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Acid-sensing ion channel receptor-3 expression in nasal mucosa and its role in allergic rhinitis

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Acid-sensing ion channel receptor-3 expression in nasal mucosa and its role in allergic rhinitis.

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Thesis submitted to The National University of Ireland (Royal College of Surgeons in Ireland) for the degree of Doctor of Medicine (MD).

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DECLARATION

I declare that the content of this thesis is the work of S.Guan Khoo, which I submit to the National University of Ireland (RCSI) for the consideration of the award of the MD higher research degree.

I declare that this work is my own personal effort, and no other degrees have been conferred to me on the basis of this work.

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S.Guan Khoo

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TABLE OF CONTENTS

		Page No.
Tables and Figures		9
Abstract		11
Abbreviations		13
CHAPTER 1 – INTRODUCTION		15
1.1	Nasal anatomy	16
1.2	Nasal physiology	17
1.3	The nasal cycle	19
1.4	The nasal epithelium	20
1.5	The nerve supply of the nose	21
1.6	Allergic rhinitis – definition	24
1.7	Allergic rhinitis – classification	25
1.8	Diagnosis of allergic rhinitis	26
1.9	Pathology of allergic rhinitis – the IgE	29
1.10	Neuropathology in rhinitis	32
1.11	Nerve activation as a mechanism in AR and neural hyper responsiveness.	33
1.12	Molecular pathophysiology of allergic rhinitis	37
1.13	The eosinophil in AR	45

CHAPTER 2 - ACID-SENSING ION CHANNELS 49

2.1 Acid-sensing ion channels (ASIC); structure and function 50

2.2 Structure of ASICs 54

2.3 Activation of ASICs 56

2.4 Role of ASICs 56

2.5 Lactic acid and its effect on acid-sensing ion channels 59

2.6 ASICs antagonism by amiloride 59

2.7 ASICs in respiratory mucosa 60

2.8 Hypothesis 62

CHAPTER 3 - MATERIAL AND METHODS 63

3.1 Beaumont Hospital Scientific Committee ethical approval 64

3.2 Patient selection 64

3.3 General materials 67

3.4 Reagent materials 68

3.5 Diagnostic skin prick allergy tests 73

3.6 Physiological nasal secretion collection and nasal challenge with isotonic saline and amiloride 76

3.7 Physiological nasal secretion collection and nasal challenge with lactic acid and amiloride 85

3.8 Inferior turbinate nasal biopsies 86

3.9 Purification of total RNA from human tissue 89

3.10 Elimination of genomic cDNA 91

3.11	Reverse transcription of cDNA	91
3.12	Polymerase chain reaction and purification of cDNA for standards	92
3.13	ASIC receptor gene quantification with polymerase chain reaction	93
3.14	ASIC receptor localisation with immunohistochemical staining	97
3.15	ASIC receptor protein identification with Western Blotting	98
3.16	IMR32 Cell cultures	101
3.17	Cell Culture: In vitro experiments with eosinophil peroxidase in co-culture with 16HBE14o cells	102
3.18	Visualization of ASIC-3 expression by epithelial cells	103
3.19	Statistical analysis and data interpretation	104
3.20	Measurement of airway surface liquid height in response to eosinophil peroxidase	105

CHAPTER 4 – RESULTS 106

4.1	Secretory responses to topical application of lactic acid	107
4.2	Real time PCR of inferior nasal turbinate biopsies	112
4.3	Immunohistochemical localisation of ASICS-3 in the nasal epithelium	115
4.4	Immunhistochemical localization of ASIC-3 to nerves	117
4.5	Induction of ASIC-3 expression by eosinophil peroxidase	119
4.6	Increased ASL in response to eosinophil peroxidase	122

CHAPTER 5 – DISCUSSION AND CONCLUSION 124

5.1 Discussion 125

5.2 Conclusion 133

APPENDICES 134

Appendix 1 Beaumont Hospital Ethical Approval 134

Appendix 2 Physiological secretions – Lactic acid and amiloride, ipsilateral measurements 135

Appendix 3 Physiological secretions – Lactic acid and amiloride, contralateral measurements 139

Appendix 4 ASIC-3 quantification 143

Appendix 5 ASIC-3 quantification graphically expressed 144

Appendix 6 ASIC-1 and ASIC-2 quantification 145

BIBLIOGRAPHY 146

TABLES AND FIGURES

Tables

Table 1	Inclusion / exclusion criteria for enrolment into the Allergic Rhinitis group	66
Table 2	Diagnostic skin prick test allergens	75
Table 3	Primers used in this study	94

Figures

Figure 1	Mast cells in the lumen of a patient with AR	39
Figure 2	CD3 positive T cell in the nasal mucosa	43
Figure 3	MBP positive eosinophils in the nasal mucosa	48
Figure 4	Model of ENaC/DEG channel complex	53
Figure 5	Structure of ASIC subunit	55
Figure 6	Structural design of clinical physiological nasal secretion weight collections	78
Figure 7	Nasal secretion weight collections following nasal challenge with isotonic saline or lactic acid	82
Figure 8	Nasal secretion weight collections following amiloride pre-treatment of nasal cavity, and following nasal challenge with isotonic saline or lactic acid	84

Figure 9	Inferior turbinate nasal mucosal biopsies	88
Figure 10	Distribution of biopsies for PCR and immunohistochemical staining	89
Figure 11A-D	Lactic acid induces a significant increase in secretion in the ipsilateral nasal mucosa	109
Figure 12A-B	Airway expression of the mRNA for ASICS 1-3 quantified by real time PCR	113
Figure 13	There is higher expression of ASIC-3 mRNA in patients with allergic rhinitis compared to controls	114
Figure 14A-D	Immunohistochemical localization of ASIC-3	116
Figure 15A-C	Immunohistochemical localization of neural ASIC-3	118
Figure 16	Induction of ASIC-3 is mediated by eosinophil peroxidase	120
Figure 17	CuFi-1 epithelial cells showing evidence of apical induction of membrane bound ASIC-3	121
Figure 18	Increased airway surface liquid in response to eosinophil peroxidase	123

ABSTRACT

Allergic rhinitis affects a large proportion of the worldwide population, and its constituent symptoms include nasal blockage, rhinorrhoea and nasal pruritus. Tissue acidosis is a component of inflammation and ischaemia, and this occurs in inflammatory conditions, such as that found in the mucosa of patients with allergic rhinitis.

Acid sensing ion channels, ASICs, are a family of ligand-gated cation channels, activated by acid (pH 7.4 – 5.5), which belong to the amiloride-sensitive degenerin/epithelial Na+ channel (ENaC) superfamily. Stimulation of these receptors on nerves leads to a variety of sensations including pain and mechano-perception, while epithelial expression linked to airway secretion has been reported in cystic fibrosis cell lines. A description and function of these receptors in human upper airways has not been described.

Our aim was to determine if the acid-sensing ion channel receptors, specifically type 3 (ASIC-3), are present, upregulated, and functional, in the nasal mucosa of patients with allergic rhinitis, and if they play a role in the allergic rhinitis constituent symptom of rhinorrhoea.

Eosinophils are almost always found in the mucosa, the submucosa and in the nasal secretions of subjects with allergic rhinitis. We hypothesised that the eosinophil has a major role to play in inflammatory conditions such as allergic rhinitis, and may have a role in acting via ASIC receptors, inducing them, or mediating them to react.

Our findings, as shown through real time polymerase chain reaction quantification of ASIC receptors in nasal biopsies, demonstrated no mRNA for ASIC-1 or ASIC-2. ASIC-3 was seen in both healthy controls and allergic rhinitis, in whom it was significantly increased ($p<0.02$, $n = 11$). Immunohistochemistry demonstrated the presence of ASIC-3 receptors on the apical surface of epithelial cells, mucous glands and nerve cells of both control and allergic rhinitis subjects, but expression was increased in the latter.

Functional experiments with lactic acid (pH 7.0) demonstrated significantly increased nasal secretary responses on the ipsilateral but not contralateral side, which were blocked by amiloride (1mM).

Eosinophils and released major basic protein (MBP) were found in association with airway nerve cells, mucous glands and epithelial cells. Eosinophil granule proteins increased the expression of ASIC-3 and showed a significant upregulation of ASIC-3 in an ERK-kinase dependent manner.

Thus, functional ASIC-3 receptors are present in nasal mucosa, and their expression is upregulated in allergic rhinitis via released eosinophil granule proteins. This data indicate a link with tissue inflammation, which may lead to enhanced nasal secretion in allergic rhinitis.

ABBREVIATIONS

AR	Allergic rhinitis
ARIA	Allergic rhinitis and its impact on asthma
ASIC	Acid-sensing ion channel
ASL	Airway surface liquid
CGRP	Calcitonin gene-related peptide
CFTR	Cystic fibrosis transmembrane receptor
DRASIC	Dorsal root acid sensing ion channel
ECP	Eosinophilic cationic protein
ENaC	Epithelial sodium channel
EPO	Eosinophil peroxidase
ERK	Extracellular-related kinase
GM-CSF	Granulocyte/macrophage colony stimulating factor
ICAM-1	Intracellular adhesion molecule 1
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IL	Interleukin
ITR	Immediate-type reactions
LPR	Late-phase responses
MAP	Mitogen-activated protein
MBP	Major basic protein
MPO	Myeloperoxidase

NANC	Non-adrenergic non-cholinergic nerves
S1P	Sphingosine-1-phosphate
SEM	Standard error of mean
TNF-α	Tumour necrosis factor alpha
TRPV1	Transient vanilloid receptor subtype V1
VCAM	Vascular cell adhesion molecule 1
WHO	World Health Organisation

CHAPTER 1 – INTRODUCTION

1.1 Nasal anatomy:

The nasal cavity extends from the nostrils anteriorly, to the posterior nasal apertures and then onto the nasopharynx. The nasal cavity is bound below by the hard palate, which separates the nasal cavity from the oral cavity. It is bound above by the cribriform plate of the ethmoid bone and body of the sphenoid bone, separating it from the cranium. The septum forms the central strut or scaffolding and separates the two nasal airways (Jones N, 2001). This divides the nasal cavity into clearly distinct right and left nasal cavities, and is a partly cartilaginous and partly bony partition (McMinn RMH, 1990). The separation of the nasal cavity is important as it is used to discriminate reflex responses from local responses (Sheahan P, 2006) and it is the basis of some work in this thesis.

The lateral wall of each nasal cavity is marked by three longitudinally oriented projections extending from anterior to posterior, one above another, called the inferior, middle, and superior turbinates. These turbinates are composed of bone covered by mucosa. They extend medially and downwards, and may occupy a significant volume of the nasal cavity. The inferior turbinate is the largest of the three and may occasionally extend as far as the nasal septum medially, and the nasal floor inferiorly. The minimum cross-sectional area of the nose is commonly found in the region of the anterior portion of the inferior turbinate. This is the nasal valve area and is the area of maximal resistance in the nasal airway. It is formed by the overlap between the upper and lower lateral cartilages, the inferior turbinate and the septum (Jones N, 2001). The space below and lateral to the

inferior turbinate is known as the inferior meatus, and receives the opening of the nasolacrimal duct.

The middle turbinate is smaller than the inferior turbinate. A narrowing in nasal cross-sectional area is usually found in the region of the head of the middle turbinate; however, the cross-sectional area here is in most cases much greater than that near the head of the inferior turbinate. The space below and lateral to the middle turbinate (the middle meatus) receives the openings of the maxillary, frontal, and anterior ethmoid sinuses (Dykewicz MS, 2003).

The superior turbinate is smaller than either the inferior or middle turbinates. The posterior ethmoid sinuses drain into the superior meatus, which is below and lateral to the superior turbinate (McMinn 1990). The sphenoid sinus drains via its own separate ostium into the nasal cavity posterosuperiorly.

1.2 Nasal physiology

The primary functions of the nose are associated with defensive and homeostatic mechanisms of the body (Sarin S, 2006), and the nose plays this role by cleaning and humidifying inhaled air (Baraniuk JN, 2008). These functions encompass olfaction, air conditioning resonance, and trapping of airborne particles, whilst the inferior, middle, and superior turbinates in the nasal cavity promote air filtration, humidification, and temperature regulation (Dykewicz MS, 2003).

Warming, humidification, and conditioning of inspired air are enhanced by blood flow through the submucosal capacitance vessels. Expansion of these vessels results in increased blood flow, which leads to increased heat exchange, as well as

swelling of the nasal lining, which enhances contact between the mucosa and inspired air (Hilberg O, 2002).

These processes are under strict physiological control, which depends on local mucosal feedback systems, and sensory and autonomic reflexes (Baraniuk JN, 2008).

The functional structures of the nasal cavity are the walls of the anterior nasal vestibule, inferior, middle and superior turbinates, and the regulatory engine of the nasal mucosa (Baraniuk JN, 2008). The considerable surface area of the mucosa overlying the turbinates render the nose ideally suited to fulfilling these roles.

Other physiological functions of the nose include sensation, immunology, mucociliary clearance, and regulation of the nasal cycle and airflow dynamics (Jones N, 2001). As air enters the nose during inspiration, it encounters two areas of narrowing: one at the internal nasal valve, near the junction of the nasal septum and the upper lateral nasal cartilages, and one in the region of the head of the inferior turbinate. These areas of narrowing convert the laminar flow of air into a turbulent flow, thus maximizing contact between the inspired air and the nasal mucosa.

A thin layer of mucus overlies the nasal epithelium. This mucus blanket functions to trap dust and other inspired particles, thus preventing them from reaching the lower airway. The mucus dynamically moves trapped matter via ciliary action to

the nasopharynx (Hilberg O, 2002) (Dykewicz MS, 2003). This is termed mucociliary clearance, and allows maintenance of the health and defence of the nose (Jones N, 2001) (Nathan RA, 2005).

Any defects in mucociliary clearance may promote mucus retention within the nasal cavity, thus reducing nasal airflow. Mucociliary function can be assessed by measures such as the saccharine test, which is in part subjective, as well as more objective tests such as the measurement of ciliary beat frequency and ciliary ultrastructure (Nathan RA, 2005).

This mucus fulfils the two roles of trapping and transporting airborne particles. Particles in the turbulent inhaled airstream stick onto nasal mucus, which lines the nasal cavity. Eighty percent of particles larger than 12.5μM are filtered from the air through this blanket of mucus, which is then moved by coordinated waves of cilia, from the front of the nose to the nasopharynx (Jones N, 2001). Here, the mucus blanket can be swallowed or expectorated.

It is thought that inflammatory mediators bring about improvement in mucociliary transport and an increase in ciliary beat frequency during an allergic nasal reaction. Experiments on patients with allergic rhinitis with allergen challenge have proven inconclusive however, in terms of clearly showing an increase in mucociliary transport (Jones N, 2001).

1.3. The nasal cycle

Airflow through the two nasal cavities is normally asymmetrical as a result of spontaneous changes in nasal airway patency, which are often reciprocal in nature.

These resulting cyclic fluctuations in nasal patency constitute the nasal cycle (Hanif J, 2000). In the "classical", or "ideal" nasal cycle, alternating, reciprocal changes in nasal patency are seen, with a periodicity ranging from 30 minutes to several hours (Gungor A, 1999) (Huang ZL, 2003). However, this classical pattern, characterized by true reciprocity and periodicity, is seen in only a minority of subjects (Gungor A, 1999) (Hanif J, 2000) (Huang ZL, 2003). On the other hand, spontaneous fluctuations in nasal patency, without contralateral reciprocity or a regular period, are seen in most individuals (Gungor A, 1999) (Hanif J, 2000) (Huang ZL, 2003).

These changes in nasal cycle are potentially important in the variations within subjects today, as seen in my study. This will be discussed later.

1.4 The nasal epithelium

The ciliated epithelium overlies a basement membrane and submucosa. The submucosa consists of serous and seromucous glands, nerves, vasculature, and other cellular elements (Dykewicz MS, 2003).

The nasal cavity is lined throughout by a ciliated pseudostratified columnar epithelium, except superiorly, near the cribriform plate (the olfactory area) and partly on the superior and middle turbinates (Jones N, 2001), where specialised olfactory epithelium is found; and at the very anterior part of the nasal cavity (the nasal vestibule), anterior to the limen nasi (the groove between the upper and lower lateral nasal cartilages), which is lined by skin (McMinn RMH, 1990).

In terms of nasal mucosa in inflammatory conditions such as allergic rhinitis, it is known that bradykinin plays a role as an important inflammatory mediator (Shirasaki H, 2009). Immunohistochemical studies have shown immunoreactivity for both B1 and B2 bradykinin receptors, in epithelial cells, submucosal glands, fibroblast, vascular smooth muscle, vascular endothelial cells, and macrophages. The B2 receptor expression was found in peripheral nerve fibers, unlike B1 expression, which was not observed in nerves (Shirasaki H, 2009).

1.5 The nerve supply of the nose

Sensory innervation of the nose arises from the olfactory nerve, the ethmoidal nerve, and the maxillary branches of the trigeminal nerve (Baraniuk JN, 1990). The bulk of the sensory nerve supply enters the nose via the sphenopalatine foramen in the nasopalatine nerve. The anterior ethmoidal nerve provided by the ophthalmic division of the trigeminal nerve provides some sensory supply to the nasal cavity.

Sensory nerves transmit signals from the mucosa causing pruritus and the motor reflexes of sneezing, and act on the sympathetic and parasympathetic reflexes that affect secretions and increased vascularity of the nasal mucosa (Sarin S, 2006).
Nasal blood flow is controlled by vasoconstricting sympathetic nerves and vasodilating nitrergic (whereby transmission is mediated by nitric oxide) nerves (Ogawa F, 2010). Stimulation of the sympathetic nerves is known to decrease the nasal blood flow in rats. In the absence of nerve stimulation, the nasal blood flow

is regulated predominantly by sympathetic nerves with vasoconstriction, rather than nitrergic nerves with vasodilation (Ogawa F, 2010).

All nasal symptoms of rhinitis can be triggered through neural pathways which can be chronically upregulated in the presence of inflammation. Stimulation of these nerves results in release of neuropeptides, causing vasodilatation, increased permeability, glandular activation and activation of other immune cells of inflammation.

The parasympathetic innervation of the nose derives from the facial nucleus of the brain stem and the superior salivatory nucleus (Sarin S, 2006). In terms of the secretomotor nerve supply, preganglionic parasympathetic nerves traverse through branches of the facial nerve. These nerves reach the pterygopalatine ganglion via the greater superficial petrosal nerve. They are joined by postganglionic sympathetic fibres from around the internal carotid artery which have relayed in one of the cervical sympathetic ganglia. The greater superficial and deep petrosal nerves reach the pterygopalatine ganglion via the nerve of the pterygoid canal. Preganglionic fibres of the greater superficial petrosal nerve and vidian nerve synapse at the sphenopalatine ganglion, with postganglionic fibres following the posterior nasal nerve to innervate serous and mucous glands.

Parasympathetic fibres synapse in the ganglion, while sympathetic fibres pass through or around these ganglia. From there, the postganglionic nerves travel to the nasal cavity in the nasopalatine nerve (Konno A, 1979). In effect this means that the local nerves of the parasympathetic division have small post ganglionic

branches which may be better able to adapt to local changes, a term called neural plasticity.

Sympathetic nerves supply blood vessels, leading to vasoconstriction and hence decongestion by releasing noradrenaline, which acts on α-receptors (Riechelmann H, 1994). Stellate ganglion blockade prevents the decrease in nasal mucosal blood flow produced by peripheral cold provocation, demonstrating the role of the sympathetic nervous system in decongestive reflexes. Parasympathetic nerves supply nasal glands, causing glandular secretion, by releasing acetylcholine, which acts on muscarinic M_3 receptors (Kaliner MA, 1992) (Okayama M, 1993) (Nakaya M, 2002).

In addition to the well defined sympathetic and parasympathetic nerves, a poorly described, as far as humans nerves are concerned, loosely termed non-adrenergic non-cholinergic (NANC) nerve supply also exists. These may play a role in diverse aspects of nasal function including recognition and response to irritant chemicals, pH changes and locally produced inflammatory mediators. Activation of these nerves may lead to responses such as pain, nasal secretion, itch and a sense of blockage and congestion (Sarin S, 2006). This thesis is focussed in part on understanding the role of these nerves.

1.6 Allergic rhinitis: definition

Allergic rhinitis is clinically defined as an immunolobulin-E mediated inflammation of the mucosal lining of the nose (Bousquet J, 2008), induced after allergen exposure via an IgE-mediated immune response (Bousquet J, 2004). This inflammation leads to the accumulation of eosinophils and T-lymphocytes (Kramer MF, 2004). This results in 3 cardinal symptoms of sneezing, nasal obstruction, and mucous discharge, in association with rhinorrhoea, nasal pruritus, and nasal congestion (Kramer MF, 2004) (Sheahan P, 2005).

By definition, symptoms should occur during two or more consecutive days, lasting greater than one hour on most days, and should be reversible spontaneously or with treatment. Both allergic rhinitis and asthma constitute systemic inflammatory conditions and often co-exist.

Allergic rhinitis is by far the most frequent manifestation of atopy (45%) (Kramer MF, 2004), and affects 15-25% of the population of the western world. The prevalence of allergic rhinitis has seen an increase over the past 50 years, with a prevalence of 3.5% each decade (van Aas K, 1997). Although the reasons for the increasing prevalence are not clear, and increasing prevalence is seen most commonly in developing countries, AR appears to be somehow associated with the Western lifestyle. Causes and risk factors for the disease can be suitably identified, be they indoor or outdoor allergens, and even exposure to occupational agents.

There is common acceptance that allergic rhinitis has a significant impact on quality of life, impairing both work and school performance and has an economic impact through medication expenses and lost time at work (Juniper EF, 1994) (Malone DC, 1997). In fact, it accounts for up to an estimated 3% of all medical visits (Fornadley JA, 1996).

1.7 Allergic rhinitis: classification

The classification and update of allergic rhinitis and its management was concluded in 1999, during a World Health Organisation (WHO) workshop. Suggestions for improvement and standardisation were tabled by experts in the field, under the Allergic Rhinitis and Its Impact on Asthma (ARIA) forum. This was based on all available literature evidence up till December 1999.

The similarities between lung and nasal mucosa highlighted the 'one airway, one disease' concept. Over 85%-95% of asthmatics have rhinitis (Togias AG, 2000), and conversely, 10-40% of patients with rhinitis have asthma. Hence, pathologic findings in the nose may also apply to asthma.

Allergic rhinitis was newly classified as intermittent or persistent, as opposed to seasonal, perennial, and occupational. This is a new classification, and not synonymous with the old classification. Severity was classified as mild or moderate/severe, emphasizing its impact on quality of life.

In intermittent allergic rhinitis, symptoms are present for either fewer than 4 days per week, or for less than 4 consecutive weeks; whereas in persistent allergic

rhinitis, symptoms are present for more than 4 days per week, and for longer than 4 consecutive weeks (Bachert C, 2002).

Mild allergic rhinitis is characterised by normal sleep, no troublesome symptoms, no impairment of daily activities, and no impairment of school or work performance. Moderate-severe allergic rhinitis is characterised by abnormal sleep or presence of troublesome symptoms or impairment of daily activities or impairment of school or work performance (Bachert C, 2002).

When comparing the new classification to the old, Sheahan et al showed good correlation of patients' symptomatic and secretory responses with the new ARIA classification, and felt that it was easier to use and more inclusive of commonly encountered patients than the traditional classification into seasonal and perennial forms (Sheahan P, 2007)

1.8 Diagnosis of allergic rhinitis:

The diagnosis of allergic rhinitis rests on the combination of a positive history of allergic symptoms, and diagnostic tests confirming that these symptoms are due to allergy (Fornadley JA, 1996) (Dykewicz MS, 2003). These diagnostic tests are based on the identification of a positive IgE-mediated skin prick test or serum-specific IgE. The skin prick test has the best positive predictive value for allergy of 48.7% (Tschopp JM, 1998). Allergic symptoms include sneezing, nasal pruritus, rhinorrhoea, and nasal congestion, and are reversible spontaneously or with treatment. There may be a temporal association between exposure to allergens and the onset of symptoms.

Diagnostic tests to confirm that the symptoms are due to allergy are directed towards the detection of free or cell-bound allergen-specific IgE antibodies. This may be achieved either through in-vivo skin testing on in vitro testing, via blood tests (Fornadley JA, 1996). Despite specific IgE antibodies to environmental allergens playing a key role in allergy, their presence, as evidenced by skin tests or in vitro tests, indicates sensitization, but it does not directly imply the occurrence of clinical allergy (Fuiano N, 2010).

The usual technique of skin testing is skin-prick testing. Skin-prick testing has superior sensitivity and specificity to skin scratch testing, and is less time and resource consuming than intradermal dilution testing, a technique which involves multiple intradermal injections of serially diluted antigen samples (Fornadley JA, 1996).

A drop of antigen is placed on the skin and a lancet needle passed through the droplet. A relatively controlled quantity of antigen can thus delivered into the dermal layer, with the distance placed on the skin reproducible each time (Fornadley JA, 1996). The advantages of skin prick testing are that it demonstrates an actual response in the patient's skin unlike in-vitro testing, which only demonstrates the presence of IgE antibodies. It is rapid and reasonably straightforward, and is highly sensitive, and allows for a wide antigen selection (Fornadley JA, 1996).

The disadvantages are that it is not suitable for children or uncooperative patients, patients with dermatographia or extensive dermatitis, or patients who are very allergic and in whom anaphylaxis is a possible risk. In addition, patients must

discontinue anti-histamines, as these may interfere with skin prick testing. Reports suggest rates of 10-20%, to up to 50% rates of negative test reactions to histamine, following different antihistamine usage (dos Santos RV, 2009).

In-vitro serum tests involve detection of allergen-specific IgE in the serum of patients. The measurement of total IgE is of little predictive value in allergic rhinitis. However, the measurement of allergen-specific IgE is of importance, and is of similar value to skin tests (Bachert C, 2002). The advantages of in-vitro testing are that there is no risk of anaphylaxis, it is suitable in uncooperative patients, and it is not affected by antihistamine treatment (Fornadley JA, 1996). However, it is expensive, and results may take several weeks. In addition, caution has to be exercised when interpreting the results of in-vitro tests, as the presence of allergen-specific IgE is not in itself sufficient to diagnose allergic disease. The demonstration that the symptoms are due to the results of IgE mediated inflammation is not possible by in-vitro testing. Thus the assessment by the physician of the clinical picture is necessary (Fornadley JA, 1996).

Another test that may be of use in future studies is the analysis of specific IgE in the nasal mucosa of patients with allergic rhinitis (Fuiano N, 2010). It is thought that some patients with rhinitis and negative skin test results may have IgE only in the nasal mucosa, probably due to local production. Techniques for measurement of nasal IgE are not routinely available. The test involves placing an allergen-coupled cellulose derivative onto a 2-hole applicator strip covered with a permeable membrane (to avoid adhesion of nasal mucosa to the substrate) and

then positioning the strip in the upper tract of the internal ostium for 10 minutes. Results are read as a colorimetric reaction and expressed on a scale of 0 (negative) to 4 (highly positive) using a calibration curve (Fuiano N, 2010). The absence of specific IgE in the nasal mucosa could account for the absence of rhinitis symptoms in most individuals sensitized to aeroallergens (Fuiano N, 2010).

Other useful assessment tests include nasal acoustic tests to detect changes in nasal cavity size, which may reflect nasal congestion. In acoustic rhinometry, a sound impulse is generated and transmitted through a tube of known dimensions into the nostrils. The reflected echoes are analysed, with the size and pattern of the reflections indicative of the structure and dimensions of the nasal airways (Nathan RA, 2005). A major advantage of acoustic rhinometry over other methods, such as rhinomanometry, is that it is a simple technique to use and requires minimal patient cooperation (Nathan RA, 2005).

Measurement of biomarkers in exhaled condensate, such as adenosine, (Vass G, 2006) have been attempted but they too have validation issues which have limited their usefulness, and as such, shall not be discussed in detail in this thesis.

1.9 Pathology of Allergic Rhinitis – The IgE

Allergic rhinitis is manifested through the interaction of cells and their mediators with cytokines, chemokines, and adhesion molecules which are coordinated in a complex network to produce local inflammation at a molecular biological level. Allergy is caused in part by IgE over-production (Watanabe M, 2008). This may be in response to common environmental antigens, food and other allergens.

Common allergens include proteins and glycoproteins in airborne dust mite faecal particles, cockroach residues, animal danders, molds, and pollens (Fornadley JA, 1996) (Dykewicz MS, 2003). Allergy can however, occur even in the absence of a specific allergen. In fact, recent studies would suggest that it may be conceivable that high levels of IgE may persistently stimulate mast cells and/or basophils to release inflammatory mediators before exposure to allergens and may modify or reinforce the subsequent allergic reactions evoked by allergen challenge (Watanabe M, 2008).

IgE itself is a product of complex interactions between mast cells, B-cells, T-cells and basophils, and is present in lymphoid tissue, nasal and bronchial mucosa. It is the aggregation of receptor-bound IgE molecules on exposure to specific allergens that results in the production and action of mediators such as histamine and leukotrienes, which in turn cause the allergic response of allergic rhinitis. IgE evokes inflammation via the activation of mast cells, which then degranulate chemical mediators, such as histamines and prostaglandins, by the interaction of IgE and allergen (Watanabe M, 2008). This results in the immediate-type reactions (ITR), such as the wheal-and-flare skin response in skin prick tests. This corresponds to the immediate phase of allergic rhinitis, which is characterized by the symptoms of sneezing, nasal discharge and nasal congestion (Okubo K, 2008). The late-phase responses (LPR) occur hours after the ITR. Late phase responses are characterized by a cellular infiltrate comprised of lymphocytes, neutrophils, and eosinophils. The LPR is also a mast cell-dependent reaction that is probably

initiated by mast cell-derived cytokines, such as TNF-α and IL-4, and possibly by several chemokines. The cytokines stimulate the expression of cell adhesion molecules on dermal endothelial cells, and the chemokines mediate the chemo-attraction of effector cells (Watanabe M, 2008). Nasal congestion is the major complaint in the delayed phase of allergic rhinitis (Okubo K, 2008).

Eosinophils are almost always found in the mucosa, the submucosa and in the nasal secretions of subjects with AR (Klemens C, 2007). Mast cells are present in increased numbers in the epithelium and the submucosa, and CD4+ T-cells are increased in number during the pollen season in patients with intermittent symptoms. Moreover, in allergic patients, there is an increase in Langerhan-like cells (CD1+) during the pollen season (Howarth, 1995). IgE-induced mast cell activation principally induces $CD4^+$ T cell recruitment into the airways (Maezawa Y, 2004). This then plays an important role in enhancing Th2 cell-mediated eosinophilic airway inflammation by recruiting Th2 cells into the site of allergic inflammation (Maezawa Y, 2004). Hence, it is likely that these cells are important in the pathogenesis and the local interaction of these cells and their mediators with the specific structures in the nose, such as the epithelium, nerves, mucous glands, and blood vessels.

Histamine has become known as the mediator of allergic and anaphylactic reactions, and is released by mast cells and basophils. Although still one of the

major effectors of the allergic reaction, many other mediators produced by different cell types are also involved.

Direct stimulation of the nose outside of neural stimulation can cause rhinorrhoea and nasal congestion, via the local phenomenon of the action of histamine acting directly on H1 and H2 receptors on nasal vasculature end organs. Another focus of this project will be to address local and neural secretory responses.

1.10 Neuropathology in rhinitis

Many symptoms of rhinitis can be mediated by nerves (Sarin S, 2006), with the nerves of the nose playing a role in the neurogenic effects of allergic rhinitis. These include sensory, sympathetic and parasympathetic nerves.

Sensory nerves transmit signals from the mucosa causing pruritus and the motor reflexes of sneezing, and act on the sympathetic and parasympathetic reflexes that affect secretions and increased vascularity of the nasal mucosa. Pruritus and sneezing represent the tactile sensation and central reflex symptoms of rhinitis (Sarin S, 2006), but reflex-type symptoms can occur even in the absence of nasal abnormality or nasal irritation. All nasal symptoms of rhinitis can be triggered through neural pathways. Increased neural activity can be chronically upregulated in the presence of inflammation.

Rhinorrhoea is also a product of the reflex activation of submucosal glands, and a contralateral secretory response to certain external stimuli is termed the neural reflex (Sarin S, 2006). In response to a sensory stimulus, neural stimulation can

also lead to vasodilatation and subsequent nasal vascular congestion via decreased sympathetic outflow in conjunction with increased parasympathetic discharge. This has been shown to occur in the contralateral nostril also. However, it is also likely that there are local effects e.g. secretion of fluid from vascular leakage and directly from epithelial cells.

1.11 Nerve activation as a mechanism in AR and neural hyper responsiveness.

When subjects with allergic rhinitis who are currently symptomatic are challenged with histamine, bradykinin, hyperosmolar saline, or capsaicin, they typically demonstrate an enhanced symptomatic and secretory response compared to that seen in normal subjects.

When the propensity of a patient to develop nasal symptoms in response to an allergic stimulus becomes abnormally exaggerated, this phenomenon is known as nasal hyper responsiveness (Nathan RA, 2005).

The mechanism for sensorineural hyper responsiveness has not been clearly identified. A major component of neural hyper responsiveness is caused by upregulation of neural activity, whereby any stimulus of even average intensity can cause symptoms. Patients with allergic rhinitis have a more marked hyper responsiveness of their nasal mucosa than those without allergic rhinitis (Nathan RA, 2005). Whether this is due to increased neural activity is debatable but can

be investigated by comparing bilateral nasal secretions attributed to a certain external stimulus challenge on a designated nostril as presented in this thesis.

Nasal hyper responsiveness may explain the exaggerated symptomatic responses of patients with allergic rhinitis to a variety of non-allergic triggers, such as cigarette smoke, noxious odours, and cold air The mechanisms of this response have been widely investigated. It is well recognised that histamine and bradykinin stimulate sensory nerves in the nasal epithelium. Immunohistochemical studies have clearly delineated expression of both B1 and B2 bradykinin receptors in epithelial cells, submucosal glands, fibroblast, vascular smooth muscle, vascular endothelial cells, and macrophages, with B2 receptor expression found in peripheral nerve fibers (Shirasaki H, 2009).

Stimulation of these sensory nerves in turn leads to the symptoms of nasal irritation / pruritus, sneezing, as well as the stimulation of local and centrally mediated neural reflexes. This then stimulates further glandular secretion and nasal congestion (Togias A, 2000).

Reflex-mediated secretion occurs on the ipsilateral side of the nose but distinguishing these from local responses is difficult. A good way to distinguish local responses from reflex responses is the study of the contralateral reflex. The contralateral reflex is an example of a centrally mediated neural reflex. This is shown by applying a chemical to one side of the nose and then measuring the contralateral secretory response to this unilateral challenge. Secretions obtained

on the contralateral side at the time of stimulation contain high levels of glandular proteins, such as secretory IgA (Raphael GD, 1989), lactoferrin (Raphael GD, 1989) (Riccio MM, 1996), lysozyme (Raphael GD, 1989) (Reynolds CJ, 1999), and low levels of albumin (Riccio MM, 1996). This would indicate that these secretions arise from nasal glandular secretion and not from vascular leakage.

In contrast, ipsilateral secretory responses to histamine and bradykinin contain high levels of both glandular proteins and albumin, suggesting that they originate from both glandular secretion and vascular leakage.

The contralateral response is significantly attenuated by applying a local anaesthetic or an anticholinergic agent (Baroody FM, 1994), and this would suggest strong evidence for a neural mechanism for the contralateral secretory response to unilateral nasal challenge.

Increased contralateral secretory reflexes are seen after unilateral nasal challenge in allergic subjects with a variety of agents including histamine and hypertonic saline (Raphael GD, 1989) (Baroody FM, 1993), and bradykinin (Baraniuk JN, 1994) (Riccio MM, 1996) (Reynolds CJ, 1999). However, such a response was not clearly identified with isotonic saline and was not evident in patients with AR who had intermittent AR, and who were studied when they were asymptomatic (Sanico AM, 1999). This data indicates that there is neural hyper responsiveness to a variety of mediators, but the mechanisms of upregulation, physiological factors, and the nerve types mediating this response are not identified.

Neural mechanisms in allergic rhinitis may also have effects through local axonal reflexes. There is evidence that stimulation of nociceptive nerves in the nasal mucosa by bradykinin, capsaicin, or hyperosmolar saline leads to the local release of neuropeptides, including substance P, neurokinin A, and calcitonin gene-related peptide (CGRP), which may then mediate glandular secretion or vascular leakage (Baraniuk JN, 1990) (Sanico AM, 1997) (Baumgarten CR, 1997) (Baraniuk JN, 1999). Increased levels of substance P-like immunoreactivity have been reported in nasal lavages of atopic subjects compared to controls, with further increases in these levels after allergen challenge (Nieber K, 1992).

Nasal challenge with capsaicin, a specific stimulus for nociceptive C-fibres, leads to increased levels of albumin and fibrinogen, markers of vascular leakage, in nasal lavage fluid in subjects with allergic rhinitis. This response is significantly diminished after pre-treatment with lignocaine, but not after pre-treatment with atropine, implying that the effects of capsaicin on vascular leakage are mediated by local neural reflexes (Sanico AM, 1998).

The effects of bradykinin on vascular leakage are not affected by lignocaine, implying that bradykinin acts directly on blood vessels (Sanico AM, 1998). However, repeated nasal challenge with bradykinin in allergic subjects leads to tachyphylaxis for induction of ipsilateral albumin levels, suggesting that these direct effects of bradykinin are augmented by a neural mechanism. Because bradykinin does not induce contralateral albumin levels, this neural contribution is suggested to reflect local axonal reflexes rather than centrally mediated reflexes (Reynolds CJ, 1999).

To summarise the relationship between nerve activation and AR, the mechanisms underlying secretory hyper responsiveness are not fully elucidated. Several possible mechanisms exist. In the first place, the sensory nerves may be hyper responsive to nociceptive stimuli. Secondly, the efferent nerves may be hyper responsive, and thus cause potentiated stimulation of the nasal glands. Thirdly, there may be centrally-mediated hyper responsiveness, with enhanced transmission of signals from afferent nociceptive fibres, and / or enhanced stimulation of efferent secretomotor nerves. Finally, the end organs (i.e. the glands and the vasculature) may themselves be altered to respond more vigorously to neural or direct stimulation (Togias A, 2000).

In studies performed in the supervising laboratory, it has been shown that nasal allergen challenge leads to increased hyper responsiveness of nerves (Sheahan P, 2005) (Sheahan P, 2006).

1.12 Molecular pathophysiology of allergic rhinitis

A coordinated response of resident inflammatory cells, which play an immunomodulatory role, recruiting profession inflammatory cells and resident non inflammatory cells, are all involved in the pathogenesis of AR. In the most typical AR response, the mast cell is considered to play a pivotal role (Figure 1). The mast cell is resident in the nasal mucosa and is coated with (sometimes several) IgE antibodies bound to mast cells via various high and low affinity FcR receptors. Inhalation of allergen leads to contact between the allergen and the

nasal mucosal mast cells. This recognition leads to activation of the mast cell and the release of substances, including histamine, leukotrienes, tryptase, and prostanoids and other fatty acid like molecules including lipoxins and sphingosine 1 phosphatase.

These released mediators have direct effects on resident cells, including blood vessels, epithelial cells, nerves and mucous glands (Togias A, 2000). These released agents also are important in the recruitment of professional inflammatory cells which then may provide a second sustained inflammation.

Figure 1.

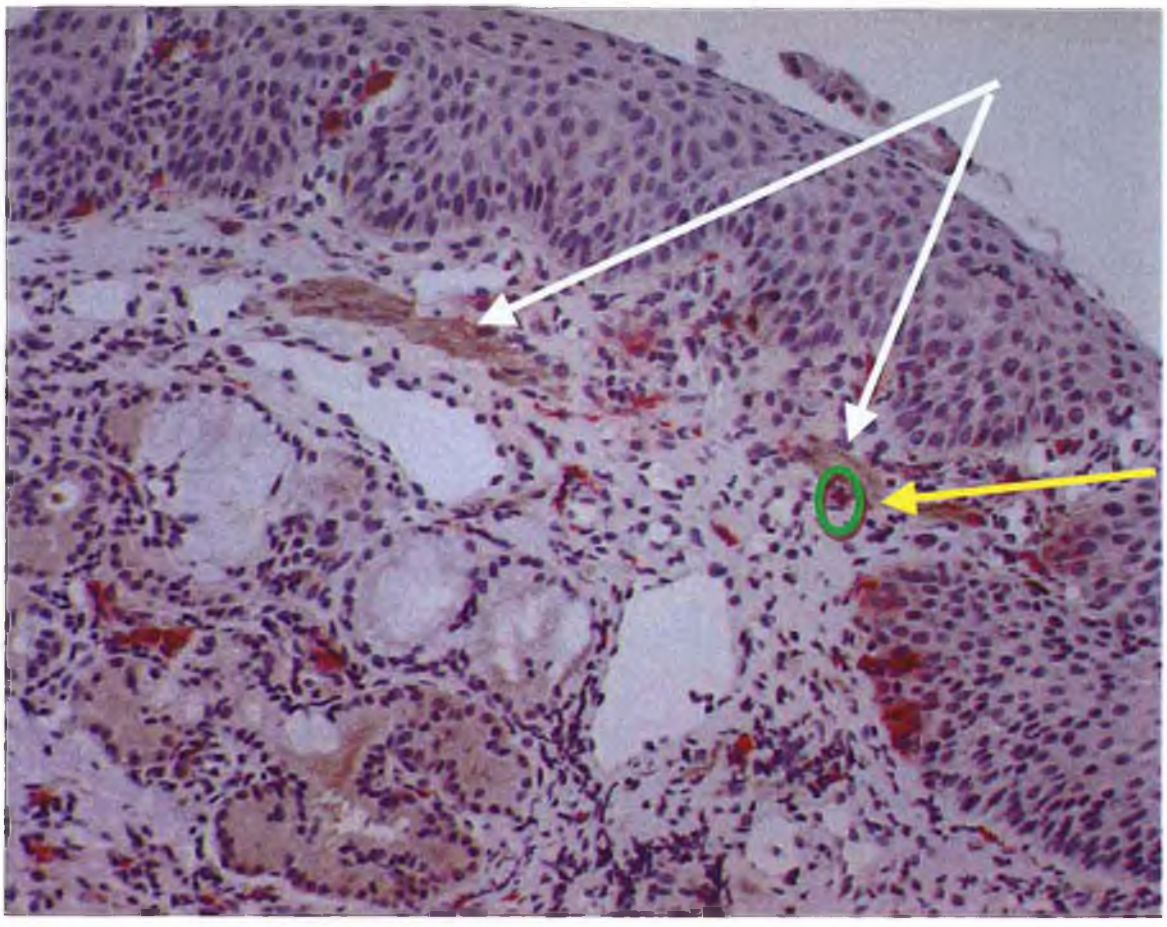


Figure 1. Mast cells in the lumen of a patient with AR is shown. Mast cells were identified with an antibody to tryptase (red) and nerves were identified with an antibody to PGP9.5 (brown). Note the presence of mast cell staining in the lumen in the epithelium, mucous glands and around the nerves. White arrow = nerve, yellow arrow = mast cell adjacent to nerve, circled in green. Picture and work by Thornton M and Costello RW.

Histamine

Histamine is released locally by mast cell degranulation after nasal allergen challenge (Baroody FM, 1994), and has multiple effects on the nasal mucosa. It directly stimulates glands and blood vessels, leading to glandular secretion, plasma extravasation, vasodilation of the capacitance vessels and plasma exudation from the post-capillary venules of the subepithelial plexus. This leads to vascular congestion (Nathan RA, 2005).

Histamine also stimulates sensory nerves, leading to sneezing, itching, and reflex-mediated glandular secretion (Togias A, 2000) (Nathan RA, 2005).

These effects are abolished after nasal pre-treatment with H1 receptor antagonists (Raphael GD, 1989) (Baroody FM, 1994). As a result, anti-histamine treatment is now central to the treatment of acute AR responses, indicating how important this mediator is in the pathogenesis of AR.

Leukotrienes

Leukotrienes released from mast cells also have strong effects on blood vessels, leading to nasal congestion (Widdecombe JG, 1990). Leukotriene receptor antagonists, such as montelukast are effective in preventing nasal obstruction due to natural allergen exposure (Philip G, 2002) (Okubo K, 2008), allergen challenge, and nasal mannitol challenge (Lee DK, 2003). Mannitol acts as a hyperosmolar stimulus causing nasal hyper-reactivity, as demonstrated by a reduction in nasal peak inspiratory flow after such a challenge.

The role of leukotrienes in AR is well demonstrated, and while they have been shown to be effective both at inhibiting the mediator's effects locally, they also prevent the recruitment of so called professional inflammatory cells by preventing the upregulation of vascular adhesion molecules.

In addition, it has been shown that activation of leukotriene receptors results in increased electrical excitability of capsaicin-sensitive (presumably nociceptive) sensory neurons, which in turn mediate trigeminal nerve activity responsible for the initiation of sensations, such as itch. These neurons also mediate central reflexes such as sneezing, and increase in parasympathetic-mediated hypersecretion from the nasal mucosa. Similarly, the effects of histamine are potentiated by leukotrienes (Taylor-Clark TE, 2008). There is little evidence however, that these medications alone are sufficient to attenuate all the subsequent inflammatory changes that occur after exposure to an allergen.

Sphingosine 1 phosphate (S1P)

This bioactive lipid produced by mast cells and platelets is important in AR. Released rapidly after mast cell activation, SIP has been shown to act directly on mast cell-bound S1P receptors, leading to activation of the mast cell, and in particular, production of cytokines. In this laboratory it has recently been shown that SIP also acts on specific eosinophil SIP receptors, SIP1,2,3, and 5, and that some of these receptors are upregulated after allergen challenge. Furthermore eosinophil chemotaxis is significantly increased after the exposure to SIP, indicating that this mediator is important in the recruitment of other important

inflammatory cells to the site of injury. SIP has also been shown to be important in the recruitment and retention of lymphocytes to specific sites of inflammation.

T-cells

In addition to releasing mediators which act directly on end-organs, mast cells also release cytokines, including IL-4, IL-5, IL-13, and TNF-α, which lead to the expression of adhesion molecules and chemo-attraction of inflammatory cells including T cells, in particular Th2 cells (Maezawa Y, 2004). Increased numbers of CD3-positive T cells in the nose have been reported in subjects with allergic rhinitis (Figure 2), particularly during times of allergen exposure (Wilson DR, 2001) (Gluck J, 2005).

Figure 2.

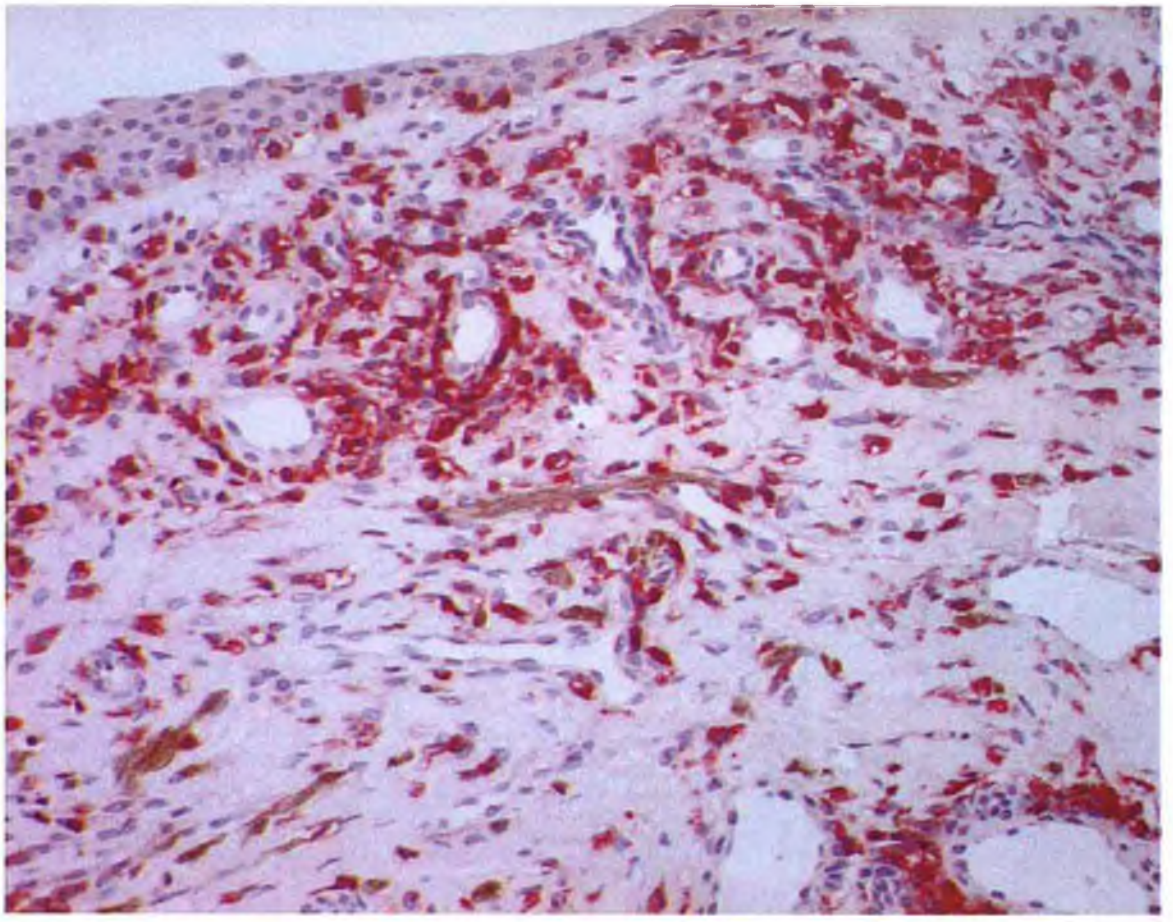


Figure 2. CD- positive T cells (stained red) in the nasal mucosa of a patient with persistent AR. Vascular and neural distribution (brown stain with antibody to the nerve marker PGP9.5), is shown. Picture and work by Thornton M and Costello RW.

T cells may be activated directly after allergen exposure through antigen-presenting cells, including dendritic cells and eosinophils which are known to express MHC11 molecules (Godthelp T, 1996). T cells in turn recruit further inflammatory cells, including progenitor inflammatory cells to promote further local mast cells, eosinophils, and memory T cells. They also recruit T regulatory cells, which may limit the damage being done by these toxic inflammatory cells. All of this is mediated through the release of cytokines, including IL-3, IL-4, IL-5, and granulocyte/macrophage colony stimulating factor (GM-CSF).

The outcome of these events is a marked cellularity of the nasal airway, which is present within 6 to 24 hours after allergen challenge. This increased cellularity may be accompanied by a recrudescence of symptoms at 8 to 12 hours after challenge (Togias A 2000).

Eosinophils, neutrophils, and T-lymphocytes are all present in the late-phase cellular response, which is accompanied by release of eosinophilic cationic protein (ECP) and generation of myeloperoxidase (MPO) and leukotriene-B_4 (Miadonna A, 1999). The presence of these inflammatory cells and their products may be a factor in rendering the nasal tissues more responsive to subsequent challenge with a smaller amount of allergen, and this is the so-called "priming effect" (Skoner DP, 2001). Nasal tissue may become more responsive to their own inflammatory cell products (e.g. histamine) (Togias A, 2000), and this is the so-called "hyper responsiveness" of the nose.

1.13 The eosinophil in AR

The eosinophil is pivotal in the pathophysiology of chronic nasal inflammation (Kramer MF, 2004), and is believed to play an important role in the late phase of the allergic reaction. Increased numbers of eosinophils in nasal submucosa and epithelium are seen in allergic rhinitis following both natural allergen exposure (Bentley AM, 1992) (Wilson SJ, 1998) and experimental allergen challenge (Miadonna A, 1999). This process is believed to be regulated, at least in part, by T_H-lymphocytes, with elevated eosinophil cationic protein (ECP) levels found in T helper lymphocyte type 2 (atopic) diseases such as allergic asthma and allergic rhinitis but also occasionally in other diseases such as bacterial sinusitis (Bystrom J 2011).

The most prominent feature of the eosinophils are their large secondary granules, each containing four basic proteins, the best known being the eosinophil cationic protein. This protein has been developed as a marker for eosinophilic disease and quantified in biological fluids including serum, bronchoalveolar lavage and nasal secretions (Bystrom J 2011).

Eosinophil recruitment is associated with increased release of eosinophil products, including major basic protein (MBP) (Figure 3), and the previously mentioned eosinophil cationic protein (Wilson SJ, 1998) (Miadonna A, 1999). These granule proteins are toxic when applied in vivo and in vitro to cells. In the lower airways the release of cationic proteins from eosinophils has also been suggested to be damaging to airway epithelium. However, this has not been demonstrated in the

nose. Hence, other possibilities as to what eosinophils may be doing in the nasal mucosa of patients with AR have been investigated, in particular in functional studies in this laboratory. These data are outlined below.

In the lower airways, localization of eosinophils around airway nerves has been demonstrated in the lungs of allergen-challenged guinea-pigs, as well as in humans dying from fatal asthma (Costello RW, 1997). Thornton et al (unpublished) showed that this occurs during the late-phase allergic response of allergic rhinitis. The mechanisms for this localization are incompletely understood. However, primary cultures of airway parasympathetic neurons express the adhesion molecules intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). Eosinophils bind to these molecules (Sawatzky DA, 2002). Airway neurons also express eotaxin. This eosinophil chemo-attractant eotaxin may be a mechanism whereby eosinophils are recruited to nerves (Fryer AD, 2006). Furthermore, co-culture of eosinophils with nerves significantly increased release of eosinophil peroxidase (EPO) and leukotriene C_4, compared to culture of eosinophils alone (Kingham PJ, 2002). There is evidence that the interaction of eosinophils with cholinergic nerves in the lower airways may upregulate neural function, so leading to airway hyper responsiveness in asthma.

Muscarinic M_2 receptors are inhibitory autoreceptors that are present pre-junctionally on postganglionic parasympathetic nerves. They are activated by

acetylcholine, released from postganglionic nerves, and act to inhibit further acetylcholine release (Minette PA, 1988) (Costello RW, 1997). Loss of M_2 receptor function may occur during the late phase of the allergic reaction as a result of the actions of eosinophil products, such as eosinophil major basic protein (MBP, see Figure 3), which has been shown to be an allosteric inhibitor of M_2 receptors (Jacoby DB, 1993) (Jacoby DB, 2001). In sensitised guinea-pigs, loss of M_2 receptor function, accompanied by hyper responsiveness to electrical stimulation of the vagus nerve, is seen 24 hours after allergen challenge. This loss of M_2 receptor function and airway hyper responsiveness is prevented by pre-treatment with antibody to MBP (Evans CM, 1997). In humans, inhalation of pilocarpine, an M_2 receptor agonist, inhibits vagally mediated reflex bronchoconstriction after inhalation of sulphur dioxide in normal subjects, but not in subjects with atopic asthma, suggesting that loss of M_2 receptor function also occurs in humans as a result of allergic inflammation (Minette PA, 1989).

Figure 3.

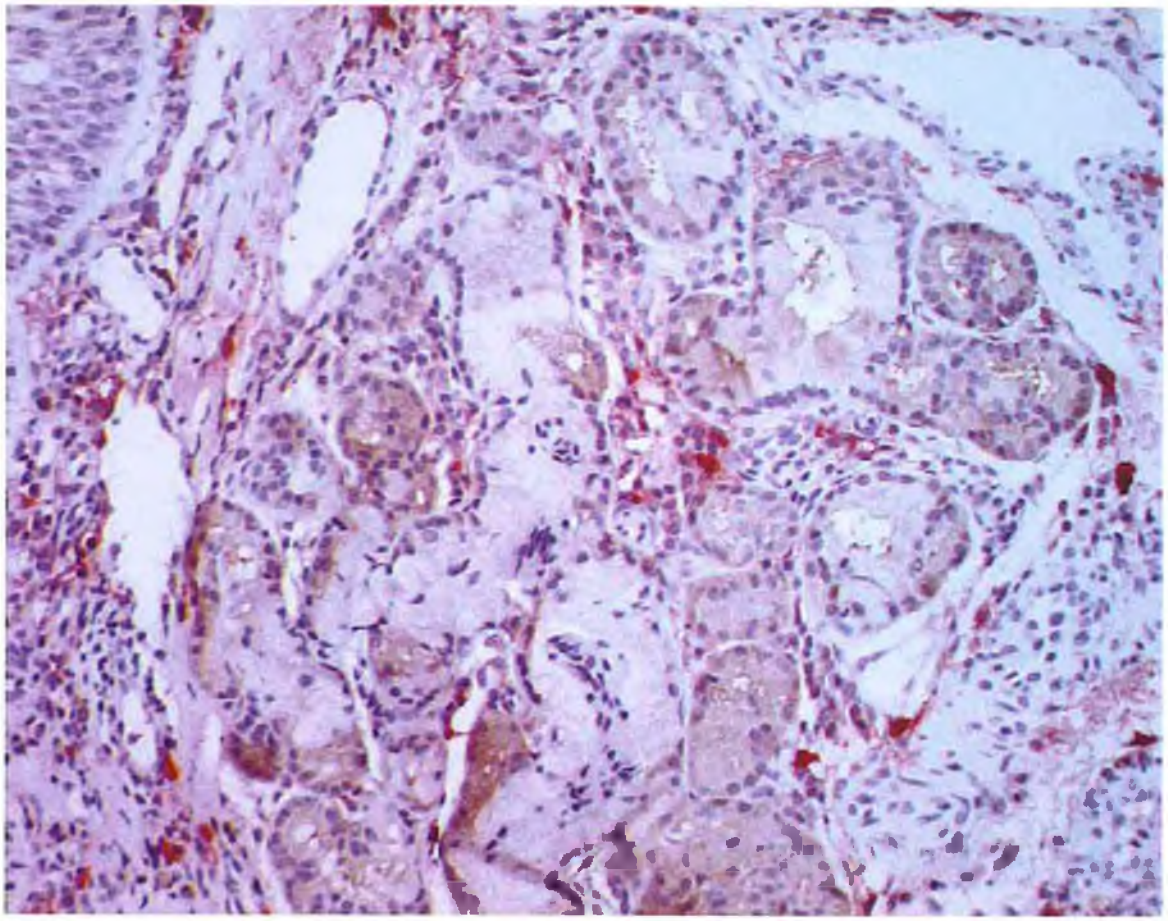


Figure 3. MBP positive eosinophils (stained red) in the nasal mucosa of a patient with persistent AR. Vascular and neural distribution (brown stain with antibody to the nerve marker PGP9.5), is shown. Picture and work by Thornton M and Costello RW.

CHAPTER 2 – ACID SENSING ION CHANNELS

2.1 Acid-sensing ion channels (ASIC); structure and function

Acid sensing ion channels, ASICs, are a family of ligand-gated cation channels, activated by acid (pH <7.4 – pH 5.5) (Waldmann R, 1998), (Sutherland SP, 2001), (Gu Q, 2006). Previously, they were referred to as dorsal root acid sensing ion channels (DRASIC) (Sluka KA, 2004). They belong to the amiloride-sensitive degenerin/epithelial Na+ channel (ENaC) superfamily (Figure 4) (Waldmann R, 1998) (Kellenberger S, 2002) (Wemmie JA, 2006) (Xu TL, 2007). H+-gated cation channels are the only ion channels known to be directly activated by a pH change (Waldmann R, 1997) and to date, the only known activators of ASICs are protons (Waldmann R, 1998)(Xu TL, 2007). With regards to other ion channels which are gated by physical or chemical conditions other than changes in pH, proton-gated ion channels use protonation for activating an ionic current (Reeh PW, 2001).

Since this novel amiloride-sensitive sodium channel was first isolated and cloned in 1995 (Waldmann R, 1995), and the proton-gated cation channel cloned (Waldmann R, 1997), at least six subunits of ASICs have been identified; ASIC1 with its two isoforms 1a (Waldmann R, 1997) and 1b (Chen CC, 1998), ASIC2 with its two isoforms 2a (Price MP, 1996), localized to the cerebellum (Jovov B, 2003), and 2b (Lingueglia E, 1997), ASIC3 (Babinski K, 1999) and ASIC4 (Grunder S, 2000) (Krishtal 2003). A role for ASIC4 has not been determined but it may be involved in downregulation of ASIC3 (Donier E, 2008). A further isoform, ASIC1b2 has since been additionally identified (Deval E, 2008). All but

two of these subunits, ASIC2b and ASIC4, have so far been shown to be activated by protons, and this is true for mammalian cases (Donier E, 2008). Of the rest of the isoforms, ASIC-3 is the most proton-sensitive, and opens when pH drops from 7.4 to 7.0 (Immke DC, 2003). However, initial earlier reports by Babinski et al suggested as low a pH as 4 to cause ASIC-3 activation (Babinski K, 1999). ASIC-1 and ASIC-2 channels have been reported to activate at a pH at or below 5 (de la Rosa DA, 2002)

The distribution of ASICs varies in different cells and under different physiological conditions, and they may function at various subcellular locations. The majority of studies have featured their location and roles in the brain, synapses, and throughout dorsal root ganglia of the peripheral nervous system (Waldmann R, 1997) (Reeh PW, 2001). Their presence in dorsal root ganglia corresponds to their function as acid sensors in nociception, but their role in the brain is as yet unclear (Waldmann R, 1997). They have also been shown to occur in epithelial tissues, such as kidney, colon, testis, ovary, pituitary, heart and lung (Waldmann R 1995) (Babinski K, 1999) (Groth M, 2006) (Yiangou Y, 2001).

Stimulation of these receptors on nerves leads to a variety of sensations including pain and mechanoperception, while epithelial expression is linked to airway secretion and has been reported in cystic fibrosis cell lines (Su X, 2006). Other functions include taste transduction (particularly sour taste), synaptic plasticity, learning / memory, acid-mediated neuronal injury, and even cochlear function

(Xu TL, 2007) (Donier E, 2008). Increasing evidence would purport a role for these receptors in neurological diseases, brain ischaemia and epileptic seizures (Xiong ZG, 2008). A description and function of these receptors in human upper airways has not yet been described.

Figure 4.

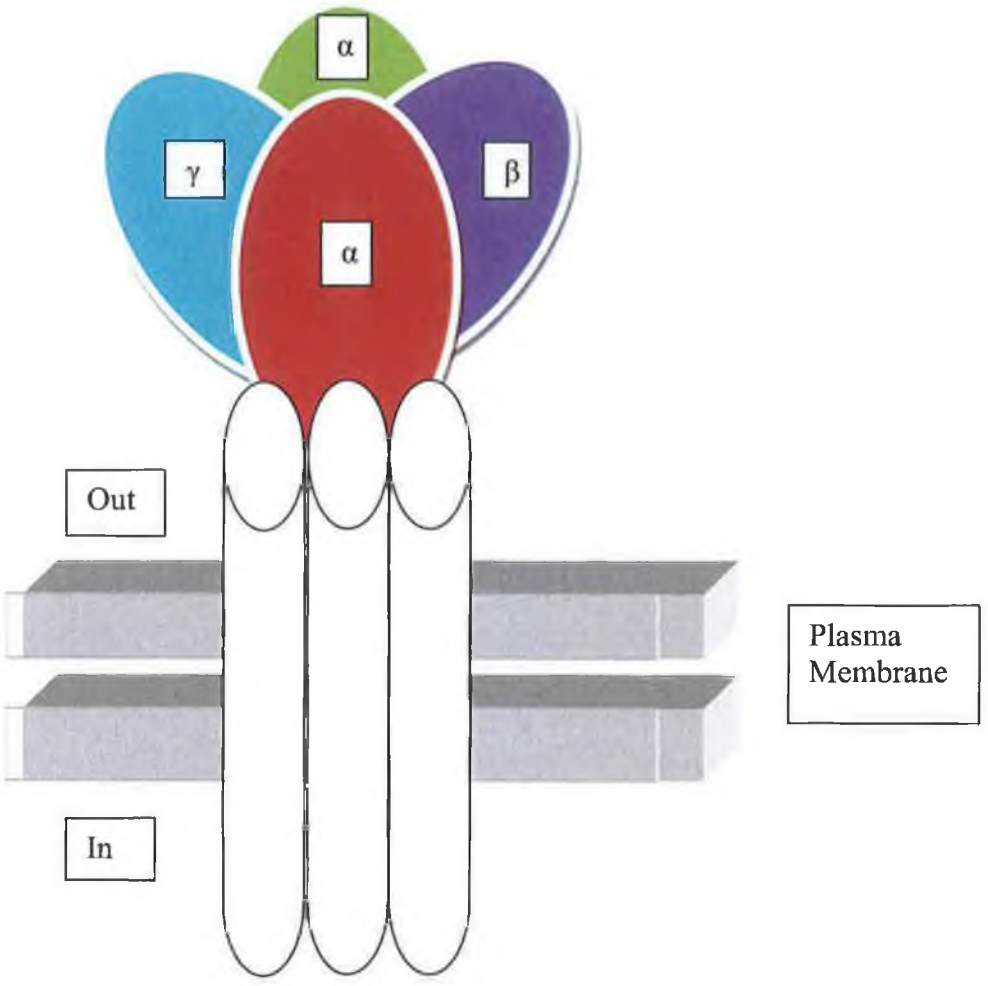


Figure 4. Model of ENaC/DEG channel complex. This is a model of the tetrameric assembly of an ENaC channel. Four subunits (2x α, β, and γ) are arranged around a central channel pore. Adapted from Kellenberger and Schild, 2002.

2.2 Structure of ASICs

Each ASIC subunit consists of two transmembrane domains, TM I and TM II (Xiong ZG, 2008) (see Figure 5). These are connected by an extracellular cystein-rich loop and intracellular N and C termini (Waldmann R, 1998). These are then assembled as tetramers of homomeric or heteromeric subunits (Lingueglia E, 1997) (Bassilana F, 1997) (Krishtal, 2003) to form amiloride-blockable proton-activated cation channels in the mammalian central and peripheral nervous systems (Ugawa S, 2005). Our interest is primarily in the structure and function of ASIC-3, whose mRNA encodes 531 amino acids, with a molecular mass of 59kDa (Babinski K, 1999).

Figure 5

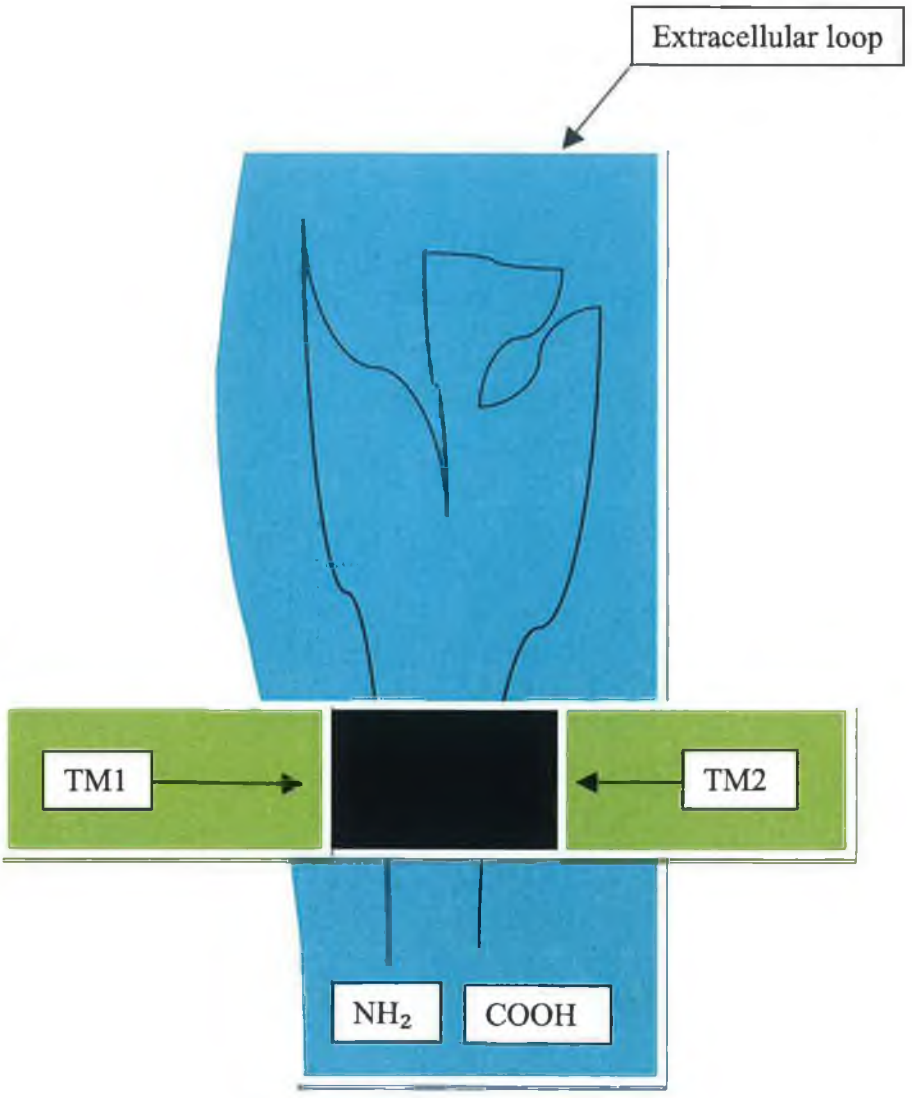


Figure 5. Structure of ASIC subunit. A single subunit comprises 2 transmembrane domains (TM1 and TM2), an extracellular loop responsible for gating, and an intracellular termini. Four subunits are assembled in the membrane to form a functional unit (Figure 4). Adapted from Krishtal O, 2003.

2.3 Activation of ASICs.

Extracellular protons activate ASICs to affect neuron function (Wemmie JA, 2006), through the simplest form of ligand binding (Reeh PW, 2001). Small to moderate changes in pH are sufficient for ASICs to respond, such as occurs during tissue inflammation (Xu TL, 2007), with ASIC-3 having a very steep activation curve (Sutherland SP, 2001). Activation however occurs only when accompanied by rapid pH drops but transient opening of ASICs can generate a long lasting effect, potentially by being modulated by other signalling molecules such as lactic acid (Xu TL, 2007). Studies have shown that ASICs are responsible for pain perception at a pH >6.0. As alluded to previously, ASIC-3 channels open with a pH drop from 7.4 to 7.0 (Krishtal 2003) (Immke DC, 2003), whereas other ASICs are less sensitive to acidity (Sutherland SP, 2001). Ugawa et al suggests ASICs are activated at a pH of 7.2 to 6.0 (Ugawa S, 2002). Contrasting earlier reports however, suggest a pH value as low as less than 4 for ASIC3 currents to be activated to cause mechanical hyperalgesia (Waldmann R, 1998) (Babinski K, 1999) (Sluka KA, 2004). Some reports suggest ASIC1 channels open at a pH of 6.9 (Waldmann R, 1997), whilst others quote figures of pH 5 and below (de la Rosa DA, 2002).

2.4 Role of ASICs

Although a variety of functions have been attributed to ASICs, there is still controversy about their actual functions in mammals (Reeh PW, 2001). They are thought to play a role in nociception (Waldmann R 1997) (Voilley N, 2001), with

studies suggesting that they modulate the perception of moderate to high-intensity acid-induced pain sensation (Chen CC, 2002). A positive correlation between pain and tissue acidity has been shown previously (Deval E 2008). The presence of ASICs, primarily in small-diameter and small-medium diameter sensory neurons, supports their involvement in these nociceptor pathways and contributes to the pH-sensitivity of nociceptors (Yiangou Y, 2001) (Ugawa S, 2005). Furthermore, it appears that hyperalgesia is primarily modulated by the presence of ASIC-3 in particular, and seems to be independent of the presence of ASIC-1 (Sluka KA, 2004). In fact, loss of ASIC-3 causes reduced responsiveness to acid (Price MP, 2001).

It is clear, through their expression throughout the peripheral and central nervous system, that ASICs have a role in signal transduction or in maintenance of cellular function (Reeh PW, 2001). Local peripheral inhibition of these receptors by ASICs antagonist amiloride has been shown to reduce nociceptive behavior in mouse experiments, from an ipsilateral but not contralateral perspective (Rocha-Gonzalez HI, 2009). Despite this, to date, it is unclear to what extent the cloned proton-sensitive ion channels contribute to pH-responses in nociception (Reeh PW, 2001).

Other receptors, such as the capsaicin receptor, transient vanilloid receptor subtype (TRPV1), also appear to modulate pain through proton responses, but among unmyelinated fibres, whilst there is a suggestion that ASIC subunits play more of a role in thin myelinated nociceptors (Leffler A, 2006). Similarly, Ugawa

et al suggests that ASICs play a bigger role in nociception at a pH of 7.2 to 6.0, whereas TRPV1 channels do not start to open till a pH of less than 6.0 is reached (Ugawa S, 2002)

ASICs also play a role in sensory and mechanoperception (Price MP, 2001). The suggestion that ASICs has a role in pilocarpine-induced limbic seizures and status epilepticus has also been studied, using the ASICs inhibitor amiloride as a potential anticonvulsant (N'Gouemo, 2008).

Interestingly, in epithelial cells ASIC-3 has been co-immunoprecipitated with the cystic fibrosis transmembrane receptor (CFTR) where their function in epithelial cells has been suggested to be involved in the maintenance of airway hydration (Su X 2006). Tissue acidification, which occurs during conditions such as inflammation and ischaemia, activates acid-sensitive ion channels. The condensate from the exhaled breath of both asthma and rhinitis patients is more acidic than that of healthy controls (Ojoo JC 2005). Hence, there may be increased activity of ASIC in a chronic inflammatory condition such as AR.

Ultimately, only inactivation of ASIC genes in animal models will perhaps clarify the physiological roles of ASICs in the central and peripheral nervous systems (Kellenberger S 2002).

2.5 Lactic acid and its effect on acid-sensing ion channels

In response to reduced oxygen supply, lactic acid is produced during inflammation and ischaemia by enhancement from aerobic to anaerobic metabolism of glucose and the release of H+ from ATP hydrolysis (Xu TL 2007) (Xiong ZG 2008). This causes a reduction in tissue pH, termed acidosis.

Lactic acid acts through chelating extracellular divalent ions such as calcium and magnesium (Xu TL 2007), decreasing them and thus increasing activity of ASICs (Immke DC 2001). These divalent ions have a significant role to play in modulating ASIC-3 function, whereby decreasing calcium opens the ASIC-3 channel, altering calcium shifts pH-dependent gating, and raising calcium blocks the channel (Immke DC, 2003). Immke & McCleskey also showed that decreasing extracellular pH to 7, in the presence of 15mM of lactic acid, depolarised ASICs-associated neurons about 70% more than at pH 7 alone (Immke DC 2001). Its effects were apparent in both ASIC-3 and ASIC1a receptors. At higher pH levels of 7.4 to 8, lactic acid caused no depolarisations of neurons, suggesting that it acts by modulating but not activating ASICs (Immke DC 2001).

2.6 ASICs antagonism by amiloride

Amiloride has been used clinically as a diuretic since 1967 (Arias RL, 2008), and is a potassium-sparing diuretic. It works primarily by inhibiting the epithelial sodium channel (ENaC) of the renal tubules (Arias RL, 2008), and is also a non-

selective blocker of ASICs (Krishtal 2003) (Hayes SG, 2007) (Arias RL, 2008) (Rocha-Gonzalez HI, 2009). It is this character that allows it to be used as a pharmacological tool to identify ASIC receptors (Xu TL 2007). As alluded to previously, ASICs are activated by acidic pH, and the inhibitory effects of amiloride are specific for acid-induced pain (Jones NG, 2004). In fact, injection of amiloride appears to significantly attenuate acid-mediated pain, suggesting that amiloride-sensitive ASIC channels are responsible for a substantial proportion of acid pain (Jones NG, 2004).

Despite it being non-specific, it has been shown that higher concentrations are required to inhibit ASIC3 receptors as opposed to ASIC1a, ASIC1b, and ASIC2a subunits (Lingueglia E 1997) (Bassilana F 1997). The sustained component of ASIC depolarisation current is certainly less responsive to amiloride (Waldmann R 1997).

2.7 ASICs in respiratory mucosa

It is known that proton-activated channels similar to ASIC, known as transient receptor potential vanilloid receptor subtype-1 (TRPV1) receptors, occur in lung and nasal mucosa, and it is postulated that they play a role in cough and pain sensation. TRPV1 has been expressed and localized to vascular endothelial cells, submucosal glands, and nerves in human nasal mucosa, and in fact appears to also have a role in secretory functions (Seki N, 2006). Almost all mammalian nociceptive C-fibre neurons express TRPV1 channels (Taylor-Clark TE, 2008).

The receptor for TRPV1 channels is capsaicin, the pungent component in chili peppers (Taylor-Clark TE, 2008).

Apart from capsaicin, TRPV1 is activated by heat and protons, and acts as a primary sensor to chemical and physical stimuli that cause pain (Tominaga M 1998). However, whilst initial application of capsaicin to human nasal mucosa was found to produce a painful response as expected, further repeated applications caused a marked attenuation of pain, demonstrating a characteristic desensitization of the nasal mucosa (Geppetti P, 1993). Stjärne in 1998 showed that topical application of capsaicin, as a desensitizing antagonist of TRPV1, to the nasal lining mucosa of patients with allergic rhinitis caused relief of nasal symptoms (Stjärne P 1998). Both TRPV1 and ASIC3 have also been shown to occur in dorsal root ganglia that project to lung and pleura mucosa of rats (Groth M 2006) (Su X 2006). It is therefore fair to postulate that, with TRPV1 expressed in nasal mucosa, similarly ASIC3 may also be expressed in nasal mucosa, and play a role in patients with allergic rhinitis. The similarities between lung and nasal epithelium add further weight to this. In experiments in both mice and rats however, there were marked species differences in expression of TRPV1 and ASIC and marked differences even within subpopulations of sensory neurons (Leffler A, 2006). Rat vagal pulmonary sensory neurons have been shown to be activated by a lower pH of between 7 and 5.5 (Gu Q, 2006). The same cannot, therefore, be extrapolated directly to humans.

2.8 Hypothesis

Increased nasal secretion and higher nasal discharge scores have been noted following application of saline, which is an acidic media, in the nose (Baraniuk JN, 2002) (Pinto JM, 2006). In the same way increased nasal secretions occur after swimming in acidic sea water, we postulate that acid-sensing ion channels, among other factors, play a role in modulating nasal secretion in this instance. ASICs have been identified in various sites of the body, including the lung. They have not been shown to be present in nasal mucosa. With striking similarities between lung and nasal mucosa, we postulate that ASICs occur in the nasal epithelia and glands, and are upregulated in patients in whom increased nasal secretion is characteristic, such as in allergic rhinitis.

We hypothesized that ASIC-3 is the functioning receptor in nasal mucosa, and this receptor would be blocked by ASIC inhibitor, amiloride, and would be functional at an acidic environment and a pH of 7.0.

With evidence of neural responses at work within the nasal cavity, causing contralateral responses in the opposite nasal cavity to that exposed to challenge substances, we hypothesized that ASIC-3 is intimately related and co-localised to neural structures and nerve bundles.

We also hypothesized that the eosinophil has a major role to play in inflammatory conditions such as allergic rhinitis, and may have a part to play in acting via ASIC receptors or inducing them or mediating them to react.

CHAPTER 3– MATERIALS AND METHODS

3.1 Beaumont Hospital Scientific Committee ethical approval

Ethical approval for this project was sought from the Scientific Committee Ethics Department at Beaumont Hospital, where the study was undertaken, on 14 August 2007. Final approval was obtained on 15 October 2007, reference 07/65 (see Appendix 1.)

3.2 Patient selection.

Patients were recruited into an AR Study group and a Control group, and were age and sex matched.

Criteria for inclusion into the study group was based on the symptoms and diagnosis of persistent allergic rhinitis as per the "Allergic Rhinitis and Its Impact on Asthma" (ARIA) criteria (Bousquet J, 2001). This defines allergic rhinitis as a symptomatic disorder of the nose after allergen exposure by an immunoglobulin E (IgE)-mediated inflammation of the membranes. Only patients with persistent allergic rhinitis based on the ARIA classification were included in the study group, (see Table 1). Skin prick tests were undertaken to confirm the allergic nature of each patient's rhinitis. The method of skin prick testing is detailed in Section 3.5.

Patients with intermittent allergic rhinitis, out of season, were omitted. Excluded were minors below the age of consent, cognitively impaired patients, and patients greater than 65 years of age. Any patient unable to understand and sign informed consent was also excluded. Women who were pregnant or breastfeeding were excluded, as were patients with a history of anaphylaxis. Patients on aspirin,

warfarin or plavix, or any other "blood thinners" were excluded, based on the fact that nasal biopsies were to be taken, thereby increasing the risk of epistaxis post-procedure. Patients with any other nasal pathology such as granulomatous diseases or malignant disease were also excluded.

Criteria for inclusion in the Control group was based on no symptoms, past or present, of nasal blockage, constant nasal secretion, and any history of atopy.

Recruitment of patients was undertaken primarily from the general Ear Nose and Throat clinics, Respiratory Allergy clinics and specialised Rhinology clinics, with the principal investigator approaching these participants in person. Further recruitment was undertaken via posters alerting the general public about the commencement and nature of the study, and these were prominently displayed around the host hospital.

Following recruitment of each participant, a full description of the reason for the project and a clear explanation of what the study entailed was related to the participant and both written and informed consent obtained. Each participant was given an information leaflet detailing the study for their perusal at their own time, prior to agreeing to the study. Participants were encouraged to take the leaflets home with them to study, prior to contacting the principal investigator with a decision as to whether they wished to participate in the study or not.

Table 1 Inclusion / exclusion criteria for enrolment into the Allergic Rhinitis Study group

Inclusion criteria	**Exclusion criteria**
Patients with persistent allergic rhinitis	Minors below the age of consent
	Cognitively impaired patients
	Patients greater than 65 years of age
	Any patient unable to understand and sign informed consent
	Women who are pregnant or breastfeeding
	Patients with a history of anaphylaxis
	Patients on aspirin, warfarin or plavix, or any other blood thinners
	Patients with any other nasal pathology such as granulomatous diseases or malignant disease

3.3 General materials

- Diagnostic skin prick allergens - Allerayde Ireland Limited, Blackrock, County Dublin
- Skin prick test lancets - Allergopharma Joachim Ganzer KG
- 1% hydrocortisone cream - Hydrocortisyl Skin Cream 1%, Aventis, Dublin, Ireland
- Cottles nasal speculum
- 'Crocodile' microforceps
- Blakesley 45 degree upward cutting upped biopsy forceps – Explorent GMBH
- Co-phenylcaine nasal spray (lignocaine hydrochloride 5% and phenylephrine) – Medisource, Dublin, Ireland
- Filter paper collection discs - Shandon Inc, Pittsburgh USA
- NUNC cryotube vials – Bio-Sciences, Dun Laoghaire, County Dublin, Ireland
- Dilvac liquid nitrogen flask – Dewar, Wolfe Laboratories, UK
- Polypropylene pellet pestle – Sigma Aldrich, USA
- DNA Engine PCR thermal cycler – Roche, USA
- pH/°C meter - Eutech EcoScan BNC Lenox, Drogheda, Ireland
- Leica DF350 FX digital camera, Leica, Milton Keynes, UK

3.4 Reagent materials

Clinical experiments

- Amiloride hydrochloride hydrate
- Alkaline sodium hydroxide 98% buffer
- 2M alkaline buffer - sodium hydroxide 98%

  All Sigma, Poole, UK
- Lactic acid free acid - Sigma Aldrich, Ireland
- Isotonic saline solution - 0.9% sodium chloride injection, Braun Medical Ltd, Dublin Ireland

PCR

- RNeasy mini kit
- QuantiTect reverse transcription kit
- QuantiTect SYBR Green PCR kit
- RNALater RNA stabilization reagent

  All Qiagen, Hilden, Germany

- ASIC1,2,3 primers
- ASIC3 antibody
- Rabbit polyclonal antibody to ASIC3

  All Abcam, Cambridge, UK

- Beta-mercaptoethanol
- 74% formaldehyde
- 0% ethanol

  All Sigma, Poole, UK

- Wizard PCR Preps DNA Purification System – Promega, Madison, WI, USA.

IMR-32 Cell cultures

Proliferation Medium:

- Dulbecco's Modified Eagles Medium with Glutamax
- 5% Foetal Calf Serum (FCS) (25 mL)
- 1 % penicillin-streptomycin (5 mL)

  All - GIBCO® Invitrogen (Paisley, UK).

- 1ul/ml gentamicin (0.5 mL) – Sigma, Poole, UK

Differentiation Medium:

- Dulbecco's Modified Eagles Medium with Glutamax
- 1 % penicillin-streptomycin (5 mL)
- 2% FCS (10 mL)

  All from GIBCO® Invitrogen (Paisley, UK).

- mM sodium butyrate (0.11g)
- 1ul/ml gentamicin (0.5 mL) – Sigma, Poole, UK

Eosinophil peroxidase in co-culture with 16HBE14o cells.

- Eosinophil peroxidase (EPO) – kindly donated by Dr Gerald Gleich, Utah, USA
- CuFi-1 cells - kindly donated by Prof Zabner, University of Iowa, USA.
- SV40-transformed human bronchial epithelial cell line 16HBE14o – kindly donated by Dr Dieter Grunert, University of Vermont, USA

- Minimal essential medium (MEM)
- 10% FCS,
- 1% L-glutamine
- 1% penicillin/streptomycin.

  All from GIBCO® Invitrogen (Paisley, UK).

Western Blotting

All reagents from Sigma, Poole, UK unless specified.

- Acrylamide
- Tris buffered saline
- SDW – sterile distilled water

- SDS – Sodium dodecyl sulphate
- APS – ammonium persulphate
- TEMED – Tetramethylethylenediamine
- B- mercaptoethanol
- Tween 20
- PBS – phosphate buffered saline.

10% Separating Gel. Buffer

Acrylamide - 3.3 mL

SDW -3.9 mL

1.5 M Tris (pH 8.8) -2.5 mL

10% SDS 200µL

10 % APS (0.1gimL) – 67µL

TEMED - 6.7µL

SDS Sample Treatment

1M Tris (pH 6.8) - 1mL

10% SDS – 2mL

0.1% Bromophenol Blue 200µL

dH_2O – 5mL

Glycerol – 2mL

β – mercaptoethanol – 1mL

4% Stacking Gel

Acrylamide - 500µL

dH20 -2.25 mL

0.5 M Tris (pH 6.8) -950µL

10% SDS -38µL

10% APS -25µL

TEMED -3.8µL

Running Buffer 5X

Tris - 7.5g

Glycine – 36g

SDS – 25g

Made up to 500mL in SDW

Transfer Buffer 1X

Tris – 2.42g

Glycine – 11.26g

10% SDS – 1mL

Made up to 200 mL in methanol,

Add 800mL SDW.

Blocking Buffer

0.2g I-Block

PBS – 100mL

0.1% Tween 20 – 100µL

Washing Buffer

1 sachet PBS

1 L SDW

Tween 20 – 1mL

Assay Buffer

SDW – 18mL

10X Assay Buffer – 2mL

Substrate/Blot	*10X Assay Buffer*
CDP-Star – 1.9ml	200mM Tris-HCL pH 9.8
Nitro Block – 100µL	10mM $MgCl_2$

Immunohistochemical staining

- 5µm formalin-fixed, paraffin-embedded (FFPE) tissue sections
- Polyclonal antibody to ASIC-3 (1:50) – Abcam, Cambridge, UK
- Monoclonal antibody to PGP9.5 (1:50) - Novocastra, Newcastle-upon Tyne, UK).
- Km Tris-EDTA (Sigma-Aldrich; pH 9.0)
- Peroxidase-labeled Envision anti-rabbit or anti-mouse secondary antibodies – Dako, Hamburg, Germany.
- TBS
- Goat anti-rabbit Alexa-488 and goat anti-mouse Alexa-568 (both 1:500) – Invitrogen, Renfrew, UK.
- 4,6-diamidino-2- phenylindole Vectashield - Vector Laboratories, Inc., Dublin, Ireland.

3.5 Diagnostic skin prick allergy tests

Diagnosis of allergen-specific persistent allergic rhinitis was confirmed with diagnostic skin prick tests. These work on the basis that IgE antibodies that recognize specific allergens are triggered by mast cells when exposed to these antigens, resulting in an allergic wheal and flare reaction.

Nineteen different common allergens were purchased in droplet form, in addition to skin prick lancets and hydrocortisone cream to dampen down any wheal and flare reaction obtained. Specific skin test mixtures are documented in Table 2.

If any participants were taking antihistamines prior to skin prick testing, these were omitted at least three days before the test. This was based on evidence suggesting that up to 10-50% of negative skin prick test results may be due to antihistamine usage (dos Santos RV, 2009). Each participant was initially interviewed as to whether they had a history, or a family history of any anaphylactic / severe allergic reactions previously. Only those who answered negatively were considered for the study. All skin prick tests were carried out in the ENT clinic, with availability of resuscitation equipment at hand.

Each participant's forearm was initially cleaned with 3 alcohol swabs. Each forearm was then delineated and prepared into three rows, allowing six skin pricks in two rows and seven skin pricks in the third. Nineteen clearly marked allergens were placed on the forearm and the skin epidermis beneath each drop was pricked with a lancet needle to allow exposure to specific allergens. As just the very surface of the skin is pricked, these were painless, and just enough to let a tiny amount of solution into the skin. A reaction was deemed to be positive, and allergy determined, when a wheal and flare reaction comparable to that obtained with the positive histamine skin prick developed following allergen exposure after

fifteen minutes. These results were duly recorded. Hydrocortisone 1% cream was spread onto the forearm to dampen down any positive inflammatory wheal and flare reactions obtained on completion of the skin prick test. Participants were encouraged to stay within the vicinity of the ENT clinic for 15 minutes after the procedure to ensure no adverse reactions. There were no recorded anaphylactic reactions or complications in any of the participants.

Table 2. Diagnostic skin prick test allergens.

Positive control (histamine)	Aspergillus fumigatus
Negative control (glycerol-saline)	Alternaria tenuis (A.alternata)
Dermatophagoides pteronyssinus	Penicillium notatum
Dermatophagoides farinae	Candida albicans
Dog hair	Duck feathers
Cat hair	Goose feathers
Horse hair	Wheat flour
Grass mix	Cow's milk
Tree mix (early blossoming)	Hen's egg (whole)
Cladasporium herbarum	

3.6 Physiological nasal secretion collection and nasal challenge with isotonic saline and amiloride

Nasal secretions may be collected via a number of different techniques. This can be through spontaneous secretion, dilution techniques, or absorption techniques (Riechelmann H, 2003) (Nathan RA, 2005). To most accurately obtain sufficient secretion for analysis, either dilution techniques or absorption techniques may be undertaken and are the most appropriate.

Dilution techniques include nasal lavage, with collection of the returned fluid (Raphael GD, 1989) (Austin CE, 1994) (Turner PJ, 2000). Absorption techniques can be performed via the placement of filter paper collection discs onto the nasal mucosa, which absorb nasal secretions directly (Baroody FM, 1993) (Riccio MM, 1996) (Sanico AM, 1999) (Sheahan P, 2005). For studies requiring assessment of the actual constituents of the secretion obtained, sampling with a polyurethane foam sampler technique is considered most useful (Riechelmann H, 2003).

As we were only measuring the weight of the nasal secretions, we used the absorption technique, in which pre-weighed, dry collection discs made from thick filter paper (Shandon, Pittsburgh, USA) were placed for a given period of time onto the anterior nasal septum. A 6mm paper punch was used to ensure that the discs were of uniform size and shape, so that they contacted a similar area of nasal mucosa on each occasion (Sheahan P, 2005). Discs were weighed using a laboratory TR208 weighing scale, accurate to 0.0001g.

Nasal secretions are absorbed from the area of mucosa with which the discs are in contact (Baroody FM, 1993) (Sheahan P, 2005). The use of thick filter paper discs was essential in order for the discs to have sufficient capacity to absorb large secretion volumes. The discs we used could absorb 40microL of fluid (Sheahan P, 2005). The filter paper disc technique thus allows measurement of nasal secretion weights, which can be calculated by subtracting the dry weight of the collection discs before placement into the nose, from the weight obtained after removal. A major advantage of this technique is that it allows secretions to be collected from both the ipsilateral and the contralateral side to the unilateral provocation, and thus allowing neurally mediated outcomes involving central reflexes to be assessed (Nathan RA, 2005). In contrast, secretion weights or volumes cannot be reliably measured using nasal lavage. The filter paper disc technique also has the advantage of being considerably better tolerated by volunteers.

All filter discs were initially pre-weighed to ascertain their baseline weights at 'Time=0'. Two discs were then placed on the anterior nasal septum of both nasal cavities following nasal challenge (described in the following section) for 2 minutes (Time = 2 minutes) and 5 minutes (Time = 5 minutes), see Figure 6. These were weighed subsequently, and their baseline weights were subtracted from their measurements to equate to nasal secretion weight.

Figure 6.

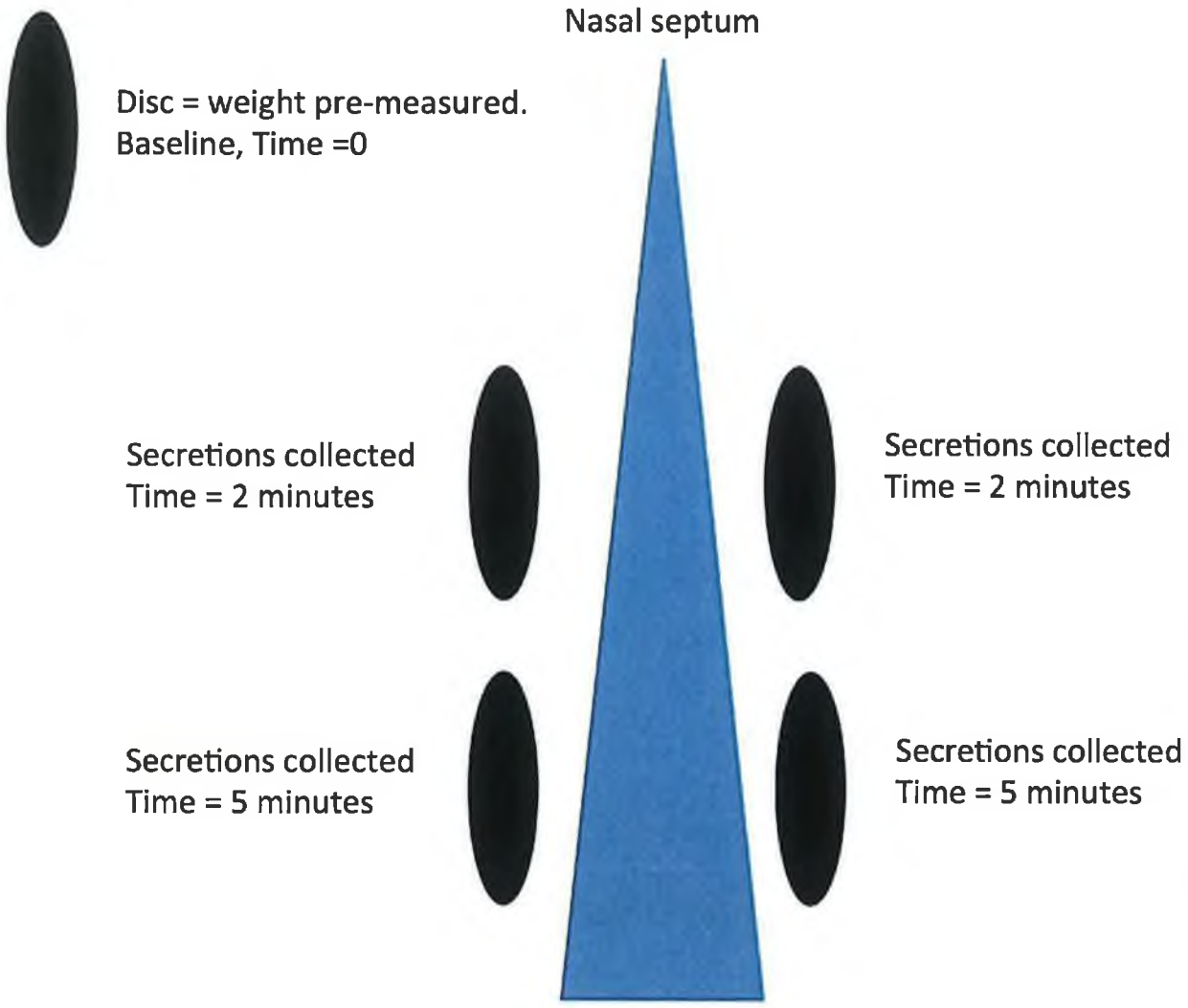


Figure 6. Structural design of clinical physiological nasal secretion weight collections. Standardised absorbable filter discs were applied to the anterior septum in both nasal cavities for 2 minutes and 5 minutes.

Technique of nasal challenge

Nasal challenge may be performed either by administration of the challenge substance into the nasal cavity by means of a nasal spray (Hofmann T, 1998) (Rodgers HC, 1999) (Turner PJ, 2000), or by placement of a filter paper disc containing a solution of the challenge substance in contact with the nasal mucosa (Baroody FM, 1993) (Riccio MM, 1996). The challenge substance then diffuses from the filter paper disc onto the nasal mucosa.

The use of a filter paper disc for nasal challenges can be advantageous, in the sense that a reproducible dose of the challenge substance may be administered on every occasion. Discs used must be of uniform size and shape, and contain a known quantity of the challenging substance. They must also be left in contact with the nasal mucosa for a uniform period of time, and if performed accurately, this technique of nasal challenge has been shown to lead to reproducible secretory responses (Baroody FM, 1993). However, the amount of challenge substance that elutes off the filter paper disc onto the nasal mucosa is unknown, and therefore, the use of nasal sprays can be equally advantageous, if the correct number of sprays is uniformly used on all occasions. We used the latter technique to good effect.

Each nasal cavity was initially assessed for patency using a Cottles nasal speculum and a headlight. This was to ensure that no marked septal deviation existed in the study participant that may prevent the challenge substance from reaching its intended target on the anterior nasal mucosa. Following assessment of

nasal patency in both nostrils, each spray was directed antero-superiorly and laterally. This targeted the nasal turbinates and anterior septum, to allow for accurate and standardised administration of the challenge substance in each and every case. It was accepted that there was always going to be the potential for variation in the amount of challenge substance delivered to the nasal mucosa not only between subjects, but also at different times within the same subject. This is due to variations in the degree of mucosal congestion, as may occur after allergen exposure in allergic subjects, or as part of the nasal cycle in normal or allergic subjects.

Two different solutions were used to promote nasal secretory response, using isotonic saline solution and buffered lactic acid in the form of aerosol sprays. Measurement of secretory response to saline and lactic acid challenges was undertaken using filter paper discs placed in both nostrils. Discs were pre-weighed in 5μL Eppendorf tubes and this constituted the baseline weight measurement

Isotonic saline solution was made up into an aerosol spray using pre-used co-phenylcaine bottles, with disposable nozzles. A pH measurement of the solution was obtained with the Eutech EcoScan pH/°C meter, which measured pH 5.5 undiluted. Standardised isotonic solution was used for all experiments from the same bottle to ensure accurate pH measurements for all participants. Three sprays of this saline solution were applied to one nostril randomly selected before the procedure and allowed to absorb into the nasal mucosa.

Following that, two fresh filter discs were placed in each nostril, once again on the anterior nasal septum just posterior to the nasal columella. Ipsilateral and contralateral nasal secretions were collected over 2 minute and 5 minute durations, measuring the ipsilateral direct nasal secretory response, and the contralateral neural-mediated reflex response, see Figure 7. These measurements were then compared with the baseline nasal secretion weights.

Figure 7.

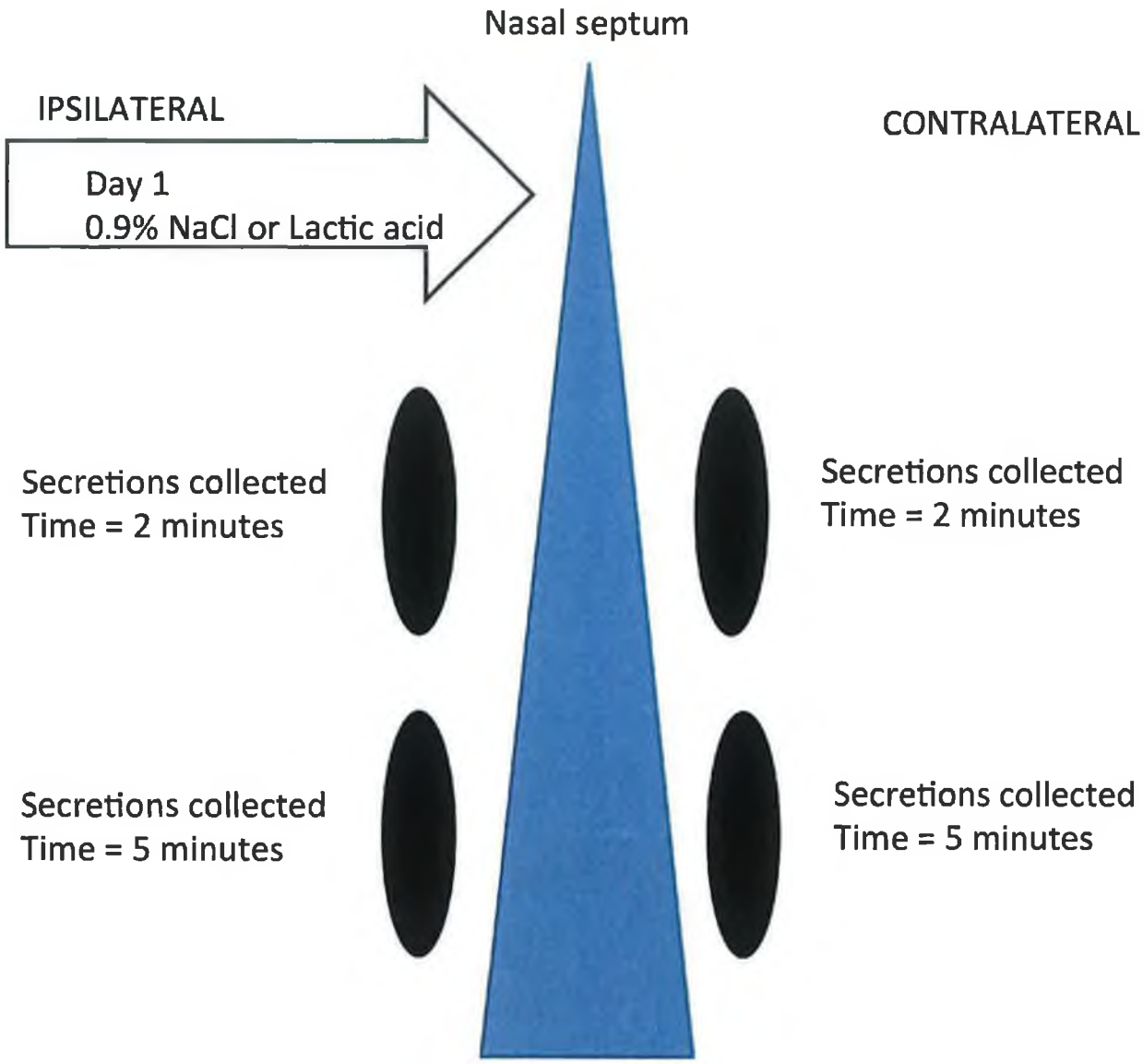


Figure 7. Nasal secretion weight collections following nasal challenge with isotonic saline or lactic acid. This was carried out on Day 1 of physiological experiments, followed on Day 2 by pretreatment with ASIC-inhibitor amiloride.

The following day, participants were invited back, and their nasal mucosa pretreated with amiloride spray. This was made up into an aerosol spray, diluted to 1x10-3M solution, at its limit of solubility with sterile water. This was calculated using the formula weight of amiloride of 266.1g/L to make 1M and its solubility in water of 50mg/mL. Therefore, 0.2661mg/mL was required for dilution to 1x10-3M, and 1g of amiloride was added to 3.757L of water to ensure this dilution.

This was introduced to the same nasal cavity that was challenged with isotonic saline the previous day. Nasal challenge was once again instituted into the same nasal cavity (termed the ipsilateral side) with isotonic saline, half an hour after amiloride pre-treatment, and weight collections at 2 minutes and 5 minutes were compared with results taken the previous day without amiloride pre-treatment, see Figure 8.

Figure 8.

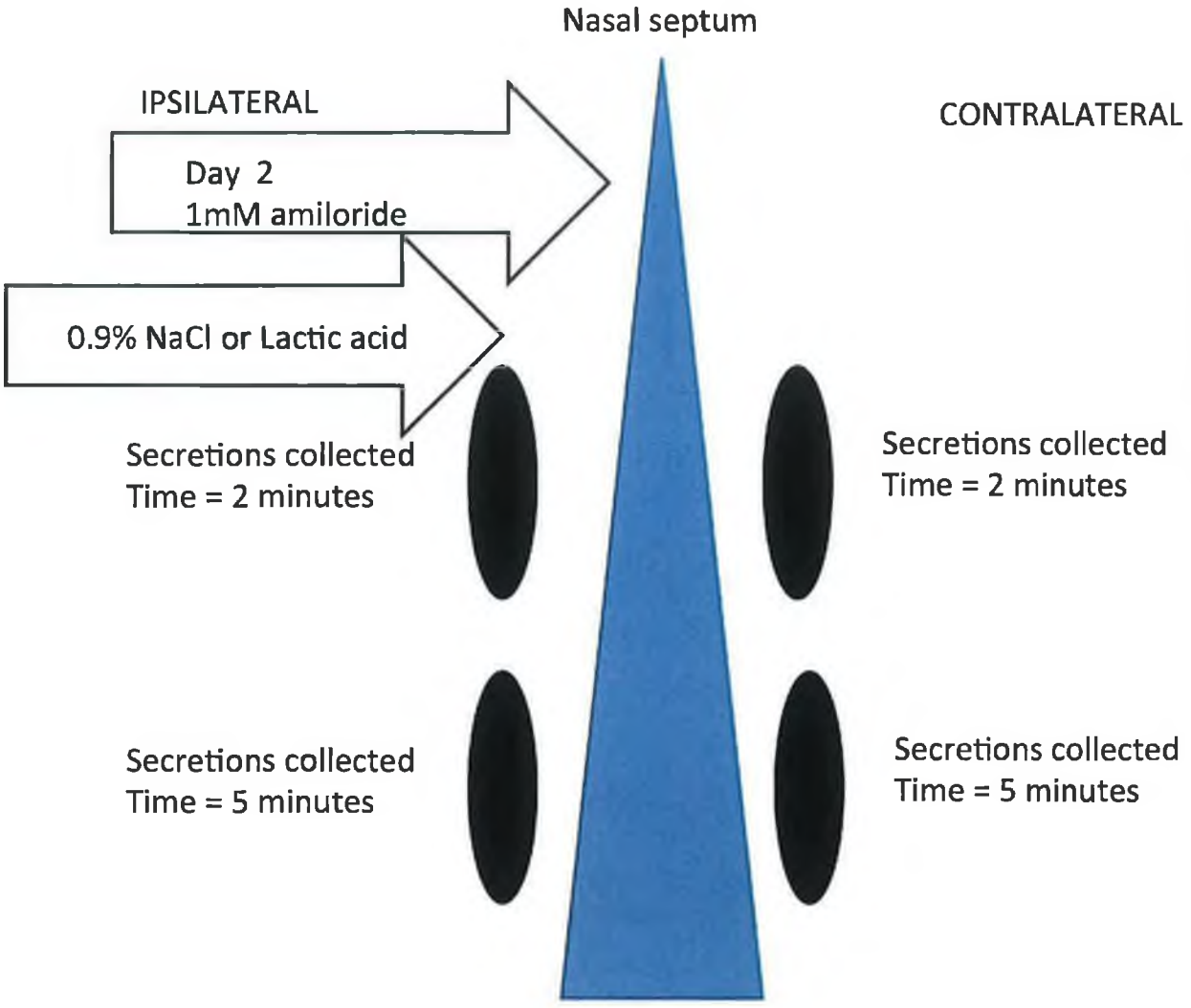


Figure 8. Nasal secretion weight collections following amiloride pre-treatment of nasal cavity, and following nasal challenge with isotonic saline or lactic acid. Amiloride was hypothesised to block ASIC receptor channels, leading to decreased nasal secretion weights compared to Day 1 collections following identical nasal challenge.

3.7 Physiological nasal secretion collection and nasal challenge with lactic acid and amiloride

The second solution used to promote nasal secretory response was 15mM lactic acid. From lactic acid's molecular weight of 90.08g/L to make 1M concentrations, 0.09008mg/mL was required to make 1mM concentrations. Further calculations derived that 1.3512mg/mL was required to make 15mM concentrations. Thus, 1g of lactic acid was diluted with 0.74L of sterile water to achieve the required concentration.

Lactic acid was then buffered using sodium hydroxide (NaOH) to achieve the desired pH range. Unbuffered lactic acid at low pH would have caused significant discomfort when sprayed onto the nasal mucosal lining. Seven point four (7.4) mL of lactic acid was buffered with 51μL of 2M alkaline buffer from a commencement pH of 4.5 to a final pH of 7.0. Final pH of the buffered solution was confirmed using the Eutech EcoScan pH/°C monitor. This was instilled as an aerosol spray in the same manner as the saline spray, into a randomly selected nostril of each participant.

Similar measurements of nasal secretion weights were undertaken using the same two-filter disc technique as previously with saline spray. Once again, participants were invited back the following day for pre-treatment with amiloride spray, and the process of nasal secretion collection repeated again, see Figures 6, 7, and 8.

3.8 Inferior turbinate nasal biopsies

All nasal mucosal biopsies were carried out within the setting of the ENT clinic with resuscitation equipment present. Three (3) nasal mucosal biopsies were taken under local anaesthetic from either the right or left anterior aspect of the inferior turbinates for each study participant. The inferior turbinate was chosen as the biopsy site for ease of access, and also due to previous studies showing that the parasympathetic regulation of glandular secretion and vasomotor tone is especially prominent in the inferior turbinate (Nakaya M, 2002). Co-phenylcaine (lignocaine hydrochloride 5% and phenylephrine) spray was used for its local anaesthetic and mucosal vasoconstrictive properties, as previously described by Thornton et al (Thornton MA, 2004). Previous descriptions of biopsy techniques using cocaine (Fokkens WJ, 1988) were not considered, with regards to the strict control and monitoring required for such a controlled substance.

Three sprays were instilled into the chosen nasal cavity, and cotton wool impregnated with co-phenylcaine was then placed adjacent to the inferior turbinate for 15 minutes thereafter (see Figure 9). Access to the inferior turbinate was enabled by a Cottles nasal speculum, under direct visualization using a headlight. Three (3) biopsies were taken from the anterior portion of the designated inferior turbinate using micro-cutting biopsy forceps once anaesthesia had been achieved. Only mucosa directly held up against the cutting forceps blade was taken, without further tearing any mucosa adjacent to the biopsy site. Haemostasis was secured post-biopsy with co-phenylcaine-impregnated pledgets held up against the biopsy site under direct vision.

Two biopsy samples were placed in cryotubes and fresh frozen in liquid nitrogen, and stored at -80 degrees Celsius for later PCR quantification of cDNA. The third biopsy was immersed and stored in formaldehyde solution at room temperature for use in immunohistochemical staining, (Figure 10).

All biopsies were adjudged to be pain free when participants were interviewed about their comfort immediately after the biopsy. All participants were encouraged to remain within the vicinity of the ENT clinics for a further 30 minutes to ensure adequate help was available should complications, such as persistent bleeding, arise.

Figure 9.

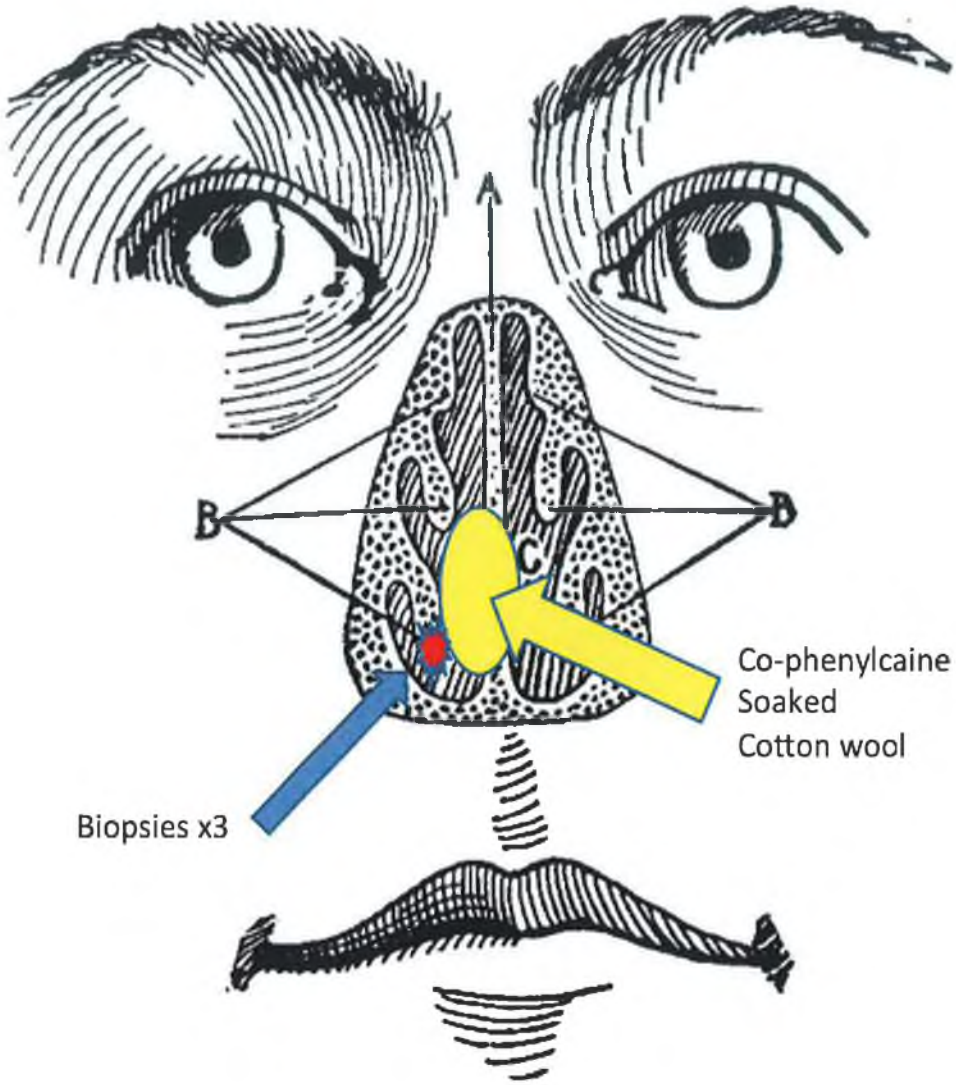


Figure 9. Inferior turbinate nasal mucosal biopsies taken under local anaesthesia. A chosen nasal cavity was prepared with co-phenylcaine spray and co-phenylcaine-soaked cotton wool until adequate anaesthesia was obtained. Three biopsies of inferior turbinate mucosa was obtained with cutting biopsy forceps.

Figure 10.



Figure 10. Distribution of biopsies for PCR and immunohistochemical staining.

3.9 Purification of total RNA from human tissue

Purification of total RNA was undertaken with the RNeasy mini kit. Fresh frozen nasal mucosal biopsy samples were thawed from -80°Cin ice. Prior to use, beta-mercaptoethanol was added to Buffer RLT in a ratio of 10μL per 1mL of Buffer RLT. Six hundred (600) μL of RLT buffer / beta β-mercaptoethanol mixture was added to each tissue specimen for disruption.

Cell wall and plasma membrane disruption to release all RNA contained within the sample was carried out with pestle and mortar for 30 seconds, and the lysate homegenised by passing the lysate 10 times through an insulin syringe and 20 gauge needle. This was then centrifuged at 4°C for 3 minutes at 10,000 revolutions per minute.

The supernatant was removed into fresh Eppendorf tubes, labeled tubes A, and 600 microlitres of 70% ethanol was added to each. This mixture was pippetted to mix.

Six hundred (600) µL of solution in tube A was then transferred to 2mL filter spin columns tubes and centrifuged for 15 seconds at 10,000 revolutions per minute at 4°C. The flow through at the bottom of the spin column tube was then discarded.

A further 600µL from tube A was again added to the filter spin column tubes and centrifuged again for 15 seconds at 10,000 revolutions per minute at 4°C. The flow through was again discarded.

Seven hundred (700) µL of Buffer RW1 was then added to the spin column tubes and centrifuged for 15 seconds at 10,000 revolutions per minute at 4°C. The flow through was again discarded.

Five hundred (500) µL of Buffer RPE was then added to the filter spin column tube and centrifuged for 15 seconds at 10,000 revolutions per minute at 4°C. The flow through was discarded.

A further 500µL of Buffer RPE was added to the filter spin column and centrifuged for 2 minutes at 10,000 revolutions per minute at 4°C to wash the spin column membrane. The collection tube was then discarded upon removal of the spin column.

The filter spin column was placed into new 1.5mL Eppendorf tubes, into which 30µL of RNAse-free water was added. This was centrifuged for 1 minute at 10,000 revolutions per minute at 4°C. Total RNA present at the base of the new collection Eppendorf tube was then stored at -20°C for cDNA preparation.

3.10 Elimination of genomic cDNA

Elimination of genomic cDNA was undertaken with a QuantiTect reverse transcription kit. RNA samples stored at -20°C were thawed on ice. Seven (7) µL of RNA were added to a master mix of 2µL of gDNA wipeout buffer and 5µL of nuclease-free water per sample in 500µL Eppendorf tubes. This solution was vortexed for a few seconds to mix. The tubes were then placed in a DNA Engine PCR thermal cycler and run on gDNA Wipe program for 2 minutes at 42°C. The tubes were then removed, centrifuged at 8,000 revolutions per minute at 4°C and stored on ice for cDNA reverse transcription.

3.11 Reverse transcription of cDNA

Following wipeout of genomic DNA, single stranded RNA was reverse transcribed into complementary DNA (cDNA) using reverse transcriptase enzyme from the QuantiTect reverse transcription kit.

Primer-buffer premix was made up with 1µL of RT primer mix to 4µL of 5X RT buffer. A total of 5µL of premix was required for each sample reaction.

A master mix comprising 5µL of premix to 1µL of RT enzyme was then made up. This was vortexed to mix thoroughly.

Six (6) µL of this master mix was added to 14µL of each single stranded RNA sample and vortexed on ice. This solution was then centrifuged at 8,000 revolutions per minute for 15 seconds.

The samples were then run on the DNA Engine thermal cycler QuantDNA program for 15 minutes at 42°C and 3 minutes at 95°C, following which samples

were centrifuged at 8,000 revolutions per minute for 15 seconds and stored at -20 °C for further quantification polymerase chain reaction.

3.12 Polymerase chain reaction and purification of cDNA for standards

High-quality nucleic acid templates for PCR amplification were obtained via purification with the Wizard PCR Preps DNA Purification System.

Direct purification from PCR amplification

One hundred (100) µL of Direct PCR Purification Buffer was aliquoted into a tube. Thirty (30) to 300µL of PCR reaction was added to this, and vortexed briefly to mix. One (1) mL of resin was added and vortexed briefly 3 times over a 1 minute interval.

PCR product purification

A Wizard™ Minicolumn was prepared for each sample to be purified. The plunger was removed from the 3mL Luer-Lok™ syringes, and the syringe barrel attached to the Minicolumn. The resin/DNA mix was added to the syringe. The plunger was inserted, and the resin/DNA slurry pushed into the Minicolumn.

Washing

The syringe was detached from the Minicolumn, and plunger removed from the syringe barrel. Two (2) mL of 80% isopropanol was added, and this isopropanol was pushed through the Minicolumn with the syringe plunger. The syringe was

Table 3: Primers used in this study

Gene	Primer sequence
β-actin	5'-GGACTTCGAGCAAGAGATGG3' (forward) 5'-AGGAAGGAAGGCTGGAAGAG-3' (reverse)
ASIC-1	5'-GCTATGGCAAAGAGCTGTCC-3' (forward) 5'-GTCCAGCACCAGGATGTTCT-3' (reverse)
ASIC-2	5'-ACAGGAGCAGAGGCTCACAT-3' (forward) 5'-CGGGTCTCACAGTCAATCCT-3' (reverse)
ASIC-3	5'-TGGAGGGACAATGAGGAGAC-3' (forward) 5'-CTGGCAAGAAACAAAGGTC-3' (reverse)

Master Mix Solution

The QuantiTect SYBR Green PCR (Qiagen) kit was used for amplification of cDNA via the Light Cycler ™ 1.0 (Roche), using SYBR Green 1, a ready to use reaction mix for PCR. These were used according to the manufacturer's instructions.

The primers were diluted first in a ration of 4microL of primer to 16μL of PCR-grade water.

A master mix was then prepared with the following components in fixed ratios:

10μL SYBR Green

0.5μL ASIC forward primer

0.5μL ASIC reverse primer

7μL PCR grade water

This made up a master mix of 18μL, which was then pipetted into a pre-cooled capillary tube in a cooling block. To the capillary tube was then added 2μL of cDNA, which was then sealed with a stopper. This tube was then centrifuged at 1000 revolutions per minute for 5 seconds in a bench-top centrifuge. The capillary tube was then placed in the rotor of the LightCycler machine, and cycled as described and compared to similar capillary tubes made up with 2μL of standards. This was repeated for each of ASIC-1, ASIC-2 and ASIC-3 primers.

Real time PCR cycling parameters

Study samples were continually monitored during PCR. The samples were initially denatured at 95°C for 15 minutes, followed by 35-40 cycles of denaturation, annealing, and extension at 95°C for 15 seconds, 55°C for 25 seconds, and 72°C for 11 seconds. For beta-actin, this was set out as 95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 20 seconds.

Characteristic melting curves were obtained at the end of the amplification process, by cooling the samples to 65°C for 15 seconds, followed by further cooling to 40°C for 30 seconds.

Serial ten-fold dilutions were prepared from individual PCR products, to be used as standards to plot against the unknown quantity study samples. Quantification of data was analysed using the LightCycler ™ analysis software, and values were normalized to the level of beta-actin expression for each sample on the same template cDNA.

Melting curve analysis

A melting curve is produced following PCR. Due to the melting behavior of DNA, fluorescence will decrease with an increase in temperature. With the SYBR Green 1 format, this is due to separation of double strands and consequent release of SYBR Green 1 molecules, resulting in a drop in fluorescence. The whole purpose of analyzing the melting curve is to determine the characteristic melting temperature of a target cDNA. This temperature supplies information useful for product identification and indentification of unwanted by-products, such as

primer-dimers. Display and analysis of this data was performed using the melting curve analysis tool of the LightCycler ™ software (www.qiagen.com/HB/_primerAssay).

3.14 ASIC receptor localisation with immunohistochemical staining

Immunohistochemistry was performed on 5μm formalin-fixed, paraffin-embedded (FFPE) tissue sections using a polyclonal antibody to ASIC-3 (1:50) and a monoclonal antibody to PGP9.5 (1:50). We specifically targeted ASIC-3 as this was the only ASIC receptor to show any significant difference in expression between Control and Allergic Rhinitis groups (as documented in the results section).

Sections were deparaffinized, and antigen retrieval was performed in 0.01 Km Tris-EDTA (pH 9.0) in a pressure cooker, at full steam pressure for 2 minutes. Primary antibodies were then incubated on sections overnight at 4°C and detected using peroxidase-labeled Envision anti-rabbit or anti-mouse secondary antibodies with DAB as peroxidase substrate. As a control, the samples were processed in the same manner but using isotype rabbit or mouse IgG, in place of the primary antibodies.

For dual-immunofluoresence, sections were subject to antigen retrieval, and then incubated overnight at 4°C in a mix of anti-ASIC-3 and anti-PGP9.5.

After washes in TBS, bound antibodies were detected by incubation in a mixture of goat anti-rabbit Alexa-488 and goat anti-mouse Alexa-568 (both 1:500) for 2 hours at 37°C.

After final rinses in TBS, sections were mounted in 4,6-diamidino-2-phenylindole Vectashield. Mounted sections were analyzed, and images were acquired using a Leica DF350 FX digital camera and processed using Leica FW4000 software.

Immunostained sections were evaluated in a blinded manner without knowledge of the clinical status of the patients. Ten non over-lapping high power fields from the surface epithelium and submucosal regions were evaluated for expression of ASICs 3. The intensity of staining was evaluated as 0, 1+ 2+ and 3+ for no expression, weak staining, medium staining and abundant staining respectively

3.15 ASIC receptor protein identification with Western Blotting

1. Gel Electrophoresis Stage

Separating gel was made up and added to 1 cm of top of gel set-up. This was layered with ethanol and allowed to set for 5 -10 min. Stacking gel was made up in the mean time. When the separating gel was set, the ethanol was removed and poured onto the stacking gel. The comb was inserted making sure no bubbles had formed. This was left to set for 30 minutes. The amount of protein in samples was measured. The hot plate was preheated to 94^0C. All samples were made up to the same amount (~10ul) using sterile distilled water (SDW). The same amount of SDS sample treatment buffer was added in the fume hood and proteins were denatured for 5 minutes.

2. Running blot

The side supports and elastic were removed from the gel and added to the chamber. Backing support was added to the other side. Three hundred and fifty (350) ml IX running buffer was made up (70 ml 5X buffer and 280 ml dH2O) and gradually added to the electrophoresis chamber, ensuring no bubbles existed. The comb was then carefully removed. Ten (10)ul of molecular weight marker and - 20ul of sample was added to the wells. This was run at 30mAmps/gel > 500 V > 20W for approximately 1 hour or until the dye had run to the bottom of gel.

3. Transferring Blot

Four blotting papers and one nitrocellulose were cut. One thousand two hundred (1200)ml of transfer buffer was added to the chamber. The blot sandwich was made up with:

- White grid
- Two foams
- Pre-wetted blotting paper
- Pre-wetted nitrocellulose
- Gel cut to size
- Pre-wetted blotting paper
- Two foams
- Black grid

Everything was clipped together and placed in the transfer chamber with the white side facing forward to the +ve side. This was run at 80 mA, 500 V, 30 W overnight.

4. Blocking Stage

The blot was removed from the sandwich, and Ponceau S solution added. This was left for a few minutes to show up proteins. This was then removed by washing under the tap. The blot was immersed in blocking buffer and left for 1 hour. The buffer was then tipped out and the sheet placed on tissue paper.

5. Washing and primary antibody stage

Primary antibody was made up in blocking buffer and 5 ~ 10 ml added to 50 m1 tubes. The blot was carefully placed in the tube, sealed with parafilm and rotated for 2 hours. The blot was removed and washed with 50 m1 washing buffer 6 times for 5 minutes on a shaker. Secondary antibody was made up in blocking buffer. The blot was immersed in antibody and shaken for 1 hour. The washing procedure was repeated to remove secondary antibody.

6. Assay & Substrate stage

Assay buffer was made up and the blot was washed with 10ml twice for 2 minutes to prepare for substrate. The substrate was made up just prior to use, and pipetted onto blot placed on a plastic sheet. This was left for 5 minutes. Excess substrate

was removed from the blot and sandwiched between the plastic folder. This was then exposed to x-ray film for 30 seconds to 1 minute.

3.16 IMR32 Cell cultures

Proliferation and differentiation media were made up (see Reagents).

Thawing:

Cells were thawed quickly by placing cryovial in a 37°C incubator. The contents of the cryovial were then transferred into 5ml medium containing 10% FCS (twice as much FCS is needed to initially boost cells; when first passage needed, ordinary 5% FCS Proliferation Medium was used) in a sterile 15mL conical tube. Cells were spun at RT, 1300 rpm for 10 minutes. The supernatant was discarded and cells resuspended in 1mL 10% FCS medium. The cells were transferred to a culture flask (T75) containing 15mL 10%FCS medium (prewarmed to 37°C). These were then labelled and incubated at 37°C, with 5% CO2.

Passaging:

Old media from the culture flask were poured off down to approximately 0.5mL. The flask was tapped until cells sheeted off from the bottom of the flask. Five (5)mL fresh Proliferation Medium (prewarmed to 37°C) was added, and remaining cells were rinsed off the bottom of the flask. The medium with suspended cells was transferred into a sterile 50mL conical tube. Approximately 10μL of cells were removed into a sterile Eppendorf tube for counting. Cells were

spun at 1300 revolutions per minute, RT, for 10 minutes. Based on cell count, cells were resuspended in an appropriate volume of proliferation media and cells were passed through a syringe with a 21G needle (green) 3-4 times to break up clumps of cells. These cells were transferred to flasks and plates as appropriate. (For example, 3x 106 cells in T75 flask will be confluent in 2-3 days).

Differentiation:

Cells were plated to an appropriate number on cell culture plates. At 24-48 hours after plating, Proliferation Medium was replaced with Differentiation Medium. Cells were allowed to grow for 7- 8 days, with the medium changed every 1-2 days.

Freezing.

Cells were removed from the bottom of the flask and spun down as in Passaging. Based on cell count, cells were resuspended in Freezing Mix (90% FCS; 10%DMSO) at approximately 5x 106 per mL and 1mL placed in each cryotube. These were labelled and placed at -80°C overnight before transfer to liquid nitrogen.

3.17 Cell Culture: In vitro experiments with eosinophil peroxidase in co-culture with 16HBE14o cells.

Eosinophils and their released granule proteins are found in association with airway epithelial cells, mucous glands and nerve cells in patients with persistent AR compared to control subjects (Godthelp T 1996 and Thornton MA et al,

unpublished). Since these are the sites at which ASIC-3 expression is also distributed, we investigated the effect of eosinophils and, in particular, their granule proteins on the expression of ASIC-3 in vitro.

16HBE14o- cells were cultured in minimal essential medium (MEM) supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin/streptomycin. Eosinophil peroxidase (EPO) was added at 1μg/mL for time points between 1 and 24 hours. In experiments designed to investigate the role of MAP-kinases, cells were pre-treated for two hours with the ERK1/2 inhibitor PD98059 (50μM). After removal of media, total RNA was isolated from the cells with TRI reagent™, according to the manufacturer's instructions. cDNA preparation from 1μg RNA and real-time PCR for ASIC-3 and β-actin from cDNA preparations was then carried out .

3.18 Visualization of ASIC-3 expression by epithelial cells.

The NuLi-1 (normal lung) and CuFi-1 line, derived from a cystic fibrosis patient with Δ508/Δ508 genotype were used for confocal and fluid secretion studies. The cell lines are able to form differentiated polarised monolayers that exhibit transepithelial resistance and maintain ion channel physiology for the genotypes. After 4-6 weeks of cell culture, the cells formed a polarised confluent monolayer with a high transepithelial resistance (TEER) of > 800 Ω/cm^2. Confluent monolayers of cells were exposed to EPO 1μg/mL applied to the basolateral surface for varying time points. The inserts were fixed with methanol and stained by incubating with the primary antibodies (rabbit polyclonal anti-ASIC-3 antibody

from Neuromics diluted to 1:50 in phosphate-buffered saline with 3% bovine serum albumin for 1 hour on ice. The samples were rinsed in phosphate-buffered saline following blocking buffer incubation, and exposed to the secondary antibodies (1:1000) for 2 hours at room temperature. The monolayers were counterstained with rhodamine phalloidin (1:200) to highlight the actin cytoskeleton in cells for 2 hours at room temperature. Control experiments were performed with rabbit IgG controls. The samples were mounted and imaged using a Zeiss LSM 710 laser scanning confocal microscope. Images were analyzed using ZEN software.

3.19 Statistical analysis and data interpretation

Comparisons of the nasal secretion weights collected was made using paired samples of values of nasal secretion weights obtained at baseline, 2 minutes and 5 minutes. Sample weights in milligrams were added to a datasheet on the statistical package Prism data base, from which means, standard deviations and standard error of the mean (SEM) as well as other basic descriptive data were derived. In this thesis the graphical representation is shown as mean with SEM. In addition the programmed allowed comparison of weights by experiment, in which case ANOVA, with Bonferroni correction for comparisons of the various time points was made, and when specific time points were compared e.g. with or without the inhibitor at 2 or 5 minutes, a paired student t-test. For the real time quantitative PCR, comparisons were made using ANOVA with corrections for multiple comparisons.

Graphs were drawn for comparison of nasal secretion weights following nasal challenge with each of the challenge substances, and nasal secretion weights following amiloride pre-treatment, to assess for any attenuation of nasal secretion following ASICs inhibitor. Direct comparisons were made between 'Time 0' and 'Time 5 minutes' time points.

Graphs were also prepared in both scatter format and column format, to show the difference in PCR quantification of ASICs in both controls and participants with persistent allergic rhinitis.

Comparison of the extent of immunohistochemical staining of ASIC-3 receptors between controls and allergic rhinitis patients was also graphically represented.

Induction of ASIC-3 mediated by eosinophil peroxidase acting via an ERK1/2 dependent pathway was also demonstrated graphically.

3.20 Measurement of airway surface liquid height in response to eosinophil peroxidase

To determine if ASIC were functional, in vitro measurements of the airway surface liquid (ASL) were carried out. These experiments were performed as described previously (Coakley RD 2001) and carried out by my colleague, Dr M al-Alawi, at the Respiratory Research lab in Beaumont Hospital. Briefly, over a period of 40 hours the ASL was intermittently measured to determine the mean plateau value of the stabilized ASL height. These results were included in this thesis as additional evidence that ASIC receptors are functional.

CHAPTER 4 – RESULTS

4.1 Secretory responses to topical application of lactic acid

Functional experiments, with isotonic saline and lactic acid, buffered to pH 7.0 were carried out to determine if ASIC-3 receptors were activated by acidic solutions, and if there were differences between patients with allergic rhinitis compared to healthy controls. In physiological experiments carried out with isotonic saline, no significant differences were noted between controls and participants with AR. These results are thus not documented.

Lactic acid was buffered using sodium hydroxide (NaOH) to achieve the desired pH range (see Methods chapter). Final pH of the buffered solution was confirmed using the Eutech EcoScan pH/°C monitor.

There were 23 subjects in the control group and 19 subjects with a clinical diagnosis of persistent allergic rhinitis, as defined by the ARIA criteria (Bousquet J 2008). Nasal challenges with lactic acid were performed before and after pretreatment with ASIC-inhibitor amiloride, ($1x10^{-3}$M), and at the specified time points, nasal secretion weights from the ipsilateral and contralateral sides of the challenge were recorded. Lactic acid administration caused no significant uncomfortable sensation, e.g. burning or irritation in either subgroup of subjects.

There was a significantly increased nasal secretory response on the ipsilateral side in patients with AR in response to lactic acid. After 5 minutes, the secretions had increased from a baseline of 3.42±0.80mg to 10.17±2.61mg, p=0.01 and these enhanced secretary responses were then blocked by amiloride (1mM). Lactic acid induced secretions were noted to be attenuated by pretreatment with amiloride, see (Figure 11A). Although there was still a significant rise in secretions after 5

minutes even after amiloride-blockade, the increase in secretions were not as high as when measured without amiloride-blockade.

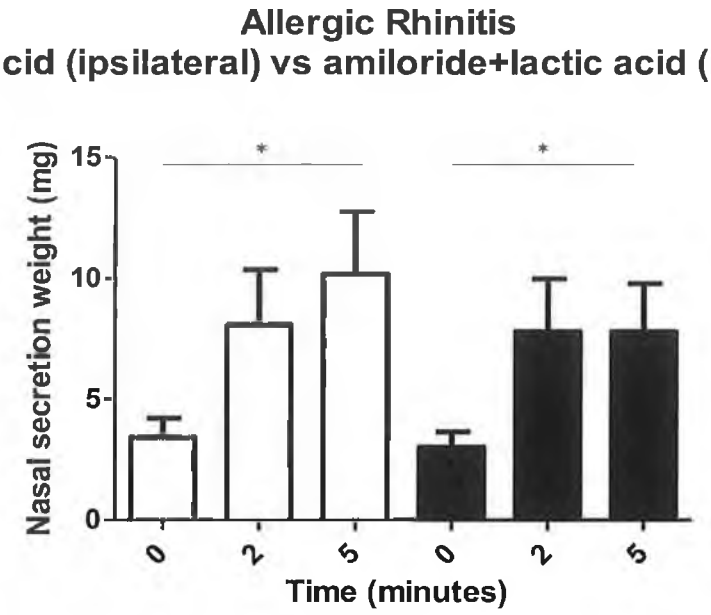
There was also a smaller but significant increase in the secretions in the control subjects after lactic acid administration, rising from a baseline of 3.76±0.65mg to 8.86±2.23mg, p=0.02. However, amiloride did not prevent this rise; in the presence of amiloride pre-treatment, secretions still rose from 3.58±0.55mg to 7.79±1.10mg, (p=0.008), which may suggest that the response might not be mediated through ASIC channels (Figure 11B).

Secretory responses were also recorded on the contralateral side to which the lactic acid had been administered, to assess if there was a reflex response to the administered lactic acid. This data showed there were no significant changes in contralateral secretions in either the AR (Figure 11C) or control groups (Figure 11D), in response to lactic acid.

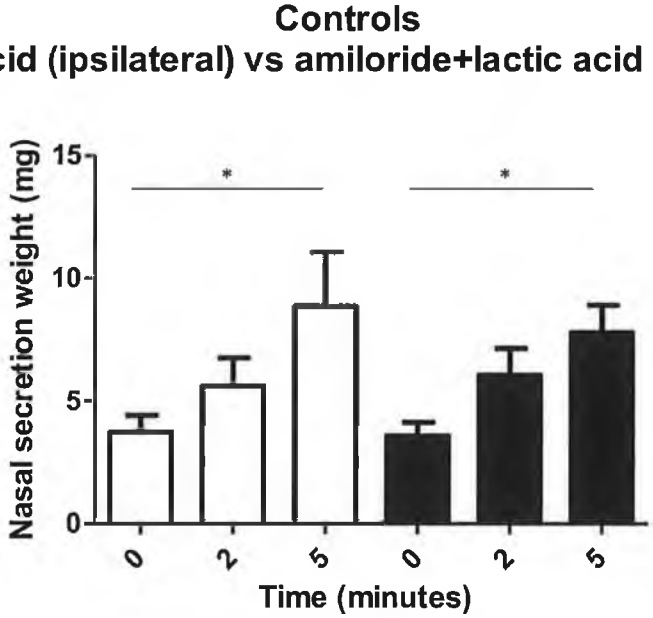
Appendices 2 and 3 record nasal secretion weights.

Figure 11 A-D

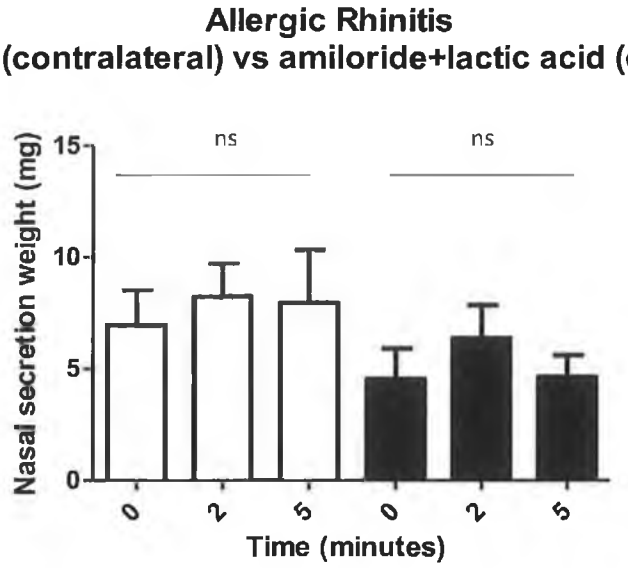
A



B



C



D

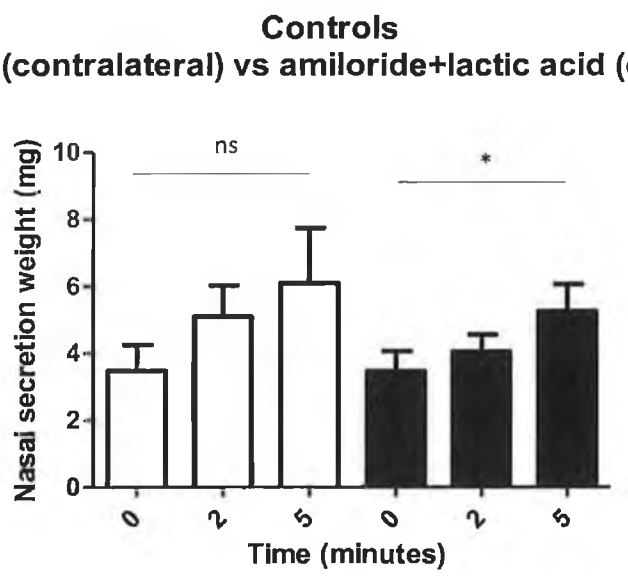


Figure 11 A-D Lactic acid induces a significant increase in secretion in the ipsilateral nasal mucosa which is attenuated by pre-treatment of the ipsilateral nasal mucosa with amiloride (1x10-3M) in subjects with allergic rhinitis but not control subjects (A and C). Graph shows the changes in nasal secretion for

subjects with allergic rhinitis who were administered lactic acid (pH=7.0)(open boxes) or pre-treated with amiloride and then challenged with lactic acid (closed boxes) as described in the Methods section. Lactic acid did not induce significant increased nasal secretions in the contralateral side in either AR or control subjects (B and D).

4.2 Real time PCR of inferior nasal turbinate biopsies

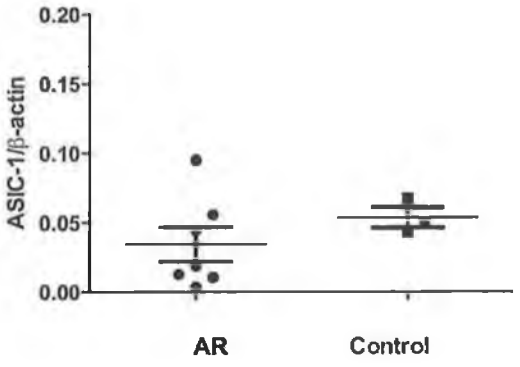
Various ASIC receptors in nasal biopsies were quantified and compared, to assess which types were upregulated, and whether these were upregulated solely in AR patients.

Real time PCR quantification of ASIC receptors in nasal biopsies demonstrated the presence of ASIC-1 in both control and AR subjects but there was no significant difference in the level of its expression between the AR and control subjects (Figure 12A). There was clear evidence of upregulation of ASIC-3 receptors in patients with AR compared to healthy control subjects (Figure 12B and Figure 13) ($p<0.02$). There was no appreciable detection of ASIC 2 in control or AR subjects (data not shown).

Appendices 4,5 and 6 record ASIC1, ASIC2 and ASIC3 quantification.

Figure 12 A-B.

A



B

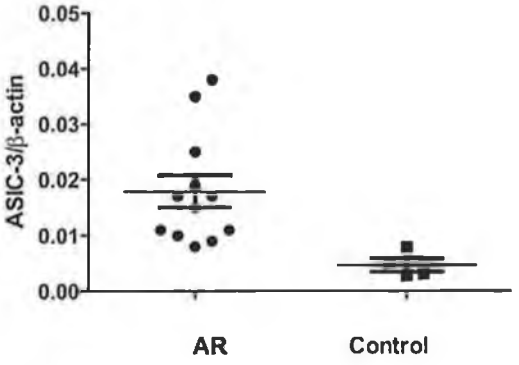


Figure 12 A-B. Airway expression of the mRNA for ASICS 1(A) and 3(B) quantified by real time PCR is shown. Nasal biopsies from the inferior turbinate of control (n=12) and allergic rhinitis subjects (n=13) were obtained as described in the Methods section. The DNA was extracted, reverse transcribed, subjected to real time PCR and the levels of expression normalized to the housekeeper b-actin. Expression of mRNA from ASIC-1 was detected in a few subjects, no ASIC-2 was detected in any subjects (data not shown) and there was expression of ASIC-3 in both control and AR subjects. The level was increased in the AR subjects, p =0.02.

Figure 13

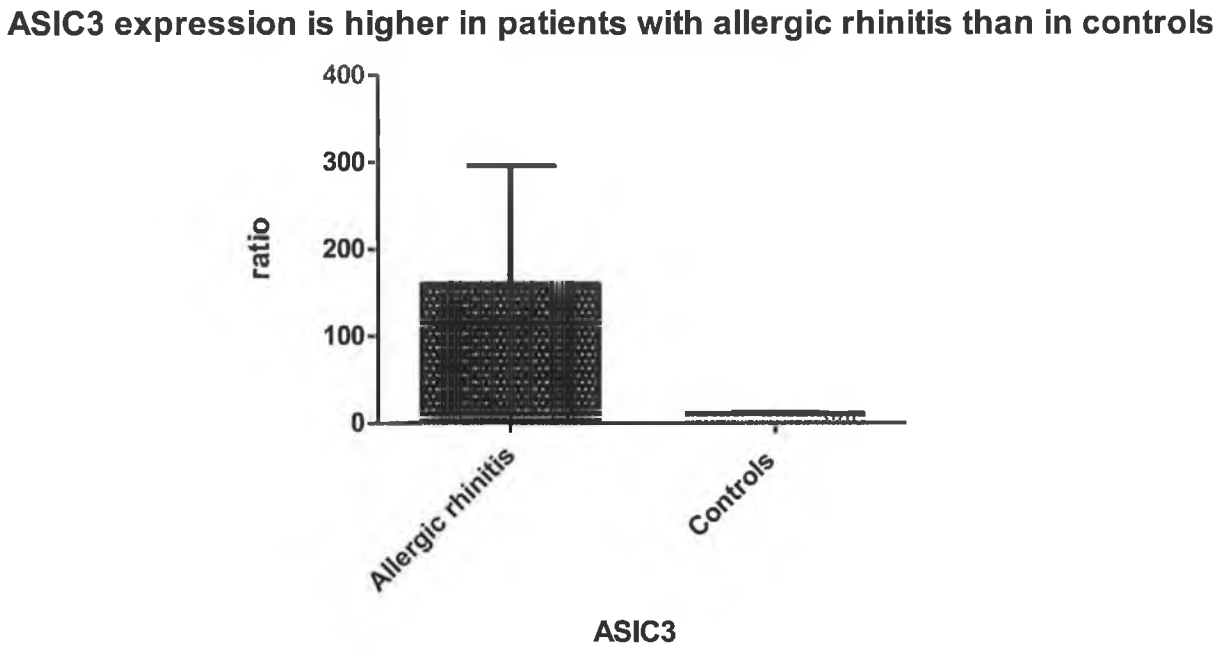


Figure 13. There is higher expression of ASIC-3 mRNA as quantified by real time PCR in patients with allergic rhinitis compared to controls.

4.3 Immunohistochemical localisation of ASICS-3 in the nasal epithelium

Since ASIC-1 transcriptional levels did not differ between AR and control subjects and because there was no appreciable detection of mRNA for ASIC -2 in the nasal biopsies, we proceeded only to localize the presence of ASIC-3, which was expressed in high levels in the AR subjects. This was alluded to in the Methods section.

There was immunostaining for ASIC-3 on the epithelial surface and epithelial glands in both AR (Figure 14A) and control subjects (Figure 14B). An isotype control antibody showed no staining (Figure 14C). There was also strong staining in the nasal mucous glands (not shown). Semi quantitative counts of the degree of epithelial cell staining intensity were performed by two independent observers. The results show an increase in the staining of ASIC-3 in the subjects with AR compared to the control subjects, $p=0.05$ (Figure 14D).

Figure 14 A-D.

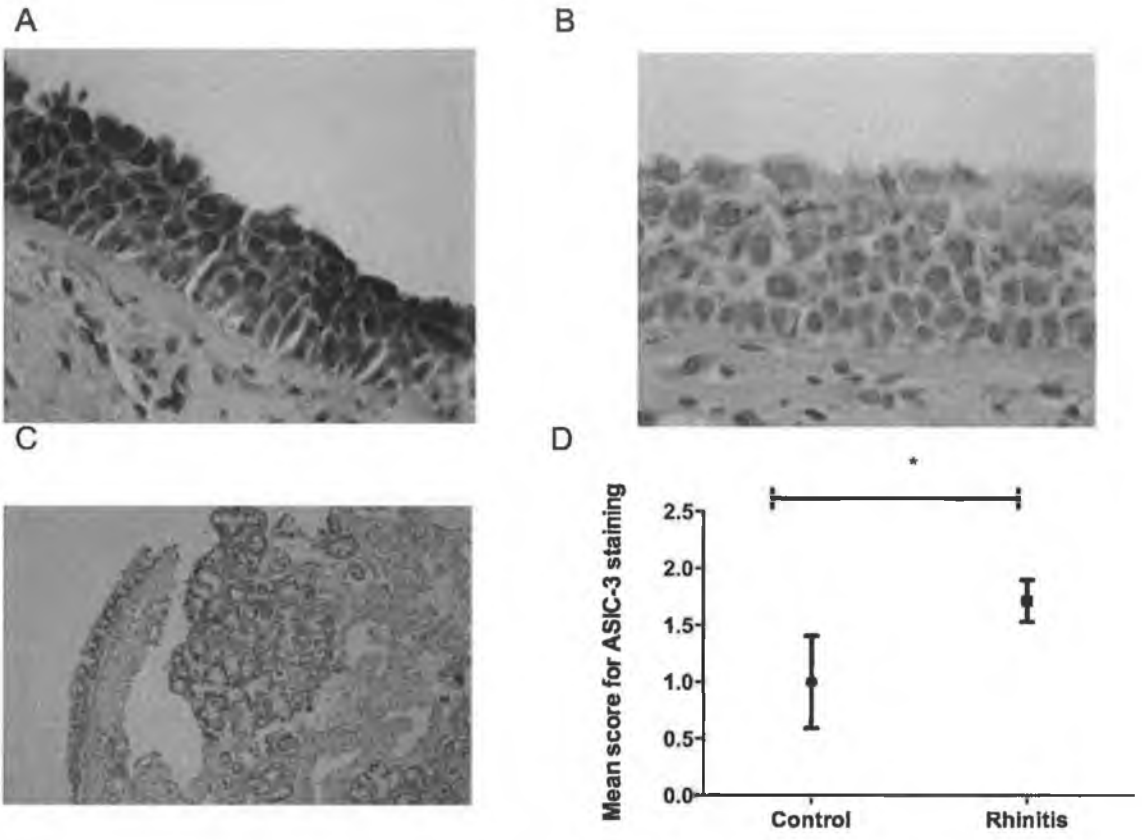


Figure 14A-D. Immunohistochemical localization of ASIC-3 on biopsies of the inferior turbinate for control and AR subjects is shown. The specificity of staining is shown in 14C where there is no staining when an isotype antibody was used. The predominant staining of ASIC-3 was by epithelial cells and mucous glands although there was also some patchy staining on mononuclear cells in the submucosa. The staining was more prominent on the surface epithelium of the subjects with AR (14A) compared to the control subjects (14B), as shown graphically (14D).

4.4 Immunhistochemical localisation of ASIC-3 to nerves

Staining of nerve cells was performed with an antibody to the nerve cell marker PGP 9.5 and the sections were then co-stained with the antibody to ASIC-3 (Figure 15).

Several areas of co-localization are clearly present in major nerve bundles and in small fibers in the airway epithelium, but overall the neural staining was not as strong as that seen in the epithelium. There was no appreciable difference in the neural staining of PGP 9.5 immunoreactive nerves between controls and AR subjects (Figure 15 A-C). Laser capture microdissection of PGP-stained sections was performed to enrich the neural tissue, to specifically quantify neural mRNA for ASIC, but there was insufficient neural tissue obtained from the nasal biopsies to extract sufficient mRNA to provide a meaningful result (data not shown).

Figure 15 A-C



Figure 15 A-C Immunohistochemical localisation of neural ASIC-3 on biopsies of the inferior turbinate for control and AR subjects are shown (A-C). Staining of ASIC-3 (green staining A and C) was seen in small nerve fibres (red staining in B) within the epithelial cells and in larger nerve bundles. Figure 14C shows the merged images showing co-localisation of the ASIC-3 within nerve fibres.

4.5 Induction of ASIC-3 expression by eosinophil peroxidase

Previous studies have shown that eosinophils and their released granule proteins are found in association with airway epithelial cells, mucous glands and nerve cells in patients with persistent AR compared to control subjects (Godthelp T 1996 and Thornton MA et al., unpublished). These are the sites at which ASIC-3 expression is also distributed. We investigated the effect of eosinophil granule proteins on the expression of ASIC-3 in vitro. IMR32 neuroblastoma cells were grown in culture and differentiated to a cholinergic phenotype.

In these in vitro studies, EPO (1 μg/mL) induced a significant upregulation of ASIC-3 at both membrane protein level and at an mRNA (transcriptional) level (Figure 16), with the increase in protein and mRNA being evident within the first hour and being sustained for 24 hours.

Previously, we have shown in the supervising laboratory that EPO induces signalling in these cells via activation of the MEK pathway. Inhibition of this pathway with the MEK-kinase inhibitor PD95805 caused a complete abolition of this induced ASIC-3 expression. This indicated dependence of ASIC-3 expression on ERK activation.

Similarly, in CuFi-1 epithelial cells (Figure 17) there was evidence of apical induction of membrane bound ASIC-3 by eosinophil peroxidase at 18 and 24 hours, (n=4)

Figure 16.

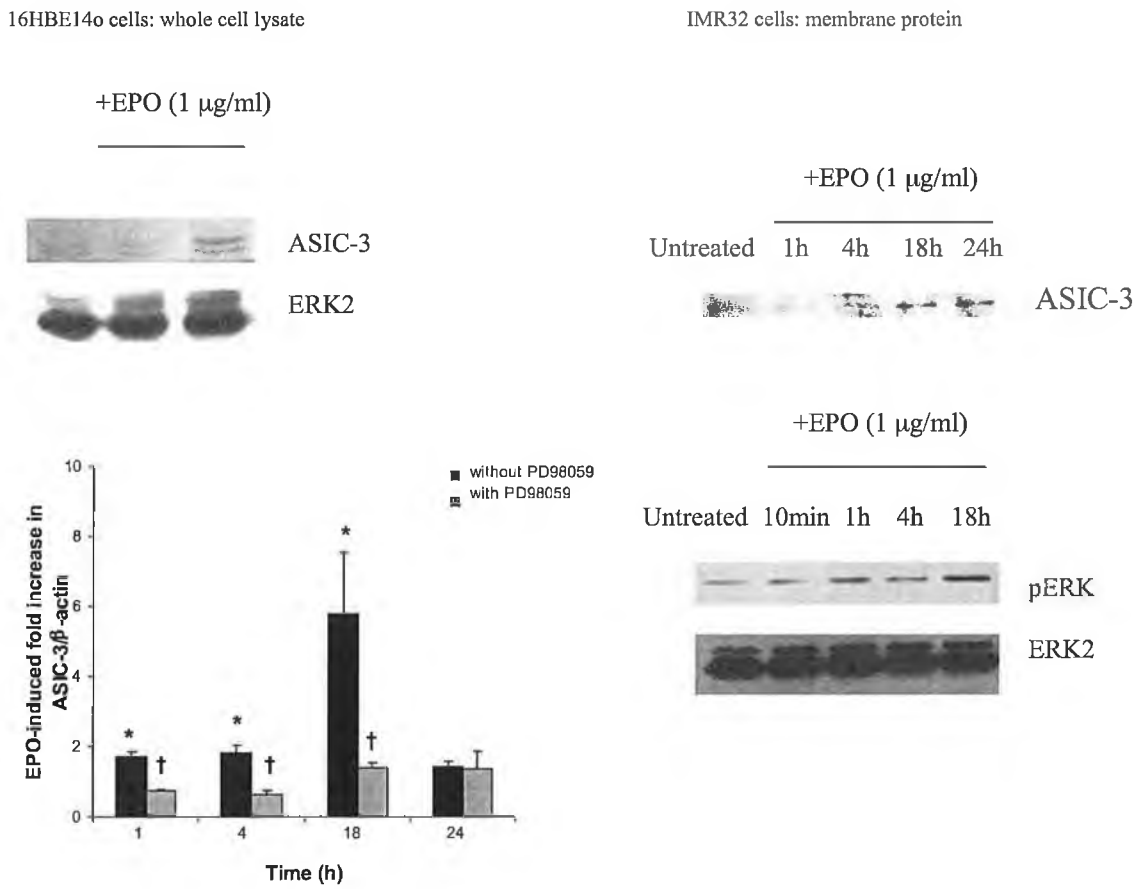


Figure 16. Induction of ASIC-3 is mediated by eosinophil peroxidase acting via an ERK1/2 dependent pathway. IMR32 cells were pretreated with EPO for times from 1 hour to 24 hours and the level of expression by mRNA and Western blot were assessed. There was a significant increase in the expression of ASIC-3 as early as 1 hour at both mRNA and protein level. This induced expression was inhibited by pretreating the cells with the MEK inhibitor PD98059.

Figure 17.

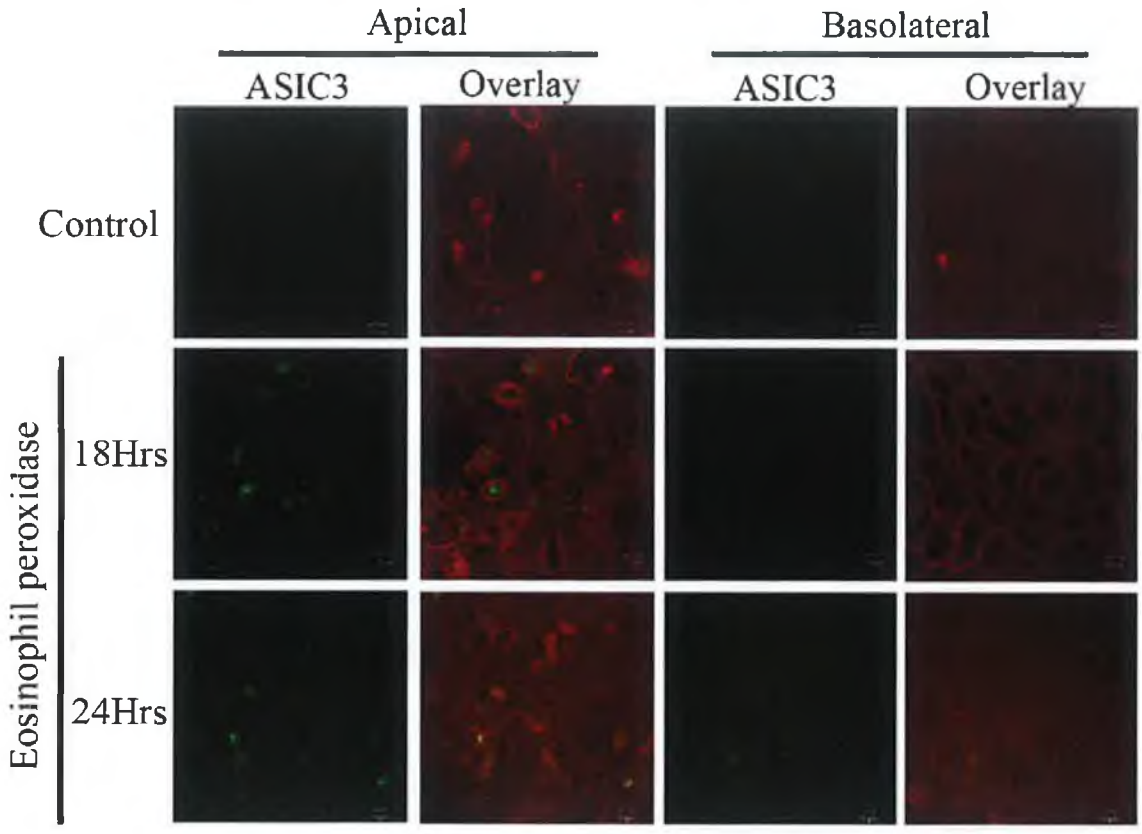


Figure 17. CuFi-1 epithelial cells showing evidence of apical induction of membrane bound ASIC-3 by eosinophil peroxidase at 18 and 24 hours, (n=4).

4.6 Increased airway surface liquid height in response to eosinophil peroxidase

To determine if ASIC were functional, in vitro measurements of the airway surface liquid (ASL) were carried out. These experiments were performed as described previously (Coakley RD 2001) and carried out in conjunction with my colleague, Dr M al-Alawi, at the Respiratory Research lab in Beaumont Hospital. Briefly, over a period of 40 hours the ASL was intermittently measured to determine the mean plateau value of the stabilized ASL height. In these experiments the plateau levels were reached at 12 hours with an average height of 7.97±0.21μm (n=27), (Figure 18). Basolateral treatment with EPO (1μg/mL) increased the baseline ASL to 19.51±0.66μm (n=6) at 18 hours and to 14.56±0.42μm (n=5) at 24 hours, p=0.0001. The observed increase was abolished using apical amiloride (1uM, n=4) dissolved in the FC-72 layer. In further experiments, basolateral bumetanide (10μM, n=4) also completely reduced the EPO-induced increase in ASL height. We were unable to dissolve the CFTR inhibitor CFTR-172 in the perfluorocarbon and the ASIC-3 inhibitor APETx2 was ineffective at inhibiting the EPO induced increase in ASL (n=3), presumably due to the residual presence of ASIC-1.

Figure 18.

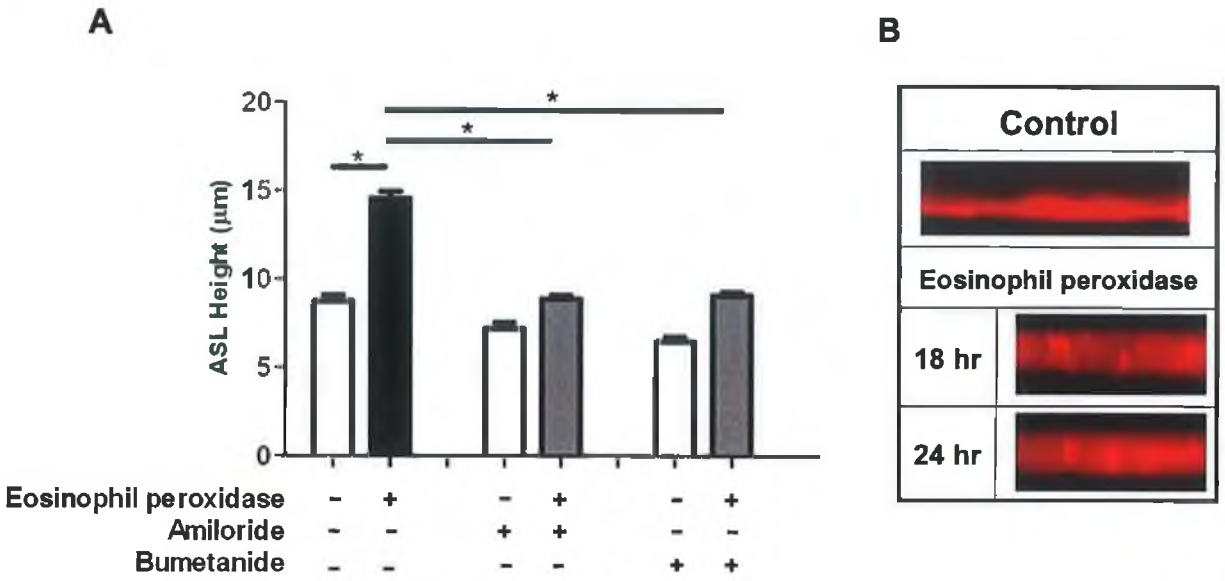


Figure 18. Increased airway surface liquid in response to eosinophil peroxidase.

CHAPTER 5 – DISCUSSION AND CONCLUSION

DISCUSSION

ASIC expression.

ASIC receptors have been noted to occur in various parts of the human body. Their presence in nasal epithelia has yet to be elucidated. In this study we have shown that among the six subunits of the ASIC family, both ASIC-1 and ASIC-3 were detected and expressed in nasal epithelial cells. This was true for both control subjects and subjects with AR. However, there was no significant difference in the level of expression of ASIC-1 between the AR and control subjects, whereas there was clear evidence of upregulation of ASIC-3 receptors in patients with AR compared to healthy control subjects, as evidenced by PCR upregulation of ASIC-3 mRNA. With control subjects having had a similar level of expression of ASIC-1 in PCR quantification to AR subjects but substantially and significantly less ASIC-3, we therefore suggest that the functional ASIC in AR subjects corresponds to ASIC-3, and it is this particular receptor subunit that has a role to play in the pathology of allergic rhinitis.

ASIC as a functional entity in the nasal epithelium

In vivo, studies showed that application of buffered lactic acid, pH 7.0, induced a significant rise in nasal secretions in AR subjects when compared to individual baseline secretions on the ipsilateral nasal cavity in which nasal challenge was introduced. This secretion was inhibited by pretreatment with the ASIC inhibitor amiloride. Although a similar phenomenon occurred in control subjects, this was

apparent only to a lesser extent. This would suggest that the ASIC receptors were functional in both AR subjects, and to a lesser extent, healthy controls, and potentially play a role in nasal secretion.

Although a reduction in nasal secretions was observed following amiloride pre-treatment, it must be noted that amiloride is not specific only to ASIC-3. It inhibits ASIC in general and ENaC. Both ASIC-1 and ASIC-3 were shown to be expressed in both AR subjects and controls on PCR experiments in the nose. However, in the experimental acidic environment of pH 7.0, ASIC-1 channels would not be functional as they only open at a pH of 6.5 (Ugawa S, 2002). Therefore, we suggest that at the experimental pH, amiloride must have been inhibiting only functional ASIC-3 receptors.

ASIC is localized to nasal epithelial cells and small nerve fibres

We undertook these experiments to investigate which ASIC were expressed in the nose of patients with AR. Having demonstrated on PCR analysis that only ASIC-3 mRNA was increased in AR subjects compared to control subjects, we investigated the sites of expression of ASIC-3.

Immunohistochemical studies indicated that ASIC-3 expression was localized to nasal airway epithelial cells, glands and nerves, and along with functional studies, there was a clear difference noted in the level of expression of ASIC-3 by epithelial cells between control and AR subjects. ASIC-3 was expressed on the apical surface of epithelial cells in both biopsy samples of AR subjects and cell culture studies after exposure to EPO.

Since many reports have also described neural expression of ASIC, we co-localized the channel to nerves by dual-immunostaining nerves with the general neural marker, PGP 9.5, and then probed for ASIC-3. In these studies, we demonstrated evidence of staining by small nerve fibers within the epithelium, where they may be ideally positioned to detect luminal acidification. There was no definite increase in expression of the ASIC-3 on nerves in the AR subjects, as judged by immunohistochemistry, when compared to control subjects. Even using laser capture microdissection of PGP 9.5 - stained sections, there was insufficient neural tissue obtained from the nasal biopsies to extract sufficient mRNA to perform quantitative analysis.

We also attempted to correlate the level of mRNA expression of ASIC-3 to a nerve marker, neurgelin, but found no difference in the expression of ASIC-3 between the two groups. Hence we cannot state definitely if there are differences in the level of expression of ASIC-3 on nerves, between AR and control subjects.

Eosinophil granule protein induces expression of ASIC-3

As little is known about the regulation of ASIC channels in the setting of allergic inflammation, we investigated a possible role for eosinophils in the induction of ASIC-3. This mechanism of induction of ASIC-3 expression relevant to AR was suggested by the finding that the eosinophil granule protein, EPO, induced the expression of ASIC-3 via ERK 1/2 in cultured epithelial cells.

In prior immunohistochemical studies, it has been demonstrated that there is a marked association of eosinophils and released eosinophil granule proteins in

association with nerves and epithelial cells in subjects with AR. These are the sites in the nasal mucosa at which ASIC-3 was most abundantly expressed. Hence, we speculated that induction of ASIC-3 may be mediated by eosinophils, and in particular, their cationic granule proteins.

We have previously used an in vitro model of granule proteins and IMR-32 cholinergic cells to study eosinophil and nerve interactions. In these studies we have shown that the eosinophil granule major basic proteins, and other eosinophil granule proteins, signal and cause activation via the MAP-kinase pathways, in particular the MEK ERK1/2 pathway (Morgan RK 2005). This leads to a variety of cellular effects, including the promotion of cell growth and survival. In other recent studies, we showed that EPO activates the expression of the Her2 receptor, and that this mediates a signalling cascade, which leads to activation of ERK1/2 (Walsh et al in review). These studies also showed that EPO induced phosphorylation of ERK as well.

We studied this model and several epithelial cell lines alone and in the presence of eosinophil peroxidase over varying time points. In all of these cell lines, there was no appreciable expression of the ASIC-3 at baseline, but there was a rapid increase in the transcriptional expression of ASIC-3 within 1 hour of exposure to EPO at both mRNA and protein level. Protein expression of ASIC-3 was most pronounced at 18-24 hours in all cell lines studied, and furthermore, ASIC-3 was localized to the cell membrane.

Increased ASIC-3 expression was inhibited when the MEK/ERK inhibitor PD95089 was used, suggesting that EPO was inducing ASIC-3 expression by

activating this ERK pathway. Hence, eosinophil localization to, and release of EPO in patients with AR, may promote the expression of ASIC-3.

Furthermore, in vitro, EPO acting via ASIC increased the airway surface liquid height, a measure of airway hydration, via a chloride mediated secretion. This data suggests that rhinorrhoea in eosinophil-associated allergic rhinitis may be due to an EPO-induced expression of ASIC-3.

EPO-induced increase in airway surface liquid height is mediated via ASIC

There have been prior reports of ASIC-3 expression by epithelial cells, and studies suggest that the CFTR and ASIC-3 are co-localised. Furthermore, in vitro reports suggest that there is a co-operative interaction of ASIC-3 with the CFTR, leading to enhanced chloride ion transport (Coakley RD 2001) (Tarran 2004) (Boucher 2007). In AR subjects, lactic acid induced an ipsilateral production of nasal secretions and pretreatment with amiloride prevented this rise in secretions. To further investigate the hypothesis that the enhanced secretion seen in the AR subjects was mediated through ASIC-3-associated epithelial fluid secretion, additional in vitro studies were performed. Measurement of the airway surface liquid height was used as a model to investigate the functional effects of EPO and epithelial ASIC-3.

Measurement of the airway surface liquid height is a functional quantitative assessment of epithelial cell ion transport. The rationale to these studies was that amiloride is both an ENaC and ASIC inhibitor. By inhibiting ENaC, amiloride inhibits sodium reabsorption, thus increasing the ASL height. By inhibiting ASIC,

amiloride inhibits chloride secretion, thus reducing ASL height. In these studies, EPO applied to the basal surface induced a significant ASL expansion at the time points that ASIC-3 protein expression was maximal, i.e. 18-24 hrs after exposure to EPO.

We suggest that this EPO-induced increase in ASL height was mediated via ASIC, and this was borne out by the finding that administration of amiloride, as an ASIC-3 inhibitor, resulted in attenuation of the ASL height. This would not have occurred if the prominent channel being inhibited was sodium absorption.

In the presence of the chloride channel inhibitor bumetanide, there was a reduction in ASL height when this chloride channel inhibitor was used. This data is in keeping with the previously described co-interaction of ASIC and CFTR (Su X 2006) (Ye JH 2007). In conjunction with immunocytochemical analysis that demonstrated increased apical ASIC-3 in response to EPO treatment, this would suggest involvement of ASIC-3 in the EPO-induced hypersecretory response observed in airway epithelia.

There is little if any secretory role to the neurally expressed ASIC-3 in the nose.

We also investigated possible functional effects of ASIC-3-expressing nerves, and we used a well characterized model of nasal reflex responses. In these experiments, a stimulus was applied on one side of the nose and nasal secretions on the ipsilateral and contralateral sides were collected and measured. Changes in contralateral secretions are mediated via a neural reflex since the nasal septum separates the two sides of the nose. In our studies, only ipsilateral nasal secretions

were enhanced following nasal challenge with lactic acid, and there was no evidence of induced secretions on the contralateral side in either the control or the AR subjects. This would suggest that there is little, if any, secretory role to the neurally expressed ASIC-3 in the nose.

Furthermore, it has been suggested that neurally expressed ASIC-3 channels in the skin mediate pain responses. In our studies there was no evidence of pain, irritation, lacrimation or burning reported by any subjects after the administration of the lactic acid, both on the ipsilateral and contralateral nasal cavities.

Hence, while there is evidence of neural expression of ASIC-3 in the nose, we have not shown a definitive function of these channels. Our physiological functional studies, using a lactic challenge, support the idea that the application of acid to the luminal surface leads to enhanced nasal secretions, only apparent on the ipsilateral side. It is not clear if these secretions represent mucous release or exudation of fluid through epithelial cells. More in vitro work is necessary to elucidate this.

The mechanism of rhinorrhoea is through EPO-mediated nasal epithelia-associated ASIC-3 expression in AR

There are several novel findings in this work, which is the first description of a functional role for ASIC in the nose. For example, this is the first study to show how an eosinophil mediator of inflammation induces epithelial and neural expression of ASIC-3, leading to enhanced nasal secretion, and it describes a novel role for EPO in the mediation of chloride secretion. This excess epithelial

fluid secretion may be relevant to the pathogenesis of rhinitis, as it may contribute to the symptom of rhinorrhoea.

In summary, these studies indicate that epithelial cells in patients with AR express ASIC-3 receptors. In vitro studies suggest that the increased expression in AR may be mediated through the eosinophil granule protein EPO. The function of nasal epithelial ASIC-3 channels appears to be that they promote nasal secretions and this may be a mechanism for rhinorrhoea, one of the common and troublesome features of particular forms of rhinitis, such as non-allergic eosinophilic rhinitis.

CONCLUSION

In this study we have shown that within the nasal mucosa there is immunohistochemical expression of the ASIC-3 ion channel, localized to the apical surface of epithelial cells, airway glands and nerves in patients with allergic rhinitis. The increased epithelial expression in allergic rhinitis is due, at least in part, to the eosinophil granule protein EPO.

The functional consequence of increased ASIC-3 expression in an acidic environment is an induction and enhancement of the nasal secretory response, as demonstrated by the application of buffered lactic acid at a pH of 7.0. These responses are inhibited by amiloride pretreatment.

We suggest that the function of nasal epithelial-associated ASIC-3 channels appears to be the promotion of nasal secretions, and this is a potential addition to the mechanism responsible for rhinorrhoea, one of the common and troublesome features of rhinitis.

APPENDICES

Appendix 1 Beaumont Hospital Ethical Approval

Ethics (Medical Research) Committee - Beaumont Hospital
Notification of ERC/IRB Approval

Investigator: Mr. S. Gunn Khoo

Protocol No.: 07/65

Protocol Title: Acid-sensing ion channel 3 (ASIC-3) and transient receptor/vanilloid subtype 1 (TVPV-1) expression in nasal mucosa and their role in allergic rhinitis.

Ethics Committee Meeting date: 31st August 2007

Final Approval Date: 15th October 2007

From: Ethics (Medical Research) Committee - Beaumont Hospital, Beaumont, Dublin 9

Document and Date	Documents Reviewed Date Reviewed	Approved
Application Form, Signed, 15/10/07	15/10/07	Yes
Control Information Leaflet Version 2	15/10/07	Yes
Control Consent Form	15/10/07	Yes
Patient Information Leaflet Version 2	15/10/07	Yes
Patient Consent Form	15/10/07	Yes
Consent Form For Retention of Tissue (Patients & Controls)	15/10/07	Yes
GP Letter, Version 2	15/10/07	Yes
Recruitment Poster, Version 2	15/10/07	Yes
CV, R. Costello	Noted	Noted
CV, S. Gunn Khoo	Noted	Noted

Professor Gerry McElvaney
ERC/IRB – Chairperson's Signature
Approval # 1, dated 15th October 2007

Appendix 2 Physiological secretions – Lactic acid and amiloride, ipsilateral measurements. Weight in grams (c = controls, ar = allergic rhinitis)

Lactic acid (ipsilateral)

Patients	*Baseline*	*2mins*	*5mins*
c23	0.002	0.002	0.003
c24	0.003	0.005	0.009
c25	0.01	0.019	0.046
c26	0.005	0.009	0.002
c27	0.002	0.004	0.005
c28	0.002	0.004	0.005
c32	0.004	0.006	0.008
c34	0.008	0.012	0.005
c35	0	0.009	0.003
c36	0.001	0.001	0.009
c37	0.002	0.001	0.001
c38	0.002	0.003	0.006
c39	0.001	0	0.005
c40	0.004	0.004	0.01
c41	0.002	0.001	0.022
c44	0.005	0.009	0.012
c45	0.011	0.005	0.004
c46	0.007	0.017	0.021

ar31	0.005	0.026	0.003
ar32	0.009	0.005	0.009
ar33	0	0.005	0.003
ar35	0.002	0.003	0.007
ar36	0.002	0.019	0.034
ar40	0.007	0.003	0.008
ar41	0.006	0.014	0.018
ar42	0.003	0.011	0.013
ar44	0.002	0.003	0.004

Lactic acid and amiloride (ipsilateral)

	baseline	*2mins*	*5mins*
c23	0.003	0.003	0.003
c24	0.002	0.009	0.018
c25	0.004	0.003	0.01
c26	0.002	0.001	0.007
c27	0.003	0.004	0.004
c28	0	0.013	0.003
c32	0.002	0.003	0.005
c34	0.006	0.015	0.002
c35	0.003	0.002	0.005
c36	0.004	0.001	0.002
c37	0.01	0.004	0.004
c38	0.002	0.001	0.012
c39	0.003	0.004	0.009
c40	0.005	0.011	0.012
c41	0	0.002	0.015
c44	0.006	0.015	0.008
c45	0.003	0.008	0.012
c46	0.007	0.007	0.004
ar31	0.007	0.001	0.002
ar32	0.006	0.008	0.002
ar33	0.001	0.015	0.013

ar35	0.004	0.002	0.004
ar36	0.002	0.004	0.004
ar40	0.002	0.008	0.009
ar41	0.002	0.019	0.015
ar42	0.001	0.004	0.007
ar44	0.002	0	0.002

Appendix 3 Physiological secretions – Lactic acid and amiloride, contralateral measurements. Weight in grams (c = controls, ar = allergic rhinitis)

Lactic acid (contralateral)

	baseline	*2mins*	*5mins*
c23	0.002	0.003	0.004
c24	0.002	0.001	0.003
c25	0.011	0.005	0.031
c26	0.003	0.013	0.008
c27	0.002	0.003	0.003
c28	0.003	0.002	0.003
c32	0.001	0.004	0.006
c34	0.002	0.019	0
c35	0.006	0	0.013
c36	0.001	0.006	0.003
c37	0.001	0.005	0.002
c38	0.001	0.001	0.002
c39	0.003	0.008	0.002
c40	0.004	0.005	0.003
c41	0.002	0.002	0.001
c44	0.004	0.007	0.002
c45	0.011	0.005	0.002
c46	0.012	0.006	0.021

ar31	0.01	0.016	0.004
ar32	0.008	0.007	0.019
ar33	0	0.004	0
ar35	0.003	0.006	0.001
ar36	0.002	0.009	0
ar40	0.007	0.005	0.009
ar41	0.002	0.004	0.002
ar42	0.003	0.009	0.011
ar44	0.001	0.005	0.007

Lactic acid and amiloride (contralateral)

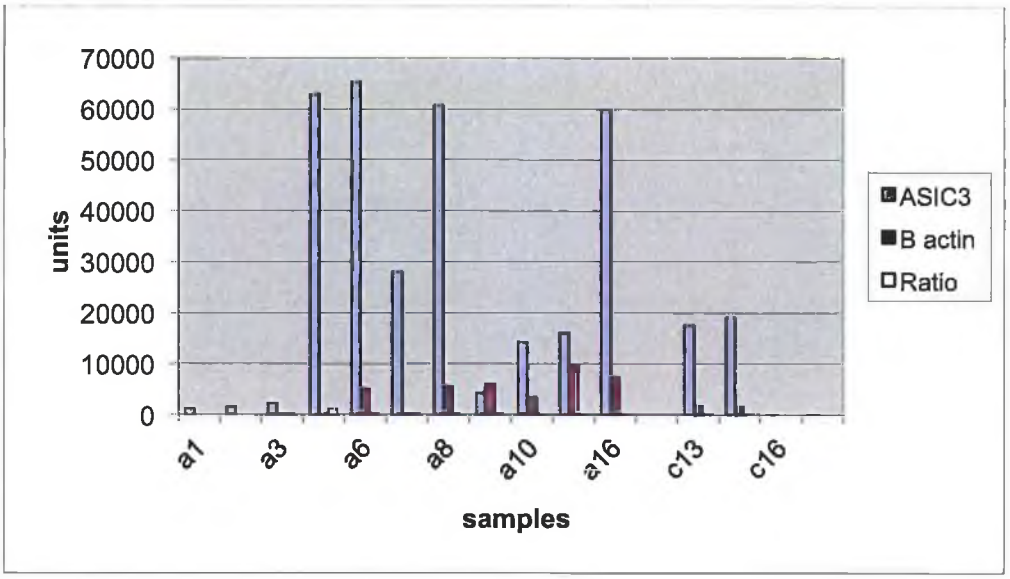
	baseline	*2mins*	*5mins*
c23	0.003	0.003	0.003
c24	0.001	0.008	0.005
c25	0.006	0.001	0.009
c26	0	0.004	0.004
c27	0.003	0.003	0.003
c28	0	0.005	0.003
c32	0.001	0.001	0.002
c34	0.003	0.002	0.006
c35	0.008	0.004	0.003
c36	0.006	0.007	0.004
c37	0.007	0.005	0.008
c38	0.002	0.005	0.012
c39	0.007	0.003	0.013
c40	0.004	0.003	0.006
c41	0.001	0.003	0.007
c44	0.003	0.007	0.008
c45	0.002	0.008	0
c46	0.007	0.001	0.003
ar31	0	0.016	0.003
ar32	0.01	0.008	0.009
ar33	0.002	0.001	0

ar35	0.006	0.002	0.003
ar36	0.001	0.007	0.007
ar40	0.002	0.003	0.004
ar41	0.001	0.01	0.001
ar42	0.001	0.002	0.002
ar44	0.014	0.009	0.01

Appendix 4 ASIC-3 quantification

Sample	ASIC3	B actin	Ratio
a1	1223		
a2	1629.5		
a3	2285.5	35.88	63.7
a5	62820	50.36	1247.42
a6	65280	5079	12.85
a7	27945	309	90.44
a8	60660	5631.5	10.77
a9	4257.5	6072.5	0.7
a10	14245	3311.5	4.3
a13	16030	9714	1.65
a16	59580	7507	7.94
c13	17560	1881.5	9.33
c14	19080	1562	12.22
c16	37.05		
c18	56.19		

Appendix 5 ASIC-3 quantification graphically expressed.



Appendix 6 ASIC-1 and ASIC-2 quantification

Ptts	ASICs 1	ASICs 2	average	B-ACTIN 1	B-ACTIN 2	average
a1	879.8	864.1	872			
a2	1297	1071	1184			
a3	1423	1974	1698.5	36.6	35.16	35.88
a4						
a5	61220	64420	62820	50.04	50.68	50.36
a6	65150	65410	65280	5170	4988	5079
a7	29880	26010	27945	312.2	305.8	309
a8	60980	60340	60660	5571	5692	5631.5
a9	4528	3987	4257.5	6176	5969	6072.5
a10	14320	14170	14245	3404	3219	3311.5
a13	9780	9405	9592.5	2748		2748
a16	8383	7081	7732	1946		1946
a11(c1)	15270	14530	14900	1865	1898	1881.5
a12(c2)	16180	16380	1280	254.2		254.2

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