Hageman Factor (Factor XII/FXII) and Zn$^{2+}$ are all important co-factors in the activation of prekallikrein in human plasma. In addition to these co-factors, proteins present on the surface of endothelial cells, such as heat shock protein 90 (HSP90) and hyaluronic acid binding protein (HABP), also aid the activation of prekallikrein. Therefore, endothelial cell homogenate, HSP90, HABP, HMWK, FXII and Zn$^{2+}$ were all added to prekallikrein activation buffers to aid the conversion of human plasma prekallikrein to human plasma kallikrein.
Chapter 6 The effect of icatibant on prekallikrein activation

6.2.2.1 The kinetics of prekallikrein activation in the presence and absence of Zn\(^{2+}\) and FXII

Hepes buffer, 105 µl, was added to the 96-well microtiter plate, followed by: 35 µl of high molecular weight kininogen (HMWK), 62.5 nM; 35 µl of human plasma prekallikrein, 62.5 nM; 35 µl of Hageman factor (FXII), 62.5 nM, or Hepes buffer. The 96-well plate was then incubated at 37°C for 15 minutes. The reaction was initiated by the addition of 20 µl of ZnCl\(_2\), 0.625 mM, or Hepes buffer and 20 µl of S-2302, 3.75 mM, dissolved in distilled water. Spectrophotometric readings were taken at 50-second intervals with Soft Max Pro3 (plate reader software).

6.2.2.2 Activation of prekallikrein by Heat Shock Protein 90

Hepes buffer, 120 µl, was added to the 96-well microtiter plate, followed by: 35 µl of high molecular weight kininogen (HMWK), 250 nM, or Hepes buffer; 35 µl of human plasma prekallikrein, 250 nM, or Hepes buffer and; 20 µl of Human Heat Shock Protein 90 (HSP90), 125 nM, or Hepes buffer. The 96-well plate was then incubated at 37°C for 15 minutes. The reaction was initiated by the addition of 20 µl of ZnCl\(_2\), 0.625 mM, or Hepes buffer and 20 µl of S-2302, 3.75 mM, dissolved in distilled water. Spectrophotometric readings were taken at 50-second intervals with Soft Max Pro3.

6.2.2.3 The effect of icatibant on prekallikrein activation in the presence of HSP90

Icatibant, aprotinin or soybean trypsin inhibitor dissolved in Hepes buffer were added to the 96-well microtiter plate followed by: 35 µl of high molecular weight kininogen (HMWK), 250 nM, or Hepes buffer; 35 µl of human plasma prekallikrein, 250 nM, or Hepes buffer; 20 µl of Human Heat Shock Protein 90 (HSP90), 125 nM, or Hepes buffer. The 96-well plate was then incubated at 37°C for 15 minutes. The reaction was initiated by the addition of 20 µl of ZnCl\(_2\), 0.625 mM and 20 µl of S-2302, 3.75 mM, dissolved in distilled water. Spectrophotometric readings were taken at 50-second intervals with Soft Max Pro3.
6.2.2.4 Activation of prekallikrein by hyaluronic acid binding protein and FXII

Hepes buffer, 85 μl, was added to the 96-well microtiter plate, followed by: 35 μl of high molecular weight kininogen (HMWK), 250 nM; 35 μl of human plasma prekallikrein, 250 nM; 35 μl of Hageman factor (FXII), 250 nM, or Hepes buffer; 20 μl hyaluronic acid binding protein (HABP), 66.6 μg/ml, or Hepes buffer. The 96-well plate was then incubated at 37°C for 15 minutes. The reaction was initiated by the addition of 20 μl of ZnCl2, 0.625 mM, or Hepes buffer and 20 μl of S-2302, 3.75 mM, dissolved in distilled water. Spectrophotometric readings were taken at 50-second intervals with Soft Max Pro3.

6.2.2.5 The effect of icatibant on prekallikrein activation in the presence of FXII

Icatibant, aprotinin or soybean trypsin inhibitor dissolved in Hepes buffer were added to the 96-well microtiter plate, followed by: 35 μl of high molecular weight kininogen (HMWK), 250 nM; 35 μl of human plasma prekallikrein, 250 nM; 35 μl of Hageman factor (FXII), 250 nM, or Hepes buffer. The 96-well plate was then incubated at 37°C for 15 minutes. The reaction was initiated by the addition of 20 μl of ZnCl2, 0.625 mM and 20 μl of S-2302, 3.75 mM, dissolved in distilled water. Spectrophotometric readings were taken at 50-second intervals for a duration of 10 minutes with Soft Max Pro3.

6.2.2.6 Potentiation of prekallikrein activation by FXII with endothelial cell lysate

Hepes buffer, 85 μl, was added to the 96-well microtiter plate, followed by: 35 μl of high molecular weight kininogen (HMWK), 250 nM; 35 μl of human plasma prekallikrein, 250 nM; 35 μl of Hageman factor (FXII), 250 nM; 20 μl endothelial cell lysate or Hepes buffer. The 96-well plate was then incubated at 37°C for 15 minutes. The reaction was initiated by the addition of 20 μl of ZnCl2, 0.625 mM and 20 μl of S-2302, 3.75 mM, dissolved in distilled water. Spectrophotometric readings were taken at 50-second intervals with Soft Max Pro3.
6.2.2.7 Prekallikrein activation with endothelial cell lysate and without FXII or Zn$$^{2+}$$

Hepes buffer, 85 µl, was added to the 96-well microtiter plate, followed by: 35 µl of high molecular weight kininogen (HMWK), 250 nM; 35 µl of human plasma prekallikrein, 250 nM; 35 µl of Hageman factor (FXII), 250 nM, or Hepes buffer; 20 µl endothelial cell lysate or Hepes buffer. The 96-well plate was then incubated at 37°C for 15 minutes. The reaction was initiated by the addition of 20 µl of ZnCl$_2$, 0.625 mM, or Hepes buffer and 20 µl of S-2302, 3.75 mM, dissolved in distilled water. Spectrophotometric readings were taken at 50-second intervals with Soft Max Pro3.

6.2.2.8 The effect of icatibant on prekallikrein activation in the presence of FXII and endothelial cell lysate

Icatibant, aprotinin or soybean trypsin inhibitor dissolved in Hepes buffer were added to the 96-well microtiter plate, followed by: 35 µl of high molecular weight kininogen (HMWK), 250 nM; 35 µl of human plasma prekallikrein, 250 nM; 35 µl of Hageman factor (FXII), 250 nM, or Hepes buffer; 20 µl endothelial cell lysate or Hepes buffer. The 96-well plate was then incubated at 37°C for 15 minutes. The reaction was initiated by the addition of 20 µl of ZnCl$_2$, 0.625 mM, or Hepes buffer and 20 µl of S-2302, 3.75 mM, dissolved in distilled water. Spectrophotometric readings were taken at 50-second intervals with Soft Max Pro3.

6.2.2.9 Activation of human plasma by endothelial cell lysate and Zn$$^{2+}$$

Hepes buffer, 123.5 µl, was added to the 96-well microtiter plate, followed by 7.5 µl of human plasma and 20 µl of endothelial cell lysate. The 96-well plate was then incubated at 37°C for 15 minutes. The reaction was initiated by the addition of 25 µl of ZnCl$_2$, 8 mM, or Hepes buffer and 25 µl of S-2302, 2.4 mM, dissolved in distilled water. Spectrophotometric readings were taken at 50-second intervals with Soft Max Pro3.
The effect of icatibant on the activation of human plasma in the presence of Zn$^{2+}$ and endothelial cell lysate

Icatibant, aprotinin or soybean trypsin inhibitor dissolved in Hepes buffer were added to the 96-well microtiter plate, followed by 7.5 µl of human plasma and 20 µl of endothelial cell lysate. The 96-well plate was then incubated at 37°C for 15 minutes. The reaction was initiated by the addition of 25 µl of ZnCl$_2$, 8 mM and 25 µl of S-2302, 2.4 mM, dissolved in distilled water. Spectrophotometric readings were taken at 50-second intervals with Soft Max Pro3.

Human pancreatic trypsin enzyme assay

Trypsin was incubated in a 96-well plate with the appropriate Tris-HCl buffer (as mention in chapter 2.5.2.3), with or without an antagonist or inhibitor present, for 15 minutes at 37°C. Reactions were initiated by the addition of the chromogenic substrate S-2765 and reading the change in optical density with a spectrophotometer at 9-second intervals and 405 nm. All reactions were performed in duplicate and were repeated at least three times.

Data analysis

Enzyme velocity graphs were fitted to a second order polynomial equation as they fitted the data better than any other function.

Polynomial equation (equation 6.1):

$$OD(t) = A + Bt + Ct^2$$

Where:

OD(t) is the optical density at time t. t is time. A, B and C are kinetic constants.

Unfortunately, the parameters of these equations could not be used to obtain a kinetic constant for the rate of the reaction.

The rate of each reaction was calculated by using a tangent to determine the gradient of the change in optical density versus time graph after any lag phase which may have occurred. The initial velocity of each assay was calculated by linear
regression using SoftMax Pro3 (96-well plate reader software) or with GraphPad-
Prism 4 software with assays exceeding 10 minutes in duration. Log₁₀[dose]-
response curves were created from the initial velocity of each assay and fitted to the
appropriate equation using GraphPad. A Wilcoxon sign-rank (nonparametric, paired
test), Friedman (nonparametric, paired test) and Dunn’s test (non-parametric, post
hoc test) were used to determine statistical differences in enzyme activity. A p value
less than 0.05 (p<0.05) was taken as statistically significant.
Chapter 6

The effect of icatibant on prekallikrein activation

6.3 Results

6.3.1 The kinetics of prekallikrein activation in the presence and absence of Zn\textsuperscript{2+} and FXII

S-2302, 0.3 mM, was cleaved in the presence of prekallikrein (8.75 nM), HMWK (8.75 nM), FXII (8.75 nM) and Zn\textsuperscript{2+} (50 μM) (figure 6.1). There were two phases to the enzyme activity, an exponential phase, followed by a linear phase with a constant rate of enzyme activity. Removal of either FXII or Zn\textsuperscript{2+} from the assay generated a 20-minute delay in observable enzyme activity, increased the duration of the exponential phase and significantly reduced the initial rate of S-2302 cleavage. The linear phase of the reaction was reduced by 87% and 89.7% with the removal of Zn\textsuperscript{2+} and FXII respectively. There was no observable enzyme activity in the absence of Zn\textsuperscript{2+} and FXII. Data for this investigation was fitted to equation 6.1.

6.3.2 Activation of prekallikrein by Heat Shock Protein 90

The cleavage of S-2302, 0.3 mM, only occurred in the presence of prekallikrein and after a delay of 45 minutes. The enzyme activity exhibited two distinct phases, an exponential phase which lasted for approximately 55 minutes, followed by a linear phase with a constant rate of S-2302 cleavage (figure 6.2A). The rate of S-2302 cleavage, during the linear phase, was greatest when Heat Shock Protein 90 (HSP90) (10 nM), Zn\textsuperscript{2+} (50 μM) HMWK (35 nM) and prekallikrein (35 nM) were present. Removal of HSP90, Zn\textsuperscript{2+}, HMWK or prekallikrein reduced the rate of kallikrein activity by 67.2%, 77.9%, 99.4% and 100% respectively (figure 6.2B). The duration of the exponential phase was not affected by any of the prekallikrein co-factors, however, the amplitude of its activity was reduced by the removal of HSP90, Zn\textsuperscript{2+} and HMWK. Data for this investigation was fitted to equation 6.1.

6.3.3 The effect of icatibant on prekallikrein activation in the presence of HSP90

The cleavage of S-2302, 0.3 mM, by active prekallikrein (plasma kallikrein), in the presence of HSP90, Zn\textsuperscript{2+} and HMWK was significantly inhibited (p<0.05, Friedman test) by aprotinin (1 μM) and SBTI (1 μM) but not icatibant (10 μM) (p>0.05, Dunn's test). Enzyme activity was reduced by 95%, 99.9% and 16.28% by aprotinin, SBTI and icatibant respectively (figure 6.3).
Chapter 6 The effect of icatibant on prekallikrein activation

Figure 6.1) The hydrolysis of S-2302 (0.3 mM) in the presence of plasma prekallikrein (8.75 nM) and HMWK (8.75 nM) and the effect of addition of FXII (8.75 nM) and Zn$^{2+}$ (50 μM). The hydrolysis of S-2302 was measured by spectrophotometry at 405 nm and 37° C for a duration of 65 minutes. Results are the mean and standard error of 3 experiments. FXII is unactive Hageman factor. HMWK is high molecular weight kininogen.
Figure 6.2) The kinetics (A) and the percentage change in the non-exponential cleavage of S-2302, 0.3 mM (B) in the presence and absence of plasma prekallikrein (35 nM), HSP90 (10 nM), Zn$^{2+}$ (50 μM) and HMWK (35 nM). The hydrolysis of S-2302 was measured by spectrophotometry at 405 nm and 37°C for a duration of 150 minutes. Results are the mean and standard error of 3 experiments. HSP90 is Heat Shock Protein 90. HMWK is High Molecular Weight Kininogen. PK is prekallikrein.
Figure 6.3) HSP90-potentiated prekallikrein (35 nM) activation in the presence of icatibant (10 μM), aprotinin (1 μM) and soybean trypsin inhibitor (1 μM). All wells contained prekallikrein (35 nM), HSP90 (10 nM), Zn²⁺ (50 μM) and HMWK (35 nM) and S-2302 (0.3mM). The hydrolysis of S-2302 was measured using spectrophotometry at 37°C and 405 nm for 150 minutes. Results are the mean and standard error of 3 experiments. SBTI is soybean trypsin inhibitor. HMWK is high molecular weight kininogen. HSP90 is Heat Shock Protein 90. *A significant reduction in enzyme activity compared to control (p<0.05, Friedman test).
6.3.4 Activation of prekallikrein by hyaluronic acid binding protein and FXII

Cleavage of S-2302, 0.3 mM, occurred in under 10 minutes after initiation of the assay with FXII (17.5 nM), Zn$^{2+}$ (50 μM), HMWK (17.5 nM), hyaluronic acid binding protein [HABP] (5.33 μg/ml) and PK (17.5 nM) (figure 6.4). Removal of HABP, FXII, PK, HMWK or Zn$^{2+}$ from the assay resulted in -7%, 99.8%, 100%, 98.9%, or 98.28% reduction in enzyme activity respectively.

6.3.5 The effect of icatibant on prekallikrein activation in the presence of FXII

The cleavage of S-2302, 0.3 mM, by active prekallikrein (plasma kallikrein), in the presence of FXII, Zn$^{2+}$ and HMWK was significantly inhibited (p<0.05, Friedman test) by aprotinin (1 μM) and SBTI (1 μM) and icatibant (10 μM). Enzyme activity was reduced by 82.6%, 95.34% and 21.2% by aprotinin, SBTI and icatibant respectively (figure 6.5).

6.3.6 Potentiation of prekallikrein activation by FXII with endothelial cell lysate

Cleavage of S-2302, 0.3 mM, in the presence of prekallikrein (8.75 nM), FXII (8.75 nM), HMWK (8.75 nM) and Zn$^{2+}$ (50 μM) was significantly augmented by the addition of whole cell lysate (1.2 mg/ml of protein), supernatant lysate (0.89 mg/ml protein) and pellet endothelial lysate (0.59 mg/ml protein) (figure 6.6A). This effect was dose-dependent (figure 6.6B). The [protein]-potentiation curves were fit to equation 6.2. The Hill coefficients for whole cell, supernatant and pellet fractions of the endothelial lysate curves were determined as 0.92 ± 0.51, 1.30 ± 0.43 and 1.18 ± 2.45 respectively.

Equation 6.2: 

\[ v_i = V_{\text{max}} \cdot \frac{[S]}{[S] + K_d} \]

\( V_{\text{max}} \) is the maximum augmentation of the initial velocity of the enzyme at saturating substrate concentrations. \( v_i \) is the initial velocity of the. \( K_d \) is the dissociation equilibrium constant.
Figure 6.4) Hydrolysis of S-2302 (0.3 mM) in the presence and absence of plasma prekallikrein (8.75 nM), HMWK (8.75 nM), HABP (5.33 μg/ml), FXII (8.75 nM) and Zn²⁺ (50 μM). The hydrolysis of S-2302 was measured by spectrophotometry at 405 nm and 37°C for a duration of 10 minutes. Results are the mean and standard error of 3 experiments. HABP is Hyaluronic acid binding protein. HMWK is High Molecular Weight Kininogen. PK is prekallikrein. FXII is inactive Hageman factor.
Figure 6.5) FXII-potentiated prekallikrein (8.75 nM) activation in the presence of icatibant (10 μM), soybean trypsin inhibitor (1 μM) and aprotinin (1 μM). All wells contained prekallikrein (8.75 nM), FXII (8.75 nM, Zn$^{2+}$ (50 μM), HMWK (8.75 nM) and S-2302 (0.3 mM). The hydrolysis of S-2302 was measured by spectrophotometry at 405 nm and 37°C for a duration of 10 minutes. Results are the mean and standard error of 3 experiments. *A significant reduction in enzyme activity compared to control (p<0.05, Friedman test). SBTI is soybean trypsin inhibitor. HMWK is high molecular weight kininogen. FXII is unactive Hageman Factor.
Figure 6.6) The rate of hydrolysis of S-2302, 0.3 mM (A) and Log_{10}[dose]-potentiation curves (B) in the presence of Zn²⁺ (50 μM), prekallikrein (8.75 nM), HMWK (8.75 nM), FXII (8.75 nM) with whole (EA.hy926) cell, supernatant and pellet lysate. The protein concentration in the whole cell lysate, supernatant and pellet of (A) are 1.2 mg/ml, 0.89 mg/ml and 0.59 mg/ml respectively. Enzyme activity was measured by spectrophotometry at 405 nm and 37°C for 10 minutes. Results are the mean and standard error of 4 experiments. HMWK is high molecular weight kininogen. FXII is inactive Hageman Factor.
6.3.7 Prekallikrein activation with endothelial cell lysate and without FXII or Zn$^{2+}$

S-2302, 0.3 mM, was cleaved at a rate of 89.4x10$^{-3}$ OD/min in the presence of FXII (8.75 nM), Zn$^{2+}$ (50 μM), HMWK and prekallikrein (figure 6.7). Removal of FXII or Zn$^{2+}$ from the assay caused an 80.5% and 82.2% reduction in amidolytic activity respectively. In the absence of Zn$^{2+}$ and FXII, S-2302, 0.3 mM, was cleaved at a rate of 15 x10$^{-3}$ OD/min. The addition of whole cell EA.hy926 lysate to the assay (1.2 mg/ml) increased the rate of prekallikrein activation 2.8-fold in the presence of Zn$^{2+}$, HMWK, FXII and prekallikrein. Removal of either FXII or Zn$^{2+}$ from the assay, with whole cell endothelial lysate present, reduced the rate of S-2302 cleavage to 21 x10$^{-3}$OD/min or less.

6.3.8 The effect of icatibant on prekallikrein activation in the presence of FXII and endothelial cell lysate

Aprotinin (1 μM), SBTI (1 μM) and the removal of the endothelial supernatant lysate (with 1.44 mg/ml of protein) from the assay significantly inhibited the cleavage of S-2302, 0.3 mM, in the presence of prekallikrein, HMWK, Zn$^{2+}$, FXII (p<0.05, Friedman test) (figure 6.8). Icatibant, 10 μM, failed to significantly inhibit the activation of prekallikrein (p>0.05, Dunn’s test).

6.3.9 Activation of human plasma by endothelial cell lysate and Zn$^{2+}$

 Amidolytic activity was generated 20 minutes after initiation of the assay and was reduced by 97.3% in the absence of Zn$^{2+}$ (figure 6.9). S-2302, 0.3 mM, was cleaved by human plasma at the rate of 23.3 x10$^{-3}$ OD/min in the presence of 3.74 μg/ml of endothelial whole cell lysate protein and Zn$^{2+}$ (1 mM). Data for this investigation was fitted to equation 6.1.

6.3.10 The effect of icatibant on the activation of human plasma in the presence of Zn$^{2+}$ and endothelial cell lysate

Icatibant (10 M), SBTI (1 μM) and aprotinin (1 μM) significantly inhibited the cleavage of S-2302, 0.3 mM, in the presence of Zn$^{2+}$ (1 mM), by human plasma (p<0.05, Friedman test) (figure 6.10). However, icatibant alone failed to significantly
Figure 6.7) The rate of S-2302, 0.3 mM, hydrolysis by prekallikrein (8.75 nM), with HMWK (8.75 nM), in the presence and absence of FXII (8.75 nM), Zn$^{2+}$ (50 μM) and 1.2 mg/ml of protein from whole (EA.hy926) cell endothelial lysate. The hydrolysis of S-2302 was measured by spectrophotometry. Readings were taken at 37°C and 405 nm. Results are the mean and standard error of 3 experiments. HMWK is high molecular weight kininogen. FXII is inactive Hageman factor.
Figure 6.8) The inhibition of prekallikrein activation in the presence of icatibant (10 μM), soybean trypsin inhibitor (1 μM) and aprotinin (1 μM). All wells also contained prekallikrein (8.75 nM), FXII (8.75 nM), Zn²⁺ (50 μM), HMWK (8.75 nM), S-2302 (0.3 mM) and 1.44 mg/ml of protein from the supernatant of EA.hy926 cell lysate. The hydrolysis of S-2302 was determined by spectrophotometry at 37°C and 405 nm for 10 minutes. Results are the mean and standard error of 3 experiments. *A significant reduction in enzyme activity compared to control (p<0.05, Friedman test). SBTI is soybean trypsin inhibitor. HMWK is high molecular weight kininogen.
Figure 6.9) The kinetics (A) and the rate of S-2302 (0.3 mM) hydrolysis (B) by human plasma activated by 3.74 mg/ml of protein from EA.hy926 whole cell lysate in the presence and absence of Zn^{2+} (1 mM). The hydrolysis of S-2302 was measured by spectrophotometry. Readings were taken at 37°C and 405nm for 50 minutes at 5 minute intervals. Results are the mean and standard error of 3 experiments. *A significant increase in enzyme activity compared to control (p<0.05, Wilcoxon sign-rank test).
Figure 6.10) The effect of icatibant (10 μM), SBTI (1 μM) and aprotinin (1 μM) on the hydolysis of S-2302 (0.3 mM) by human plasma activated in the presence of the protein (3.74 mg/ml) from EA.hy926 whole cell lysate and Zn$^{2+}$ (1 mM). The hydrolysis of S-2302 was measured at 37°C and 405 nm for 50 minutes at 5 minute intervals. Results are the mean and standard error of 4 experiments. HMWK is high molecular weight kininogen. *A significant reduction in enzyme activity compared to control (p<0.05, Friedman test). FXII is unactive Hageman factor. SBTI is soybean trypsin inhibitor.
inhibit the enzyme activity of human plasma (p>0.05, Dunn's test).

6.3.11 The effect of S-2765 concentration on human pancreatic trypsin enzyme activity

Trypsin, 1.81 nM, displayed Michaelis-Menten kinetics in the presence of increasing concentrations of S-2765 (figure 6.11A). Enzyme activity was saturable, with a single value for $V_{\text{max}}$ and $K_M$. Data was fitted to the Michaelis-Menten equation:

$$v_i = \frac{V_{\text{max}} \cdot [S]}{[S] + K_M}$$

$V_{\text{max}}$ is the maximum augmentation of the initial velocity of the enzyme at saturating substrate concentrations. $v_i$ is the initial velocity of the. $K_d$ is the Michaelis-Menten constant. $[S]$ is the concentration of the substrate in the assay.

$V_{\text{max}}$, $K_M$ and $k_{\text{cat}}$ were calculated to be $109.2 \pm 1.58 \triangle \text{optical density x 10}^{-3}/\text{min}$, $40.66 \pm 2.01 \mu\text{M}$ and $148.19 \text{s}^{-1}$ respectively.

6.3.12 The effect of kinin receptor ligands on human pancreatic trypsin enzyme activity

The cleavage of S-2765, 20 µM, by human pancreatic trypsin was determined in the presence of the following kinin receptor ligands: Icatibant, NPC 567, [Ad]-Bk, WIN 64338 (kinin B$_2$ receptor antagonists); bradykinin (a kinin B$_2$ receptor agonist); des-arg$^{10}$-kallidin (a kinin B$_1$ receptor agonist); des-arg$^{10}$-leu$^{9}$-kallidin (a kinin B$_1$ receptor antagonist) (figure 6.11B). The concentration of S-2765 was chosen on the basis of the $K_M$ obtained from figure 6.11A. Trypsin enzyme activity was not affected by the presence of any of the kinin receptor ligands at ligand concentrations up to and including 1 µM. At ligand concentrations above 1 µM trypsin enzyme activity was potentiated.
Figure 6.11) Human pancreatic trypsin, 1.81 nM, activity in the presence of increasing concentrations of S-2765 (A) and in the presence of kinin receptor ligands (B). Percentage changes were normalised to the control (mean of 30.01 x10^{-3} OD/minute, standard error of the mean 16.6 OD x10^{-3}/minute. Data represent the means from 3 experiments with duplicate samples.
6.4 Discussion

Incubation of prekallikrein with HMWK, Zn$^{2+}$ and FXII resulted in the immediate hydrolysis of S-2302 (figure 6.1). Removal of either FXII or Zn$^{2+}$ from the assay significantly reduced the rate of prekallikrein activation, while removal of HMWK from the assay completely abrogates prekallikrein activation (figure 6.2).

The mechanism by which HMWK acts as a cofactor in prekallikrein activation is not known but it is likely to be aided by (1) HMWKs ability to bind simultaneously to prekallikrein and cell surface receptors, such as the receptor for the globular head of complement fragment 1q (gC1qR) (Thompson et al., 1979; Kaplan et al., 2002), and (2) HMWKs ability to potentiate the activation of FXII (Meier et al., 1977). The latter observation would suggest that FXII binds directly to HMWK, however, to date, no binding sites for HMWK have been mapped out on FXII or vice versa.

Zn$^{2+}$ is also an essential cofactor for the activation of prekallikrein in vitro. It is required for the binding of FXII and HMWK to cell surface receptors such as gC1qR (Joseph et al., 2002). It has been reported that Zn$^{2+}$ induces a conformational change in gC1qR which results in the re-arrangement of hydrophobic amino acids present within its HMWK-binding site. This conformational change increases the affinity of HMWK for gC1qR (Kumar et al., 2002). Zn$^{2+}$ also appears to be important in the activation of prekallikrein which occurs independently of cell surface receptors (figure 6.1; figure 6.2; figure 6.4). Therefore, it is likely that Zn$^{2+}$ also binds to one or more of the proteins involved in the activation of prekallikrein to aid prekallikrein activation.

Initial studies on the activation of prekallikrein in human plasma using artificial surfaces, such as kaolin, suggested that FXII was responsible for the activation of prekallikrein in plasma as FXII-deficient plasma did not activate upon contact with artificial surfaces (Proud and Kaplan, 1988). However, studies on endothelial surfaces have revealed that FXII-deficient human plasma is activated when incubated with Zn$^{2+}$ and HUVECs (Joseph et al., 2001) and (2) purified prekallikrein is activated when incubated with HMWK, Zn$^{2+}$ and HUVECs in the absence of FXII (Rojkjaer et al., 1998). Consequently, FXII is now believed to be a potentiator, and not activator, of prekallikrein in vivo. As a result of this it is also believed that
another protein found on cells may be responsible for the activation of prekallikrein in vivo. One candidate is Heat Shock Protein 90 (HSP90).

Prekallikrein (35 nM) is autoactivated after a delay of 40 minutes when incubated with Zn$^{2+}$ (50 μM) and HMWK (35 nM) (figure 6.2). The addition of HSP90, 10 nM, to the assay significantly enhanced the rate of prekallikrein activation without altering the 40-minute delay in plasma kallikrein activity. Previous studies on the activation of prekallikrein in the presence of HSP90 suggest that HSP90 is an activator, and not potentiator, of prekallikrein activation as prekallikrein was not activated in the absence of HSP90, it was only activated in the presence of HSP90 (Joseph et al., 2002). In addition to activating prekallikrein, HSP90 also plays a role in signal transduction and protein degradation (Sreedhar et al., 2004). These effects are believed to be dependent on its ability to induce protein folding within cells. However, the mechanism behind HSP90's ability to potentiate the activation of prekallikrein is not known, but it may be dependent on its protein folding ability as the binding of HMWK to prekallikrein requires a conformational change in the structure of HMWK (Lin et al., 1996). Alternatively, HSP90 may display proteolytic activity similar to that of FXIIa. This theory is supported by the fact that HSP90's ability to activate prekallikrein, like FXII, is potently inhibited by the protein corn trypsin inhibitor (Joseph et al., 2002). Secondly, HPS90, like FXII, requires Zn$^{2+}$ and an intact single chain of HMWK to potentiate the activation of prekallikrein (figure 6.2; figure 6.4)(Joseph et al., 2002).

Unsurprisingly, the serine protease inhibitors aprotinin and soybean trypsin inhibitor (SBTI) significantly inhibited the hydrolysis of S-2302, 0.3 mM, in the presence of prekallikrein, Zn$^{2+}$, HMWK and HSP90 (figure 6.3). However, icatibant, 10 μM, failed to inhibit HSP90-induced potentiation of prekallikrein activation.

Although FXII may not be responsible for the activation of prekallikrein in vivo it does play a major role in the generation of plasma kallikrein activity in vitro (figure 6.4). Incubation of icatibant, 10 μM, with prekallikrein, HMWK, Zn$^{2+}$ and FXII failed to significantly impede the activation of prekallikrein (figure 6.5) suggesting that icatibant does not reduce the recovery of kinin from human nasal airways by impeding the progression of FXII-potentiated activation of prekallikrein.
Chapter 6 The effect of icatibant on prekallikrein activation

The cell surface receptors gC1qR, cytokeratin 1 (CK1) and urokinase plasminogen activator receptors (u-PAR) are now regarded as physiological activators of prekallikrein (Joseph et al., 1996; Hasan et al., 1998; Joseph et al., 1999; Mahdi et al., 2001). The precise mechanism by which activation is brought about is not understood but of the three proteins gC1qR (also known as hyaluronic acid binding protein [HABP]) is responsible for the majority of HMWK binding to HUVECs (Joseph et al., 2001). The addition of cloned gC1qR to enzyme assays containing prekallikrein, Zn\(^{2+}\), HMWK and FXII, dose-dependently potentiates S-2302 hydrolysis. Potentiation of prekallikrein activation by gC1qR is not seen in the absence of FXII (Joseph et al., 2001). Unfortunately, these results could not be reproduced with HABP. It failed to potentiate the activation of prekallikrein in the presence of FXII, Zn\(^{2+}\) and HMWK (figure 6.4). This lack of efficacy could be due to an insufficient concentration of HABP, a lack of purity or species variation (as HABP was obtained from affinity-purified bovine nasal cartilage).

Replacement of HABP with endothelial cell lysate, resulted in significant potentiation of prekallikrein activation in the presence of FXII, Zn\(^{2+}\) and HMWK (figure 6.6). Removal of either FXII or Zn\(^{2+}\) from reactions assays with endothelial lysate, prekallikrein and HMWK significantly attenuated the amidolytic activation observed (figure 6.7). This result is corroborated by other reports in which the activation of prekallikrein on the surface of HUVECs is only potentiated in the presence of FXII, Zn\(^{2+}\) and HMWK (Joseph et al., 2001). Icatibant, 10 \(\mu\)M, was incubated with this assay to determine if it was capable of inhibiting prekallikrein activation by interfering with the interaction between the FXII or Zn\(^{2+}\) and the endothelial lysate proteins. However, icatibant, 10 \(\mu\)M failed to inhibit the activation of prekallikrein (figure 6.8).

Human plasma was activated when incubated with endothelial cell lysate and Zn\(^{2+}\) (figure 6.9). Compared to assays with purified proteins, a relatively high concentration of Zn\(^{2+}\) was required to induce amidolytic activity. This is likely to be due the presence of plasma proteins such as albumin which act as chelators of Zn\(^{2+}\). This result is corroborated by reports which have shown that high concentrations of Zn\(^{2+}\) are required for human plasma to be activated on the surface of HUVECs (Rojkjaer et al., 1998). Incubation of icatibant, 10 \(\mu\)M, also failed to significantly inhibit the activation of human plasma (figure 6.10).
The enzyme or cofactors responsible for the activation of tissue prokallikrein within the human nasal airway have not been identified. Investigations in vitro have revealed that the serine proteases trypsin and human plasma kallikrein are capable of activating tissue prokallikrein (Takada et al., 1985). However, the inhibition of human plasma kallikrein by icatibant is not likely to account for the reduction in recovery of kinins from the nasal airway of PAF-challenged individuals, as shown in chapter 4. In addition the inability of icatibant (and other kinin B₂ receptor antagonists) to inhibit the enzyme trypsin (figure 6.11B) suggests that icatibant does not reduce the recovery of kinin kinins from the nasal airway by inhibiting trypsin or trypsin-like enzymes thought to be present within the human airway.

In summary, icatibant, 10 μM, failed to significantly inhibit the activation of prekallikrein or human plasma in vitro. Icatibant, 10 μM, also failed to inhibit the enzyme activity of trypsin. This suggests that inhibition of prekallikrein activation or trypsin-like enzyme activity in vivo is not likely to be a plausible explanation for icatibant's ability to inhibit the recovery of kinins from human nasal airways as previous reports have shown that icatibant, 10 μM, abolishes the binding of [¹²⁵I]-icatibant to kinin B₂ receptors on human nasal turbinates with an inhibition constant of 0.48 ± 0.02 nM (Dear et al., 1996).
6.5 Summary

(1) The activation of human plasma prekallikrein and human plasma were measured by the cleavage of S-2302, 300 µM (at pH 7.4, 37°C and 405 nm).

(2) The activation of human plasma prekallikrein (35 nM) was potentiated by presence of Heat Shock Protein 90 (10 nM) in the presence of Zn²⁺ (50 µM) and HMWK (35 nM).

(3) Heat shock protein 90-induced potentiation of prekallikrein activation was not inhibited by incubation with icatibant (10 µM) but it was significantly inhibited by the plasma kallikrein inhibitors aprotinin (1 µM) and SBTI (1 µM).

(4) The activation of human plasma prekallikrein (8.75 nM) was potentiated by presence of FXII (8.75 nM) in the presence of Zn²⁺ (50 µM) and HMWK (8.75 nM).

(5) Hageman Factor-induced potentiation of prekallikrein activation was not inhibited by icatibant (10 µM) but it was significantly inhibited by the plasma kallikrein inhibitors aprotinin (1 µM) and SBTI (1 µM).

(6) The activation of human plasma prekallikrein (8.75) was potentiated by EA.hy926 cell lysate in the presence of FXII (8.75 nM), Zn²⁺ (50 µM) and HMWK (8.75 nM).

(7) The activation of human plasma prekallikrein (8.75 nM) was not inhibited by icatibant (10 µM) but it was significantly inhibited by the plasma kallikrein inhibitors aprotinin (1 µM) and SBTI (1 µM).

(8) The activation of human plasma in the presence of Zn²⁺ (1 mM) and EA.hy926 cell lysate (3.74 mg/ml) was not inhibited by icatibant (10 µM) but it was significantly inhibited by the plasma kallikrein inhibitors aprotinin (1 µM) and SBTI (1 µM).
Chapter 6 The effect of icatibant on prekallikrein activation

(9) The kinin B₂ receptor antagonists, WIN 64338, NPC-567, icatibant and [1-adamantaneacetyl-D-Arg⁰, Hyp³-(2-thienyl)-Ala⁵⁸⁹, D-Phe⁷]-bradykinin did not inhibit the enzyme activity of human pancreatic trypsin.

(10) The inhibition of human plasma prekallikrein activation or trypsin-like enzyme activity is not likely to be a plausible mechanism by which icatibant impedes the recovery of kinins from the nasal airway of non-atopic individuals challenged with PAF.
CHAPTER 7

THE ROLE OF KININ B₁ AND B₂ RECEPTORS IN AIRWAY HYPER-REACTIVITY IN THE HUMAN NASAL AIRWAY

7.1 Introduction

Intranasal challenge with PAF mimics the symptoms of allergic rhinitis by causing nasal blockage, nasal discomfort, sneezing and an increase in vascular permeability. PAF is found in the nasal lavages of allergic individuals challenged intranasally with allergen (Touqui, 1994; Chilton et al., 1996). At doses considerably lower than that required to mimic symptoms of allergic rhinitis PAF also causes an increase in kinin production (Turner et al., 2000b) and induces airway hyper-reactivity to allergen (Andersson et al., 1988), histamine and bradykinin (Austin and Foreman, 1993; Turner et al., 2000b). Intranasal pre-treatment with the kinin B₂ receptor antagonists, icatibant and [1-adamantaneacetyl-D-Arg⁹, Hyp³, beta-(2-thienyl)-Ala⁸,D-Phe⁹]-bradykinin abolishes all of these PAF-induced effects. Therefore, the capacity of kinins to induce hyper-reactivity in human nasal airway is thought to be of significance.

Nasal lavages recovered, from individuals with perennial allergic rhinitis and challenged with house dust mite have been found to contain kinins (Dear et al., 1996). It has been established that these kinins are also subject to further degradation by aminopeptidase enzymes which cause them to form high affinity kinin B₁ receptor agonists (Proud et al., 1987). The use of kinin B₁ receptor agonists and antagonists in models of allergic asthma augments and impedes, respectively, the development of airway hyper-reactivity (Huang et al., 1999; Gama Landgraf et al., 2003; Sugahara et al., 2003). However, kinin B₁ receptors have not been found in the airway of non-atopic individuals, they have only been found within the upper airway of atopic individuals (Christiansen et al., 2002). If, indeed, kinin B₁ receptors play a role in allergic rhinitis, the ability of icatibant to block airway hyper-reactivity may also result from its ability to weakly antagonize kinin B₁ receptors (Burkard et al., 1996; Stewart et al., 1999).
Unilateral allergen challenge of individuals with seasonal allergic rhinitis causes bilateral rhinorrhea and an elevation in the secretory IgA content of nasal lavage fluid recovered from both nasal cavities (Raphael et al., 1991). This is primarily believed to be due to the activation of sensory neurones by mediators such as histamine and bradykinin which are produced as a result of intranasal allergen challenge (Raphael et al., 1989b; Riccio and Proud, 1996). However, unilateral challenge of non-atopic individuals with bradykinin and histamine only causes ipsilateral blockage, implying that its congestive-inducing effects are restricted to the cavity to which it is introduced. It is yet to be determined whether this remains true after pre-treatment with PAF.

In this chapter the role of PAF, bradykinin and des-arg^10^-kallidin are investigated in human nasal airway hyper-reactivity and the effect of PAF on mediator production is also determined.
7.2 Experimental protocol

All experiments were performed single-blind, on non-atopic individuals.

7.2.1 The effect of bradykinin and captopril on histamine-induced nasal blockage

The aim of this study was to determine whether inhibition of angiotensin converting enzyme (ACE), in conjunction with intranasal challenge with bradykinin, would enhance the half-life of bradykinin in the nasal airway and subsequently enhance histamine-induced nasal blockage. Subjects were challenged bilaterally with a 100 µl spray of saline, histamine, 200 µg, and then bradykinin, 250 µg. The minimum cross-sectional area of the nasal airway ($A_{min}$) was measured immediately prior to, 2, 5, and 10 minutes after all three challenges. Immediately after the last acoustic rhinometric measurement subjects were given captopril orally, 12.5 mg, or nothing (negative control). Both treatments were given randomly in a single blind cross-over manner at least 72 hours apart. One, two, four and six hours after receipt of captopril or control, subjects were re-challenged with bradykinin, 250 µg. Histamine, 200 µg, was re-administered 6.5 hours after receipt of captopril or control. $A_{min}$ was measured immediately prior to, 2, 5, and 10 minutes after all five challenges apart from the 2 and 4 hour time-points. The protocol of this investigation is summarized in figure 7.1:

Figure 7.1) Protocol for investigating the effect of captopril and bradykinin on histamine-induced nasal blockage.
7.2.2 The duration of PAF-induced airway hyper-reactivity

The aim of this study was to determine the duration of PAF-induced nasal airway hyper-reactivity to histamine. Subjects were challenged bilaterally with a 100 μl spray of saline, followed by a bilateral 100 μl spray with histamine, 200 μg, thirty minutes later. The minimum cross-sectional area of the nasal airway (A_{min}) was measured immediately prior to, 5, 10 and 15 minutes after both challenges. Half an hour after receipt of histamine, subjects were treated intranasally with a 100 μl spray of PAF, 60 μg, or saline. Both treatments were administered randomly in a single blind cross-over manner at least a week apart. Four, six and eight hours after treatment with PAF or saline, subjects were re-challenged bilaterally with histamine. A_{min} was measured immediately prior to, 5, 10 and 15 minutes after challenge with histamine at all three time points. The doses and time-course used were based upon pilot studies and previous publications (Austin and Foreman, 1993). The protocol of this investigation is summarized in figure 7.2:

![Figure 7.2](image)

**Figure 7.2** Protocol for investigating duration of PAF-induced airway hyper-reactivity.

7.2.3 The role of kinin B_{1} receptors in PAF-induced nasal airway hyper-reactivity

The aim of this study was to determine whether intranasal pre-treatment with PAF alters nasal responsiveness to challenge with the kinin B_{1} receptor agonist, des-arg^{10}-kallidin. Subjects were challenged bilaterally with 100 μl sprays of saline; bradykinin, 100 μg; des-arg^{10}-kallidin, 200 μg; and des-arg^{10}-kallidin, 400 μg. Each challenge was performed in a random order and at thirty-minute intervals. Immediately prior to, 2, 5, 10 and 15 minutes after each nasal challenge, the
Chapter 7  

Kinin receptors and airway hyper-reactivity

minimum cross-sectional area ($A_{\text{min}}$) of the nasal airway was determined by acoustic rhinometry. Half an hour after receipt of the last drug subjects were treated intranasally with a 100 $\mu$l spray of PAF, 60 $\mu$g, or saline. Both treatments were administered randomly in a single blind cross-over manner at least a week apart.

Four hours after receipt of PAF or saline subjects were re-challenged bilaterally with 100 $\mu$l sprays of bradykinin, 100 $\mu$g; des-arg$^{10}$-kallidin, 200 $\mu$g; and des-arg$^{10}$-kallidin, 400 $\mu$g. Each challenge was performed in a random order and at thirty-minute intervals. $A_{\text{min}}$ were determined immediately prior to, 2, 5, 10 and 15 minutes after all three challenges. The protocol of this investigation is summarized in figure 7.3:

![Figure 7.3](image)

**Figure 7.3** Protocol for investigating the effect of PAF pre-treatment on challenge with a kinin $B_1$ receptor agonist. DAKD is des-arg$^{10}$-kallidin.

### 7.2.4 The effect of unilateral pre-treatment with PAF on contralateral histamine-induced nasal blockage

The aim of this study was to determine whether unilateral pre-treatment with PAF alters histamine-induced nasal blockage in the contralateral nostril. Subjects were challenged unilaterally (in the left nostril) with a 100 $\mu$l spray of saline. The minimum cross-sectional area of the nasal airway ($A_{\text{min}}$) was measured immediately prior to, 2, 5, 10 and 15 minutes after challenge with saline. Thirty minutes after receipt of saline, subjects were treated in the contralateral nostril (right nostril) with a 100 $\mu$l spray of PAF, 80 $\mu$g, or saline. It must be noted that the dose of PAF used in this investigation does not cause nasal congestion (Maniscalco et al., 2000). Subjects were then re-challenged in the left nostril (contralateral to the nostril which PAF or saline was administered to 6 hours prior) with a 100 $\mu$l spray of histamine, 200 $\mu$g. $A_{\text{min}}$ was measured (in both nostrils) immediately prior to, 2, 5, 10 and 15 minutes
after challenge with histamine. This protocol was repeated at least a week apart in a single blind crossover manner (Part 1).

This investigation was repeated again in a single blind cross-over manner at least one week apart but with each subject receiving PAF, 80 μg, or saline in the right nostril, and histamine in the left nostril (Part 2). The protocol of these investigations are summarized in figure 7.4A and 7.4B respectively:

![Diagram A and B]

**Figure 7.4A and 7.4B** Protocol for investigating the effect of unilateral pre-treatment with PAF on contralateral histamine-induced nasal blockage.

### 7.2.5 The effect of unilateral pre-treatment with PAF on contralateral bradykinin-induced nasal blockage

The aim of this study was to determine whether unilateral pre-treatment with PAF alters bradykinin-induced nasal blockage in the contralateral nostril. Subjects were challenged unilaterally (in the left nostril) with a 100 μl spray of saline. The minimum
cross-sectional area of the nasal airway ($A_{\text{min}}$) was measured immediately prior to, 2, 5, 10 and 15 minutes after challenge with saline. Thirty minutes after receipt of saline, subjects were treated in the contralateral nostril (right nostril) with a 100 µl spray of PAF, 80 µg, or saline. It must be noted that the dose of PAF used in this investigation is not sufficient enough to nasal congestion (Maniscalco et al., 2000). Subjects were then re-challenged in the left nostril (contralateral to the nostril which PAF or saline was administered to 6 hours prior) with a 100 µl spray of bradykinin, 100 µg. $A_{\text{min}}$ was measured (in both nostrils) immediately prior to, 2, 5, 10 and 15 minutes after challenge with bradykinin. This protocol was repeated at least a week apart in a single blind crossover manner (Part 1).

This investigation was repeated in a single blind cross-over manner at least a week apart but with each subject receiving PAF, 80 µg, or saline in the right nostril, and bradykinin in the left nostril (Part 2). The protocol of these investigations are summarized in figure 7.5A and 7.5B respectively:

**Figure 7.5A and 7.5B** Protocol for investigating the effect of unilateral pre-treatment with PAF on contralateral bradykinin-induced nasal blockage.
Figure 7.5A and 7.5B) Protocol for investigating the effect of unilateral pre-treatment with PAF on contralateral bradykinin-induced nasal blockage.

7.2.6 The effect of bilateral challenge with PAF on unilateral histamine-induced nasal blockage

The aim of this study was to determine whether bilateral pre-treatment with PAF alters nasal patency in the unchallenged nostril after unilateral challenge with histamine. Subjects were challenged unilaterally (in the right nostril) with a 100 μl spray of saline. The minimum cross-sectional area of the nasal airway ($A_{min}$) was measured immediately prior to, 2, 5, 10 and 15 minutes after challenge with saline. Thirty minutes after receipt of saline, subjects were treated bilaterally with a 100 μl spray of PAF, 80 μg, or saline. It must be noted that the dose of PAF used in this investigation is not sufficient enough to nasal congestion (Maniscalco et al., 2000). Six hours later subjects were re-challenged in the right nostril with a 100 μl spray of histamine, 200 μg. $A_{min}$ was measured (in both nostrils) immediately prior to, 2, 5, 10 and 15 minutes after challenge with histamine. This protocol was repeated at least a week apart in a single blind crossover manner (Part 1).

This investigation was repeated in a single blind, cross-over manner at least a week apart but with each subject receiving a bilateral pre-treatment with PAF, 80 μg, or saline, and challenge with histamine in the left nostril (Part 2). The protocol of these investigations are summarized in figure 7.6A and 7.6B respectively:
7.6A) Protocol for investigating the effect of bilateral pre-treatment with PAF on nasal blockage after unilateral challenge with bradykinin.

7.6B) Protocol for investigating the effect of bilateral pre-treatment with PAF on nasal blockage after unilateral challenge with bradykinin.

7.2.7 Data analysis

The minimum cross-sectional area ($A_{min}$) of each nasal cavity was determined by acoustic rhinometry. Values of $A_{min}$ obtained after each nasal challenge were normalised against the basal value of $A_{min}$. A normalised $A_{min}$ versus time graph was plotted and the area under the curve (AUC) was calculated. Data were analyzed statistically using a Wilcoxon sign-rank test. A p value less than 0.05 ($p<0.05$) was taken as statistically significant.

Note: Data in figure 7.9B were calculated by determining the area under the "area under $A_{min}$ vs time curve" (figure 7.9A) at time points 4, 6 and 8 hours after challenge with PAF or saline.
Data in figure 7.7B were calculated by determining the area under the "area under A_{min} vs time curve" (figure 7.7A) at time points 1 and 6 after treatment with captopril or placebo.
7.3 Results

7.3.1 The effect of bradykinin and captopril on histamine-induced nasal blockage

Intranasal challenge with bradykinin, 250 µg, caused a significantly greater reduction in nasal patency compared to the change in nasal patency caused by challenge with saline (p<0.05, Wilcoxon sign-rank test) (figure 7.7). Re-challenge with bradykinin 1 hour after receipt of captopril, 12.5 mg, resulted in significant potentiation of nasal blockage (p<0.05, Wilcoxon sign-rank test). Nasal blockage caused by intranasal challenge with histamine 6.5 hours after pre-treatment with captopril and repeated challenges with bradykinin was not significantly different from the histamine-induced nasal blockage generated after repeated challenges with bradykinin without oral captopril (figure 7.8).

7.3.2 The time-course of PAF-induced airway hyper-reactivity

Nasal challenge with 200 µg of histamine, 0, 4, 6 and 8 hours after pre-treatment with saline caused a significant reduction in nasal patency compared to challenge with saline (figure 7.9A) (p<0.05, Wilcoxon-sign rank test). Histamine-induced nasal blockage generated 4, 6 and 8 hours after pre-treatment with PAF, 60 µg, was significantly greater than the nasal blockage obtained after pre-treatment with saline (figure 7.9B) (p<0.05, Wilcoxon-sign rank test).

7.3.3 The role of kinin B₁ receptors in PAF-induced nasal airway hyper-reactivity

Bradykinin, 100 µg, caused a reduction in nasal patency which was significantly enhanced by pre-treatment with PAF, 60 µg, 6 hours prior, compared to the reduction in nasal patency observed after pre-treatment with saline (p<0.05, Wilcoxon sign-rank test). Des-arg^{10}-kallidin, 200 µg and 400 µg,
Figure 7.7) (A) A graph showing the change in nasal patency after challenges with bradykinin, 250 μg, prior and post receipt of captopril or placebo. A\textsubscript{min} was measured in triplicate before 2, 5 and 10 min after each challenge. Values were normalised by expressing A\textsubscript{min} as a percentage decrease in A\textsubscript{min} from baseline for each subject, following which the area under normalised A\textsubscript{min} versus time curve (AUC) was determined. (B) Area under "area under A\textsubscript{min} vs time curve" at time points 1 and 6 hours. Data are means from 5 subjects, displayed as mean ± standard error of the mean. *Significant reduction in the AUC post captopril treatment and challenge with bradykinin (at time points 1 and 6 hours) compared with the reduction in AUC caused by challenge with bradykinin(at time points 1 and 6 hours) (p<0.05, Wilcoxon sign-rank test). *Significant reduction in the AUC caused by bradykinin challenge compared with the reduction in AUC caused by challenge with saline (p<0.05, Wilcoxon sign-rank test). The dotted line corresponds to unchanged nasal patency.
Figure 7.8) A graph showing the change in nasal patency after challenge with histamine, 200 µg, 6.5 hours after receipt of captopril, 12.5 mg, or control followed by repetitive challenges with bradykinin, 250 µg, 6.5 hours after receipt of captopril or control. $A_{\text{min}}$ was measured in triplicate before 2, 5 and 10 min after each challenge. Values were normalised by expressing $A_{\text{min}}$ as a percentage decrease in $A_{\text{min}}$ from baseline for each subject, following which the AUC was determined. Data are means from 5 subjects, displayed as mean ± standard error of the mean. The dotted line corresponds to unchanged nasal patency.
Figure 7.9(A) A graph showing the change in nasal patency after challenge with histamine, 200 μg, at 0, 4, 6 and 8 hours after treatment with saline or PAF, 60 μg. $A_{\text{min}}$ was measured in triplicate immediately prior, 5, 10 and 15 min after each challenge. Values were normalised by expressing $A_{\text{min}}$ as a percentage decrease in $A_{\text{min}}$ from baseline for each subject, following which the area under the normalised $A_{\text{min}}$ versus time curve (AUC) was determined. (B) Area under "area under $A_{\text{min}}$ vs time curve" at time points 4, 6 and 8 hours. Data are means from 7 subjects, displayed as mean ± standard error of the mean. * Significant reduction in the AUC caused by histamine after PAF pre-treatment compared with the reduction in AUC caused by histamine after pre-treatment with saline (p<0.05, Wilcoxon sign-rank test). * Significant reduction in the nasal patency following histamine challenge and pre-treatment with PAF, 60 μg, compared with the reduction in patency caused by challenge with histamine after pre-treatment with saline (p<0.05, Wilcoxon sign-rank test). The dotted line corresponds to unchanged nasal patency.
Figure 7.10) A graph showing the change in nasal patency after challenge with 100 µg of bradykinin and des-arg^{10}-kallidin, 200 µg and 400 µg, 4 hours after saline or PAF, 60 µg, treatment. A_{min.} was measured in triplicate immediately prior, 2, 5, 10 and 15 min after each challenge. Values were normalised by expressing A_{min.} as a percentage decrease in A_{min.} from baseline for each subject, following which the area under normalised A_{min.} versus time curve (AUC) was determined. Data are means from 8 subjects, displayed as mean ± standard error of the mean. *Significant reduction in the AUC caused by challenge with bradykinin post treatment with PAF compared with the reduction in AUC caused by challenge with bradykinin post treatment with saline (p<0.05, Wilcoxon sign-rank test). DAKD is des-arg^{10}-kallidin. The dotted line corresponds to unchanged nasal patency.
Chapter 7 Kinin receptors and airway hyper-reactivity

did not have a significant effect on nasal patency 4 to 5 hours patency after pre-treatment with PAF compared to pre-treatment with saline (figure 7.10).

7.3.4 The effect of unilateral pre-treatment with PAF on contralateral histamine-induced nasal blockage

Part 1: Histamine challenge in the left nostril and PAF pre-treatment in the right nostril

Unilateral challenge of the left nostril with saline did not cause a significant change in the patency of either nostril (figure 7.11). Unilateral challenge of the left nostril with histamine, 200 μg, 6 hours after contralateral pre-treatment with saline caused a significant reduction in the left nostril's patency (p<0.05, Wilcoxon sign-rank test). There was no significant effect on the patency of the unchallenged (right) nasal cavity. Unilateral challenge of the left nostril with histamine 6 hours after contralateral pre-treatment with PAF, 80 μg, did not have a significant effect on the patency of the left nasal cavity. The unchallenged nasal cavity (right nostril) exhibited a significant reduction in nasal patency 6 hours after ipsilateral pre-treatment with PAF, 80 μg (p<0.05, Wilcoxon sign-rank test). The reduction in patency of the unchallenged nasal cavity occurred as early as 2 minutes after contralateral challenge with histamine and peaked at the 10-minute mark (figure 7.12). The reduction in patency of the left nasal cavity occurred as early as 2 minutes after challenge with histamine and began to resolve immediately.

Part 2: Histamine challenge in the right nostril and PAF pre-treatment in the left nostril

Unilateral challenge of the right nostril with saline did not cause a significant change in the patency of either nostril (figure 7.13). Unilateral challenge of the right nostril with histamine, 200 μg, 6 hours after contralateral pre-treatment with saline caused a significant reduction in the right nostril's patency (p<0.05, Wilcoxon sign-rank test).
Figure 7.11) A graph showing the change in nasal patency after challenge of the left nasal cavity with histamine, 200 μg, 6 hours after contralateral treatment with saline or PAF, 80 μg. Amin was measured in triplicate before 2, 5, 10 and 15 min after each challenge. Values were normalised by expressing A_{min} as a percentage decrease in A_{min} from baseline for each subject, following which the AUC was determined. Data are means from 9 subjects, displayed as mean ± standard error of the mean. (R) is challenge or treatment of the right nostril. (L) is challenge or treatment of the left nostril. * Significant reduction in AUC of challenged nostril compared to the AUC of the unchallenged nostril. The dotted line corresponds to unchanged nasal patency.
Figure 7.12) A normalised $A_{\text{min}}$ vs. time graph showing the change in nasal patency of the left and right nasal cavity after challenge with histamine, 200 $\mu$g, in the left nasal cavity and treatment with PAF, 80 $\mu$g, in the right nasal cavity 6 hours earlier. $A_{\text{min}}$ was measured in triplicate before, 2, 5, 10 and 15 min after challenge with histamine. Values were normalised by expressing $A_{\text{min}}$ as a proportion of baseline $A_{\text{min}}$ for each subject. Data are means from 9 subjects, expressed as mean $\pm$ standard error of the mean. The dotted line corresponds to unchanged nasal patency.
There was no significant effect on the patency of the unchallenged (left) nasal cavity. Unilateral challenge of the right nostril with histamine 6 hours after contralateral pre-treatment with PAF, 80 µg, caused a significant reduction in the patency of the right nasal cavity (p<0.05, Wilcoxon sign-rank test). There was no significant effect on the patency of the unchallenged (left) nasal cavity 6 hours after ipsilateral pre-treatment with PAF, 80 µg.

7.3.5 The effect of unilateral pre-treatment with PAF on contralateral bradykinin-induced nasal blockage

Part 1: Bradykinin challenge in the left nostril and PAF pre-treatment in the right nostril

Unilateral challenge of the left nostril with saline did not cause a significant change in the patency of either nostril (figure 7.14). Unilateral challenge of the left nostril with bradykinin, 100 µg, 6 hours after contralateral pre-treatment with saline caused a significant reduction in the patency of the left nasal cavity (p<0.05, Wilcoxon sign-rank test). There was no significant effect on the patency of the unchallenged (right) nasal cavity. Unilateral challenge of the left nostril with bradykinin 6 hours after contralateral pre-treatment with PAF, 80 µg, did not have a significant on the patency of the left nasal cavity. The unchallenged nasal cavity (right nostril) exhibited a significant reduction in nasal patency 6 hours after ipsilateral pre-treatment with PAF, 80 µg (p<0.05, Wilcoxon sign-rank test). The reduction in patency of the unchallenged nasal cavity occurred as early as 2 minutes after contralateral challenge with bradykinin and then slowly began to resolve (figure 7.15). The reduction in patency of the left nasal cavity occurred as early as 2 minutes after challenge with bradykinin and began to plateau at the 5-minute mark.
Figure 7.13) A graph showing the change in nasal patency after challenge of the right nasal cavity with histamine, 200 μg, 6 hours after contralateral treatment with saline or PAF, 80 μg. $A_{\text{min.}}$ was measured in triplicate before 2, 5, 10 and 15 min after each challenge. Values were normalised by expressing $A_{\text{min.}}$ as a percentage decrease in $A_{\text{min.}}$ from baseline for each subject, following which the AUC was determined. Data are means from 9 subjects, displayed as mean ± standard error of the mean. (R) is challenge or treatment of the right nostril. (L) is challenge or treatment of the left nostril. * Significant reduction in AUC of challenged nostril compared to the AUC of the unchallenged nostril. The dotted line corresponds to unchanged nasal patency.
Figure 7.14) A graph showing the change in nasal patency after challenge of the left nasal cavity with bradykinin, 100 µg, 6 hours after contralateral treatment with saline or PAF, 80 µg. A\textsubscript{min} was measured in triplicate before 2, 5, 10 and 15 min after each challenge. Values were normalised by expressing A\textsubscript{min} as a percentage decrease in A\textsubscript{min} from baseline for each subject, following which the AUC was determined. Data are means from 8 subjects, displayed as mean ± standard error of the mean. (R) is challenge or treatment of the right nostril. (L) is challenge or treatment of the left nostril. * Significant reduction in AUC of challenged nostril compared to the AUC of the unchallenged nostril. The dotted line corresponds to unchanged nasal patency.
Figure 7.15) A normalised $A_{\text{min}}$ vs. time graph showing the change in nasal patency of the left and right nasal cavity after challenge with bradykinin, 100 μg, in the left nasal cavity and treatment with PAF, 80 μg, in the right nasal cavity 6 hours earlier. $A_{\text{min}}$ was measured in triplicate before, 2, 5, 10 and 15 min after challenge with histamine. Values were normalised by expressing $A_{\text{min}}$ as a proportion of baseline $A_{\text{min}}$ for each subject. Data are means from 9 subjects, expressed as mean ± standard error of the mean. The dotted line corresponds to unchanged nasal patency.
Chapter 7

Part 2: Bradykinin challenge in the right nostril and PAF pre-treatment in the left nostril

Unilateral challenge of the right nostril with saline did not cause a significant change in the patency of either nostril (figure 7.16). Unilateral challenge of the right nostril with bradykinin, 100 µg, 6 hours after contralateral pre-treatment with saline caused a significant reduction in the right nostril’s patency (p<0.05, Wilcoxon sign-rank test). There was no significant effect on the patency of the unchallenged (left) nasal cavity. Unilateral challenge of the right nostril with bradykinin 6 hours after contralateral pre-treatment with PAF, 80 µg, caused significant reduction in patency of the right nasal cavity (p<0.05, Wilcoxon sign-rank test). There was no significant effect on the patency of the unchallenged (left) nasal cavity 6 hours after ipsilateral pre-treatment with PAF, 80 µg.

7.3.6 The effect of bilateral challenge with PAF on unilateral histamine-induced nasal blockage

Part 1: Histamine challenge in the right nostril

Unilateral challenge of the right nostril with saline did not cause a significant change in the patency of either nostril (figure 7.17). Unilateral challenge of the right nostril with histamine, 200 µg, 6 hours after bilateral pre-treatment with saline caused a significant reduction in the right nostril’s patency (p<0.05, Wilcoxon sign-rank test). There was no significant effect on the patency of the unchallenged (left) nasal cavity. Unilateral challenge of the right nostril with histamine 6 hours after bilateral pre-treatment with PAF, 80 µg, caused a significant reduction in the patency of the right nasal cavity compared to the change in patency caused by challenge of the right nasal cavity with histamine after bilateral saline pre-treatment (p<0.05, Wilcoxon sign-rank test). The unchallenged nasal cavity (left nostril) exhibited a significant reduction in nasal patency 6 hours after ipsilateral pre-treatment with PAF, 80 µg, compared to the change in patency caused by unilateral challenge with saline (p<0.05, Wilcoxon sign-rank test).
**Figure 7.16** A graph showing the change in nasal patency after challenge of the right nasal cavity with bradykinin, 100 μg, 6 hours after contralateral treatment with saline or PAF, 80 μg. A_{min} was measured in triplicate before 2, 5, 10 and 15 min after each challenge. Values were normalised by expressing A_{min} as a percentage decrease in A_{min} from baseline for each subject, following which the AUC was determined. Data are means from 8 subjects, displayed as mean ± standard error of the mean. (R) is challenge or treatment of the right nostril. (L) is challenge or treatment of the left nostril. * Significant reduction in AUC of challenged nostril compared to the AUC of the unchallenged nostril. The dotted line corresponds to unchanged nasal patency.
Figure 7.17) A graph showing the change in nasal patency after challenge of the right nasal cavity with histamine, 200 μg, 6 hours after bilateral treatment with saline or PAF, 80 μg. $A_{\text{min}}$ was measured in triplicate before 2, 5, 10 and 15 min after each challenge. Values were normalised by expressing $A_{\text{min}}$ as a percentage decrease in $A_{\text{min}}$ from baseline for each subject, following which the AUC was determined. Data are means from 7 subjects, displayed as mean ± standard error of the mean. (R) is challenge or treatment of the right nostril. (L) is challenge or treatment of the left nostril. * Significant reduction in AUC of challenged nostril compared to the AUC of the unchallenged nostril. The dotted line corresponds to unchanged nasal patency.
Part 2: Histamine challenge in the left nostril

Unilateral challenge of the left nostril with saline did not cause a significant change in the patency of either nostril (figure 7.18). Unilateral challenge of the left nostril with histamine, 200 µg, 6 hours after bilateral pre-treatment with saline caused a significant reduction in the left nostril's patency (p<0.05, Wilcoxon sign-rank test). There was no significant effect on the patency of the unchallenged (right) nasal cavity. Unilateral challenge of the left nostril with histamine 6 hours after bilateral pre-treatment with PAF, 80 µg, caused a significant reduction in the patency of the left nasal cavity (p<0.05, Wilcoxon sign-rank test). There was no significant effect on the patency of the unchallenged (right) nasal cavity 6 hours after ipsilateral pre-treatment with PAF, 80 µg.
Figure 7.18) A graph showing the change in nasal patency after challenge of the left nasal cavity with histamine, 200 µg, 6 hours after bilateral treatment with saline and PAF, 80 µg. \(A_{\text{min}}\) was measured in triplicate before 2, 5, 10 and 15 min after each challenge. Values were normalised by expressing \(A_{\text{min}}\) as a percentage decrease in \(A_{\text{min}}\) from baseline for each subject, following which the AUC was determined. Data are means from 7 subjects, displayed as mean ± standard error of the mean. (R) is challenge or treatment of the right nostril. (L) is challenge or treatment of the left nostril. *Significant reduction in AUC of challenged nostril compared to the AUC of the unchallenged nostril. The dotted line corresponds to unchanged nasal patency.
7.4 Discussion

PAF-induced airway hyper-reactivity is thought to be dependent on PAF's ability to induce kinin production within the human nasal airway. First because the kinin B₂ receptor antagonists, icatibant and [1-adamantaneacetyl-D-Arg⁶, Hyp⁷, beta-(2-thienyl)-Ala⁵,8-D-Phe⁷]-bradykinin, inhibit PAF-induced airway hyper-reactivity in non-atopic individuals (Turner et al., 2000b) and, secondly because, long-term use of ACE inhibitors, by patients with cardiovascular complications, is associated with an increased occurrence of airway hyper-reactivity and coughing (Overlack et al., 1992; Dicpinigaitis and Dobkin, 1996). Additionally, challenge of unsensitised guinea-pigs with bradykinin induces airway hyper-reactivity to ACh in the lower airways (Omini et al., 1989). However, repetitive challenge of non-atopic human subjects with bradykinin does not appear to induce airway hyper-reactivity to histamine in the nasal airway (Turner et al., 2000b). This was suspected to be due to bradykinins short half-life in the human nose.

Intranasal challenge with bradykinin, 250 μg, caused a significant reduction in the AUC, which is experienced subjectively as nasal blockage (figure 7.7). Re-introduction of bradykinin into the nasal airway 1 and 6 hours after treatment with the control produced a consistent degree of nasal congestion. This implies that there was no tachyphylaxis of the congestive response. Intranasal challenge with bradykinin, 250 μg, one hour after receipt of captopril, 12.5 mg, significantly enhanced the bradykinin-induced congestive response and its effects were still evident a further 5 hours later. However, the reduction in nasal patency caused by challenge with histamine, 200 μg, after repetitive challenge with bradykinin and receipt of captopril was not significantly different from the congestive response obtained in the presence of control (figure 7.8). This finding is in agreement with previous studies performed in the upper and lower human airways. Short-term treatment with the ACE inhibitors, captopril and lisinopril, failed to potentiate airway reactivity to histamine (Overlack et al., 1992; Dicpinigaitis and Dobkin, 1996) and the symptoms of the immediate response in the nasal airway of allergen-challenged atopic individuals (Proud et al., 1990). However, it must be noted that potentiation of bradykinin-induced nasal blockage does not necessarily correspond to an increase in the kinin content of the nasal airway. It has been reported that there are some circumstances, such as organ bath preparations, in which an accumulation of
kinins in response to inhibition of ACE cannot be detected even though there is an icatibant-sensitive potentiation of the response to bradykinin (Busse and Lamontagne, 1991; Hecker et al., 1994). Moreover, reports have shown that captopril potentiates the effects of bradykinin in vitro, at concentrations of bradykinin expected to desensitise kinin B\textsubscript{2} receptors (Hecker et al., 1994). Therefore, it is believed that captopril is also capable of potentiating the actions of kinins in vivo, independent of kinin-accumulation. However, reports have shown that captopril does enhance the recovery of kinins from the nasal airway of allergen-challenged atopic individuals (Proud et al., 1990). Nonetheless, captopril does potentiate the activity of kinins in vivo but this does not appear to alter nasal reactivity to intranasal challenge with histamine.

Intranasal challenge with histamine, 200 µg, caused a significant reduction in nasal patency (figure 7.9A). Re-challenge with histamine 4, 6 and 8 hours after pre-treatment with saline caused a significant congestive response. Intranasal challenge with histamine 4, 6 and 8 hours after pre-treatment with PAF, 60 µg, significantly potentiated the congestive response (figure 7.9B). This is consistent with previous studies on the ability of PAF to induce airway hyper-reactivity in non-atopic individuals (Austin and Foreman, 1993; Turner et al., 2000b).

Pre-treatment with PAF, 60 µg, significantly enhanced the congestive response to bradykinin, 100 µg (figure 7.10). This also concurs with previous investigations (Austin and Forman, 1993). However, des-arg\textsuperscript{10}-kallidin, 200 µg and 400 µg, failed to produce a significant change in nasal patency after pre-treatment with PAF, 60 µg. This suggests that PAF-induced AHR within non-atopic nasal airways is not mediated by the expression or activation of kinin B\textsubscript{1} receptors.

The expression of kinin B\textsubscript{1} receptors in atopic individuals is induced by inflammation of the nasal airway. Mediators such as PAF, which is only detectable in the lavages of allergen-challenged atopic individuals (Touqui et al., 1994) and known to cause inflammation of the nasal airway, may be capable of inducing the expression of functional kinin B\textsubscript{1} receptors in non-atopic airways, as found in rat hind-paws (Fernandes et al., 2003). However, intranasal challenge of atopic individuals with des-arg\textsuperscript{10}-kallidin does not induce any subjective or objective changes in symptom severity, even though the receptors were functional (Christiansen et al., 2002). In
order for kinin B1 receptors to directly cause nasal blockage they need to be expressed on the vascular endothelium. Unfortunately, the cellular location of their expression within the human nose has not been determined.

Intranasal unilateral (left nostril) challenge with saline had no effect on the patency of the left nostril (figure 7.11). Unilateral challenge of the left nostril with histamine, 200 µg, 6 hours after pre-treatment of the contralateral cavity (right nostril) with saline produced significant ipsilateral (left nostril) nasal blockage, with no significant change in patency of the contralateral (right nostril) nasal cavity and a similar result was obtained when bradykinin, 100 µg, was used in the place of histamine (figure 7.14). Previous studies have produced similar findings. Unilateral challenge with histamine consistently causes ipsilateral nasal blockage, which is likely to be due to the activation of local vascular receptors (Kirkegaard et al., 1983; Birchall et al., 1993; Sheahan et al., 2005). However, the response of the contralateral nasal cavity to challenge with histamine appears to vary. A reduction (Shelton et al., 1994), increase (Birchall et al., 1993) and no change (Kirkegaard et al., 1983) in nasal patency have been observed with unilateral challenges with histamine. Therefore it appears that not all histamine’s effects on nasal patency may be exerted locally. This is not inconceivable as receptors for histamine are also situated on nerves within the human nasal airway (Nakaya et al., 2004). In addition to affecting contralateral nasal patency, unilateral challenge with histamine also produces a bilateral increase in nasal secretions (Raphael et al., 1989b; Riccio and Proud, 1996). Nasal lavage fluid of the unchallenged nasal cavity exhibits a significant increase in the production of glandular proteins, indicating that the serous glands were activated by the parasympathetic nervous system, CGRP or sensory neuropeptides (as mentioned in chapter 1.3.1.2.6; 1.3.1.2.7.1; 1.3.1.2.7.4).

Unilateral (left nostril) challenge with histamine, 200 µg or bradykinin, 100 µg, 6 hours after contralateral pre-treatment with PAF, 80 µg, failed to cause statistically significant blockage in the left nasal cavity (figure 7.11; figure 7.14). The reason for this lack of blockage is not known. The PAF-treated (right) nasal cavity exhibited a congestive response only once the contralateral cavity was challenged with histamine or bradykinin (figure 7.11; figure 7.14). It must be noted that PAF, 80 µg, does not cause nasal congestion in the absence of challenge with histamine or bradykinin (Maniscalco et al., 2000). The transient nature of this response suggest
that it may be neuronal in origin (figure 7.12; figure 7.15). This is supported by the fact that intranasal challenge with high doses of PAF causes nasal discomfort which is indicative of C-fibre activation. Alternatively, nasal blockage could be caused by mast cell degranulation in response to the release of tachykinins by C-fibres (Devillier et al., 1989; Austin et al., 1996; Baumgarten et al., 1997). However, receptors for PAF have not been identified on human nasal neurons (Shirasaki et al., 2006) and intranasal challenge with PAF does not cause the production of substance P within non-atopic human nasal airways (Turner et al., 1999).

Unfortunately, these results were not reproducible. When investigation 7.24 and 7.26 were repeated on the same individuals in a cross-over manner, unilateral challenge with histamine or bradykinin caused ipsilateral (right nostril) blockage, while the PAF-treated (left) nostril did not exhibit any change in nasal patency (figure 7.13; figure 7.16).

Previous investigations in which PAF, 60 μg, has been introduced bilaterally into the nasal airway of non-atopic individuals have failed to show any sign of tachyphylaxis (Austin and Foreman, 1993). Therefore, investigation 7.2.4 was repeated but with bilateral pre-treatment with PAF, 80 μg, as opposed to contralateral pre-treatment to determine whether PAF’s effects on the nasal airway were reproducible.

Unilateral challenge (right nostril) with histamine, 200 μg, 6 hours after bilateral intranasal pre-treatment with PAF, 80 μg caused significant bilateral nasal blockage. Histamine-induced reduction of the right nostril’s patency was potentiated by pre-treatment with PAF. This concurs with figure 7.11 and previous studies (Austin and Foreman, 1993; Turner et al., 2000b). The unchallenged (left) nostril also became congested in response to contralateral challenge with histamine, as found in figure 7.11. This implies that PAF’s hyperresponsive-inducing effect on the nasal airway is not nostril selective. However, when this investigation was repeated on the same individuals in a cross-over manner, PAF failed to induce airway hyper-reactivity in the left nostril (which was challenged with histamine) or congestion in the unchallenged (right) nostril in response to contralateral challenge with histamine (figure 7.18).
Chapter 7 Kinin receptors and airway hyper-reactivity

One possible reason for the lack of reproducibility could be tachyphylaxis. Tachyphylactic responses to PAF have previously been reported in animal and human airways (Cuss et al., 1986; Rubin et al., 1987; Kioumis et al., 1988; Roberts et al., 1988; Popovich et al., 1988; Block et al., 1990; Coyle et al., 1990). The reason for its occurrence has not been fully established but it is believed to be platelet-dependent, as PAF-induced contraction of canine tracheal strips only occurs in the presence of fresh platelets and tachyphylaxis of this response is only overcome by replenishment of old platelets (Popovich et al., 1988). Additionally, depletion of functional platelets in vivo with an anti-rabbit platelet serum inhibits PAF-induced airway hyper-reactivity in rabbit lungs (Coyle et al., 1990). This suggests that platelets are not only involved in the onset of tachyphylaxis but they may also be involved in inducing airway hyper-reactivity (Pitchford and Page, 2006). Alternatively, assuming that PAF's airway hyper-reactivity inducing-effects are mediated by sensory neurons, tachyphylaxis may simply occur due to the depletion of neuropeptide stores, as observed with the use of high doses of capsaicin or repetitive stimulation of sensory neurones (Lundblad et al., 1983; Manzini et al., 1993).
7.5 Summary

(1) Intranasal challenge with bradykinin, 250 μg, caused a significant reduction in nasal patency in comparison to the change in patency caused by challenge with saline. Re-challenge with bradykinin, 250 μg, 1 and 6 hours later did not show any sign of tachyphylaxis.

(2) Pre-treatment with captopril orally, 12.5 mg, potentiated bradykinin-induced nasal congestion.

(3) Nasal congestion caused by intranasal challenge with histamine, 200 μg, after 6 hours of repetitive challenge with bradykinin, 250 μg, was not potentiated by pre-treatment with captopril, 12.5 mg.

(4) Intranasal pre-treatment with PAF, 60 μg, significantly potentiated histamine-induced nasal blockage at the 4, 6 and 8 hour time points.

(5) Intranasal challenge with des-arg^{10}-kallidin, 200 and 400 μg, failed to induce a significant change in nasal patency prior or post intranasal pre-treatment with PAF, 60 μg. Intranasal challenge with bradykinin, 100 μg, 4 hours post intranasal pre-treatment with PAF, 60 μg significantly potentiated the congestive response.

(6) Intranasal unilateral challenge with histamine, 200 μg, caused bilateral nasal blockage 6 hours after pre-treatment of the contralateral nasal cavity with PAF, 80 μg. Nasal blockage was only observed in the challenged cavity when the investigation was repeated in a cross-over manner. A similar result was obtained when bradykinin, 100 μg, was used in place of histamine, 200 μg.

(7) Intranasal unilateral challenge with histamine, 200 μg, caused bilateral nasal blockage, 6 hours after intranasal bilateral pre-treatment with PAF, 80 μg. Nasal blockage was only observed in the challenged cavity when the investigation was repeated in a cross-over manner.
CHAPTER 8
GENERAL DISCUSSION AND FUTURE AIMS

Intranasal pre-treatment of non-atopic individuals with the kinin B₂ receptor antagonist, icatibant, dose-dependently inhibits the recovery of kinins from non-atopic individuals intranasally challenged with PAF. The inhibitory effect of icatibant on the recovery of kinins from nasal lavage fluid \textit{in vivo} is extremely potent and is comparable to its ability to antagonise bradykinin-induced nasal blockage \textit{in vivo} (Dear et al., 1996).

In chapter 3, icatibant failed to inhibit PAF-induced albumin production within the nasal airway and the kallikrein inhibitor, aprotinin, also failed to inhibit PAF-induced albumin production, which implies that kinins do not play a major role in the induction of PAF-induced increases in vascular permeability. It also suggests that icatibant is not likely to inhibit PAF-induced kinin production \textit{in vivo} by impeding the influx of plasma proteins which promote kinin synthesis, such as FXII and HMWK, into the nasal airway. Thus, it appears that icatibant’s effect on the recovery of kinins from nasal lavage fluid occurs independently of the activation of vascular kinin B₂ receptors. However, kinin production in rats which have been used to model pancreatitis, by intravenous administration of caerulein, is inhibited by pre-treatment with icatibant (Griesbacher et al., 2003). This was attributed to a reduction of vascular permeability, as icatibant also inhibited the recovery of α₂-macroglobulin (a marker of increased vascular permeability) and kininogen content of rat pancreas.

Reports have shown that pre-treatment of individuals, who suffer from perennial allergic rhinitis, with a non-selective kallikrein inhibitor impedes the congestive response to house dust mite allergen, as well as kinin production (Katori et al., 1996). Pre-treatment of individuals who suffer from perennial allergic rhinitis with the kinin B₂ receptor antagonist, icatibant, also potently inhibits the congestive response to allergen and inhibits the recovery of kinins from the nasal airway (Dear et al., 1996). Consequently, it was speculated that icatibant may act as an inhibitor of kallikrein in addition to being a kinin B₂ receptor antagonist. However, it is evident
from chapter 4 that icatibant is a significantly better antagonist of kinin B₂ receptors than it is an inhibitor of kallikrein. This means it is very unlikely that icatibant inhibits the recovery of kinins from PAF-challenged non-atopic individuals or house dust mite challenged individuals with perennial allergic rhinitis by inhibiting tissue or plasma kallikrein.

The inability to detect an increase in kallikrein-like enzyme activity in the nasal lavage fluid of PAF-challenged non-atopic individuals could be due to the possibility that kallikrein is not responsible for PAF-induced kinin production. Therefore, in chapter 5, the enzyme activity of crude nasal lavage fluid was compared to that of purified tissue and plasma kallikrein. Crude nasal lavage fluid exhibited similar enzyme kinetics and an inhibitory profile to that of human plasma kallikrein, suggesting that the human plasma kallikrein is the enzyme present in crude nasal lavage fluid which is responsible for the cleavage of the chromogenic substrates S-2302 and S-2266. However, this does not necessarily imply that the enzyme present in crude nasal lavage fluid is responsible for PAF-induced kinin synthesis. Confirmation of the identity of the enzyme in crude nasal lavage fluid could be provided by the use of a western-blot and a kininogenase assay (with HMWK) on purified nasal lavage fluid obtained from non-atopic individuals challenged with PAF.

In chapter 6, the effect of icatibant on prekallikrein activation was determined. Icatibant failed to significantly inhibit the activation of prekallikrein in vitro at a dose known to abolish the binding of [¹²⁵I]-icatibant to kinin B₂ receptors of human nasal turbinates (Dear et al., 1996). This makes it unlikely that icatibant inhibits the recovery of kinins from PAF-challenged individuals in vivo by inhibiting the activation of prekallikrein.

As intranasal challenge with PAF causes an increase in vascular permeability, shown in chapter 3, it is quite feasible that plasma kallikrein is responsible for PAF-induced kinin synthesis within the nasal airway of non-atopic individuals. However, the inability of icatibant to inhibit: (1) PAF-induced albumin production (2) the activation of prekallikrein and (3) plasma kallikrein enzyme activity (with high affinity) suggests that there must be another source of kinins within the nasal airway which is sensitive to pre-treatment with icatibant. This other source may be tissue
kallikrein (Poblete et al., 1993; Proud and Vio, 1993). Tissue kallikrein is stored within submucosal glands of the nasal airway and could be released in response to challenge with PAF. However, there are no reports present within the literature to suggest that PAF is capable of doing so and markers of glandular activation are not produced in response to challenge of non-atopic individuals with PAF, as shown in chapter 3. Nonetheless, receptors for PAF have been identified on human nasal submucosal glands (Shirasaki et al., 2005). Alternatively, submucosal glands may be activated by kinins synthesized by plasma kallikrein in response to challenge with PAF. While, kinin B$_2$ receptors are not present on submucosal glands within the human nasal airway but they are present on nerves within the nasal airway (Baraniuk et al., 1990d) and it has been shown that intranasal challenge with bradykinin causes glandular activation, via the parasympathetic nervous system, in hyper-responsive individuals (Riccio et al., 1996). If this theory holds true, then it is likely that PAF-induced kinin production is predominantly synthesised by tissue kallikrein which is released in response to indirect innervation of submucosal glands (by the sensory-parasympathetic reflex). This theory could be tested by determining whether SBTI, an inhibitor of plasma kallikrein, but not tissue kallikrein, inhibits the recovery of kinins from PAF-challenged non-atopic individuals. Unfortunately, this investigation could not be performed for ethical reasons. Additionally, it would be interesting to determine whether intranasal challenge of non-atopic individuals with PAF (and atopic individuals with allergen) causes the release of tissue kallikrein (identified western blotting and a kininogenase assay) into the nasal airway as well as determine whether its release is sensitive to pre-treatment with icatibant.

The ability of icatibant to inhibit PAF-induced kinin production and AHR suggests that kinins may play a role in the induction of AHR. However, repetitive intranasal challenge of non-atopic individuals with bradykinin does not alter nasal responsiveness to challenge with histamine and there is no sign of tachyphylaxis to bradykinin-induced congestion, as shown in chapter 7. This remains true even in the presence of an ACE inhibitor, as shown in chapter 7.

Two possible explanations for bradykinins inability to induce AHR to histamine in vivo are firstly, that it is not responsible for the development of AHR, it may simply be responsible for the maintenance of AHR while another mediator, such as PAF, initiates the hyper-reactive response or secondly, bradykinin is not the only
mediator responsible for initiating AHR, it may need to work in conjunction with another mediator to induce airway AHR.

As shown in chapter 7, unilateral challenge with histamine (or bradykinin) results in bilateral nasal blockage when the contralateral nasal cavity is pre-treated with PAF. However, the inability to reproduce these results in individuals which have already been treated with PAF suggests these results are questionable. One possible reason for the lack of reproducibility could be tachyphylaxis. An ideal experiment to address this possibility would be to reproduce these investigations 7.2.4 – 7.2.6 in an unpaired, as opposed to paired, manner.

In conclusion this study has shown that PAF induces kinin production within the human nasal airway which is impeded by pre-treatment with the high affinity kinin B₂ receptor antagonist icatibant. Multiple enzymes appear to be involved in the production of kinins within the nasal airway, one of which is plasma kallikrein. This study has also shown that icatibant does not inhibit the enzyme activity of tissue or plasma kallikrein potently enough to suggest that it may play a role in relief of symptoms of nasal allergy in vivo. Further characterisation of the effects of PAF in vivo would aid the understanding of how PAF induces AHR and kinin production within the human nasal airway, as well as how icatibant impedes these effects.
CHAPTER 9

REFERENCES


DYKEWICZ MS (2003). Rhinitis and sinusitis. Journal of Allergy and Clinical Immunology, 111: S520- S529.
Chapter 9 References


JOSEPH K, GHEBREHIWET B & KAPLAN AP (1999). Cytokeratin 1 and gC1qR mediate high molecular weight kininogen binding to endothelial cells. *Clinical Immunology*, 92(3): 246-55.


MOSIMANN BL, WHITE MV, HOHMAN RJ, GOLDRICH MS, KAULBACH HC & KALINER MA (1993). Substance P, calcitonin gene-related peptide, and vasoactive
intestinal peptide increase in nasal secretions after allergen challenge in atopic patients. *Journal of Allergy and Clinical Immunology*, 92(1 Pt 1): 95-104.


PAGE JD, YOU JL, HARRIS RB & COLMAN RW (1994). Localization of the binding site on plasma kallikrein for high-molecular-weight kininogen to both apple 1 and apple 4 domains of the heavy chain. *Archives of Biochemistry and Biophysics*, 314(1): 159-64.


276


PROUD D, TOGIAS A, NACLERIO RM, CRUSH SA, NORMAN PS &


REFERENCES


280


Immunopharmacology, 33(1-3): 51-60.


VAN MEGEN YJ, KLAASSEN AB, RODRIGUES DE MIRANDA JF, VAN GINNEKEN CA & WENTGES BT (1991b). Alterations of muscarinic acetylcholine receptors in the nasal mucosa of allergic patients in comparison with nonallergic individuals. *Journal of Allergy and Clinical Immunology*, 87(2): 521-529.


