



The Role of Tachykinins in Airway Inflammation and Bronchial Hyper-responsiveness

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For my wife, Carol,
and my daughters, Bianca and Chelsea.

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Abstract

Tachykinins, principally substance P (SP) and neurokinin A (NKA), are neuropeptides present in sensory nerves in the airways. Release of these peptides into the local milieu leads to several responses including, the stimulation of smooth muscle contraction, vasodilatation, increased vascular permeability, mucus secretion, smooth muscle and epithelial cell proliferation, chemotaxis and activation of inflammatory cells. Thus, tachykinins are implicated in the mediation of airway inflammatory responses and may have roles in airway remodeling and healing. The actions of tachykinins are mediated by specific receptors, designated NK1, NK2 and NK3. Tachykinin degradation, an important mechanism for limiting the effects of these peptides, is principally mediated by neutral endopeptidase (NEP). In this thesis, I have investigated the role of tachykinins, *in vivo*, in an ovine model and in human airway epithelium. The ovine studies showed that tachykinins contribute to the acute lung resistance response to allergen, an action inhibited by a specific NK1 receptor antagonist and enhanced by NEP blockade. Studies using exogenous SP showed that this tachykinin acts on lung resistance via a cholinergic mechanism, and that the effects were modulated by rapid receptor desensitisation. The response was again inhibited by the specific NK1 antagonist, illustrating the potential therapeutic utility of such agents. Studies of human airway epithelium *in vivo* demonstrated gene expression for all three tachykinin receptors, NEP and preprotachykinin-A (PPT-A, the precursor for SP and NKA) in epithelial brush biopsy samples. Previously, only NK1 and NEP expression had been described in human airway epithelium, and the new finding of PPT-A mRNA at this site indicates the potential for local production of tachykinins in the epithelium itself in addition to release from nerves. Further, incubation of the epithelial samples with SP showed some evidence of a pro-inflammatory response because interleukin 8 mRNA levels were increased. The net effect of tachykinins in the airway will depend on the relative balance between the expression of receptors, tachykinins and NEP. Assessment of these molecules in the airway epithelium from subjects with normal lungs or chronic bronchitis showed that PPT-A gene expression was relatively higher in the disease group whereas NEP and NK1 receptor levels were unchanged. These studies provide new insights into the role of tachykinins in airways disease.

Submission Statement:

This work contains no material which has been accepted for any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Paul N. Reynolds

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Chapter One

Introduction - Tachykinins and C-fibre afferent nerves in airways disease.

Airway inflammatory diseases account for a substantial fraction of the budget of health care services and result in enormous economic losses. In Australia, asthma prevalence in adults is approximately 12% of the population and in children, between 20 and 30% report asthma symptoms¹. In 1996, 730 people died of asthma. The cost to the Australian economy of this disease has been estimated at \$720 million per year (National Asthma Campaign report). Other airway inflammatory diseases such as chronic bronchitis add even more to these costs.

The approach to the treatment of asthma and chronic obstructive airways disease has improved in recent years, with the appreciation of the inflammatory basis for these disorders^{2,3}. In response to the realisation of the importance of inflammation in these diseases, treatment for asthma in particular is clearly focused on the use of anti-inflammatory agents, with inhaled corticosteroids being the foundation of pharmacological therapy in adults⁴. Nevertheless, the use of broad spectrum anti-inflammatory agents such as corticosteroids can be associated with unwanted side effects, and some patients remain resistant to this treatment⁵. In addition, problems with the overuse of inhaled β_2 agonists have also been recognised⁶. There is clearly a need for further development of more specific anti-inflammatory and immunomodulatory therapies. A better understanding of the role of the nervous system and neuroimmune interactions in the pathogenesis of airway inflammatory disorders will help in the rational development of new therapeutic approaches.

Afferent neural pathways in the lungs mediate the protection of the pulmonary tree from

noxious agents⁷. Reflexes generated by the stimulation of sensory nerves are both protective (eg. apnoea, laryngeal narrowing and bronchoconstriction) and defensive (sneezing, coughing and aspiration and expiration reflexes). The nervous system modulates other important defensive strategies operative in the lung including mucociliary clearance⁸, vasodilatation^{9,10}, increased vascular permeability¹¹ and the influx of inflammatory cells¹² and may play a role in the healing processes following airway epithelial injury¹³.

There is evidence that perturbation of neurologically mediated defence mechanisms may be an important factor in the pathogenesis of inflammatory airways diseases¹⁴. For example, asthma is characterised by reversible airways obstruction, airway inflammation and enhanced bronchoconstrictor responses to specific and non-specific stimuli (bronchial hyper-responsiveness). The features of asthma suggest a relative hyperactivity of pulmonary protective strategies resulting in a counter-productive physiological and clinical outcome. There is evidence that this hyperactivity may include relative hyperactivity of sensory nerve functions¹⁴. Other pulmonary diseases such as chronic bronchitis are also associated with airway epithelial inflammation and varying degrees of reversible and irreversible airways obstruction¹⁵. The central theme of this thesis is an investigation of the role of sensory afferent nerves, and the neuropeptides they contain, in the mediation of bronchoconstriction and airway epithelial inflammation.

Sensory innervation of the lungs

Respiratory protective reflexes are mediated predominantly by vagal nerve fibres, although there is some sensory innervation from the thoracic dorsal roots. There are both myelinated and unmyelinated afferents⁷.

Myelinated afferents transmit impulses from slowly adapting (SAR) and rapidly adapting (RAR) stretch receptors located in the airway wall. It is believed that the SARs mediate the Hering - Bruer reflex, and SAR activation leads to reduction in smooth muscle tone and bronchodilatation⁷. There is some evidence that they are involved in cough generation, although on balance it appears that they may have an indirect role by facilitating the reflex activity of expiratory muscles rather than a direct effect. There is more evidence for a direct effect of RAR activation in cough generation and bronchoconstriction. For example, evidence for the involvement of myelinated nerves in these responses has been obtained from studies conducted in rabbits, whereby the vagus nerve was cooled to a temperature selective for myelinated nerve conduction block (7°C), which resulted in inhibition of cough and a substantial reduction in the bronchoconstrictor response to histamine⁷. However, although this procedure is designed not to impair unmyelinated conduction, absolute selectivity is uncertain. Local anaesthetics inhibit cough and reflex bronchoconstriction to differing degrees, suggesting different receptors may be involved in these responses.

Unmyelinated (C-fibre) afferents constitute the majority of vagal fibres originating from the airways. The nerve cell bodies are located predominantly in the jugular and nodose ganglia with lesser numbers in the thoracic dorsal root ganglia¹⁶. C-fibre nerves have been identified widely throughout the airway tissues including, within and under the epithelium, around exocrine glands and ganglion cells, within smooth muscle and around arteries, arterioles and venules¹⁶.

Two populations of C-fibre endings have been described - pulmonary and bronchial - based on their delays to activation following the intravenous injection of stimulatory agents. These two types of nerve endings differ in their responses to mechanical and chemical stimuli. Their endings are referred to as 'polymodal' receptors although they predominantly respond to nociceptive stimuli. Unlike myelinated fibres, which discharge spontaneously, C-fibres are normally silent. These nerve fibres have many important roles in the modulation of pulmonary responses in disease states. Much of the initial information about the role of C-fibres in the airways has come from experiments using the agent capsaicin¹⁷.

The effects of capsaicin in the airways

C-fibre nerves are sensitive to capsaicin, a pungent agent from red peppers, which has been used extensively in the study of C-fibre responses^{17,18}. Capsaicin binds to the membrane anchored vanilloid receptor, resulting in the opening of a cation channel which allows entry of sodium and calcium ions with subsequent nerve impulse generation and impulse propagation centrally to the CNS. In addition, capsaicin causes release of the neuropeptides contained within C-fibres, with subsequent manifestation of neuropeptide effects locally in the airway. It has been proposed that this local release may be mediated both by axon reflexes and a direct effect of capsaicin on the nerve terminal. The concept of an axon reflex was initially proposed to explain the flare response which occurs following cutaneous noxious stimulation. This flare (vasodilatation) extends far beyond the point of the initial stimulus and is dependent on local sensory innervation as it does not occur in denervated skin and is blocked by local anaesthetics. It is believed to result from antidromic conduction of impulses down axonal branches of the nerve fibre initially stimulated, which then results in the release of neuropeptides from the distal

terminal of the axon branch. Distal vasodilatation resulting from stimulation of the proximal ends of severed sensory nerves was demonstrated over a hundred years ago and lead to the initial concept of sensory nerves having local effector functions in addition to central conduction of sensory impulses. The lack of tonic discharge in C-fibres may be essential in allowing them to mediate axon reflexes as in theory two impulses travelling in different directions would cancel each other out. Although axon reflexes may play some role in the effector functions of C-fibre nerves, whether they contribute to the capsaicin induced release of neuropeptides is unclear, particularly as there are reports that this response is resistant to inhibition by both tetrodotoxin and local anaesthetics¹⁷.

Following excitation, capsaicin induces an immediate reversible refractory state on the nerve (capsaicin desensitisation). Chronic administration of capsaicin leads to neuropeptide depletion (which occurs some time after conduction inhibition). Hence if a particular airway response under question is reduced after chronic capsaicin, the implication is that C-fibre nerves and neuropeptides are involved in the generation of that response. The adequacy of the initial capsaicin treatment can be assessed by quantifying residual neuropeptide content in the lung, however, in the case of certain neuropeptides (eg the tachykinins) no amount of capsaicin will totally eliminate these peptides from tissues as some is contained in capsaicin insensitive nerves. High dose capsaicin treatment leads to C-fibre nerve degeneration and has been used to permanently abolish C-fibre function in neonatal animals. Clearly this will not only abolish axon reflex mechanisms but will also affect the sensory afferent role of C-fibres and their role in classical centrally mediated reflexes. It should be noted that capsaicin will also stimulate a small number of myelinated fibres including the small A δ fibres from RARs (to a weaker extent than C-fibres), and that a threshold dose of capsaicin for C-fibre selectivity exists.

Following capsaicin desensitisation the flare response in the skin is abolished, which

implicates neuropeptides in the mediation of this response. Lundberg in 1983¹⁹ first proposed a role for axonal reflex release of neuropeptides in airways disease after demonstrating that capsaicin desensitisation reduced the increase in vascular permeability seen in rat airways following noxious stimulation but not stimulation with the neuropeptide substance P. The increase in permeability could also be inhibited by local anaesthetics and could be stimulated by electrical stimulation of the vagus below the nodose ganglion. A definite anatomical correlate to the axon reflex hypothesis in the airways (ie. 2 separate branches of the same nerve fibre supplying epithelium and blood vessels) has not been demonstrated however.

Many experiments using capsaicin have provided evidence for a role for C-fibre afferents in the genesis of cough and bronchoconstriction, although there are important species differences. For example capsaicin inhalation in guinea pigs initially causes cough and significant bronchoconstriction^{7,20}. Pretreatment with capsaicin to deplete neuropeptides markedly reduces the cough and bronchoconstriction response. However, the cough (but not the bronchoconstriction) induced by nicotine (acting via RARs and myelinated nerves) is not reduced, thereby indicating that myelinated fibres also play a role in the cough reflex. Protein extravasation in response to other irritants such as sulphur dioxide, toluene diisocyanate, acid and formalin is also reduced following capsaicin desensitisation, as is the bronchoconstriction and some elements of the inflammatory response to allergen^{21,22}. Thus, these experiments provide evidence that the neuropeptides contained within the C-fibre nerves contribute to inflammatory responses.

In humans, capsaicin inhalation caused cough and a rapid decrease in airway conductance lasting sixty seconds which was partly inhibited by ipratropium bromide, implying the involvement of a cholinergic reflex²³. The brevity of the bronchoconstrictor response has lead some to hypothesise that neuropeptide containing nerves may not be significant in the

pathogenesis of asthma in man²⁴. However, capsaicin desensitisation has been shown to reduce nasal symptoms in vasomotor rhinitis following allergen challenge in man¹⁸ and to reduce the flare response to allergen in human skin. In the acute phase capsaicin application to the nasal mucosa in man leads to an influx of inflammatory cells¹². In contrast to the stimulation of bronchial C-fibres by aerosolised noxious agents, the stimulation of pulmonary C-fibres by intravenous agents in most experimental animals does not cause coughing⁷, although in one human study, one of three subjects did have paroxysmal coughing in response to intravenous capsaicin²⁵.

In summary, studies using capsaicin have established that C-fibre nerves have a role in the modulation of cough and pulmonary inflammatory responses. Recent studies using specific neuropeptide agonists and antagonists have begun to more precisely define the roles of individual peptides.

Tachykinins

The results of capsaicin desensitisation experiments imply that the neuropeptides contained within C-fibre nerves have pro-inflammatory effects, hence the role of C-fibre nerves and their neuropeptide contents in the pathogenesis of airways disease has received considerable attention. Perhaps the most studied neuropeptides in this area are the tachykinins²⁶⁻²⁹. Studies of the effects of exogenous tachykinins in various tissues, along with studies using specific tachykinin antagonists, have provided a substantial body of evidence that this group of neuropeptides are important mediators of inflammatory responses¹⁸. Different tachykinins may co-localise to the same nerve, often with other neuropeptides such as calcitonin gene related peptide (CGRP). The name tachykinin derives from an ability to rapidly contract extravascular

smooth muscle (as opposed to the delayed and slow response to bradykinin). The first recognised mammalian tachykinin was substance P (SP), initially described by von Euler and Gaddum in 1931³⁰. They described a substance derived from equine brain and intestine which caused hypotension and smooth muscle contraction. The origin of the name “substance P” derives from the “powder” they obtained. Lembeck initially hypothesised that SP may be a sensory neurotransmitter in 1953²⁶, suggested by the observation that dorsal spinal roots contain more SP than ventral roots. The neurotransmitter functions of SP were not confirmed until the 1970’s.

Determination of the amino acid sequence of SP, which was shown to be an undecapeptide, and synthesis of SP, was achieved by Chang and Leeman in 1971³¹. In the early 1980’s other mammalian tachykinins were described by several groups²⁶ including Maggio et al³² (substance K), Kimura et al³³ (neurokinin α and neurokinin β), Nawa et al³⁴ (substance K), Matsuo et al^{35,36} (neuromedin L and neuromedin K). It was subsequently shown that substance K, neuromedin L and neurokinin α were an identical decapeptide, and since 1986 the term neurokinin A (NKA) has been used for this peptide. Similarly, neurokinin β and neuromedin K are an identical decapeptide, now termed neurokinin B (NKB). Subsequently, amino-terminally extended forms of NKA, termed neuropeptide K (NPK) and neuropeptide γ (NP γ)^{28,37} have also been described. The term substance P has been retained.

Mammalian tachykinins have a common carboxy terminus, Phe-X-Gly-Leu-Met-NH₂, where X is either Phe or Val and the terminal methionine is amidated (Figure 1-1). It is this common terminus that is required for most of the tachykinin transmitter functions whereas the N terminal of the peptides confers receptor specificity³⁸. The non-mammalian tachykinins derived from amphibians including physaleamin, kassinin and eledoisin also share the above carboxy terminal sequence. SP and NKA (along with NPK and NP γ) are derived from a single gene,

preprotachykinin A (PPT-A). NKB is derived from a separate gene, preprotachykinin B which evolved from a common ancestor gene by duplication²⁷. The derivation of the individual peptides from the PPT-A gene occurs by differential mRNA splicing of the primary transcript, which leads to the production of three distinct precursor proteins. In human tissues the β transcript dominates (80-85% of PPT-A mRNA), followed by γ (15-20%), with no α PPT-A detected in the basal ganglia³⁹. Post-translational processing of these precursor proteins by proteolytic enzymes (convertases) results in production of the individual peptides (Figure 1-2). Although acute alterations in mRNA splicing patterns might offer a means to control tachykinin production, studies in the rat have not noted differences in the PPT-A transcript proportions in response to various stimuli⁴⁰. Evidence for transcriptional regulation of tachykinin production is seen in studies investigating the mechanism of dopamine antagonist-induced decreases in the levels of SP and NKA in basal ganglia⁴⁰. In this setting, the reduction in tachykinins is preceded by a decrease in PPT-A mRNA. In keeping with these findings, augmented dopamine release increases both PPT-A mRNA and tachykinin levels. These studies serve to validate the use of PPT-A mRNA levels as a means to investigate tachykinin production.

Peptide Sequence of the Mammalian Tachykinins

Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH ₂
Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH ₂
Neuropeptide K	Asp-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu- Lys-Ala-Leu-Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys- Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH ₂
Neuropeptide γ	Asp-Ala-Gly-His-Gly-Gln-Ile-Ser-His-Lys- Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH ₂
Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH ₂

Figure 1-1. Peptide sequence of the mammalian tachykinins, showing homologous sequences at the carboxy termini, the regions responsible for interaction with tachykinin receptors.

In human airways, tachykinin containing nerves have been detected within and beneath the airway epithelium, around blood vessels and in the smooth muscle¹⁶. SP content in human lung is approximately 3pmol per gram of tissue, a value similar to that for the guinea pig. NKA immunoreactivity has also been detected in C-fibres in the lungs, often co-localised with SP, with tissue levels of 0.3pmol per gram detected⁴¹. Significant levels of NKB have not been reported in the lung.

Tachykinin receptors

Tachykinins mediate their effects by binding to specific receptors. These receptors are designated neurokinin 1, 2, or 3 (NK1, NK2 or NK3) on the basis of whether their preferred endogenous ligand is SP, NKA or NKB respectively²⁹. There is considerable cross reactivity of each of the ligands for each of the receptors, although in each case the preferred ligand has an affinity at least one to two orders of magnitude higher for its specific receptor than the other ligands (NK1 - SP > NKA > NKB; NK2 - NKA > SP > NKB; NK3 - NKB > NKA > SP). Structurally the receptors consist of an extracellular amino terminus, seven hydrophobic transmembrane helices with alternating extracellular and cytoplasmic loops, and a cytoplasmic carboxy terminus. This basic structure of the tachykinin receptors places them in the superfamily of G-protein linked receptors which also includes the adrenergic, rhodopsin and muscarinic receptors.

The sequence of the NK1, NK2 and NK3 tachykinin receptors has been determined. The first receptor to be characterised in this way was the bovine substance K (NK2) receptor⁴². In brief, this was achieved by first extracting mRNA from bovine stomach, using reverse transcription to generate a cDNA library, then inserting cDNA fragments into an RNA

expression vector, transcribing RNA, then injecting this RNA into *Xenopus* oocytes. Oocytes were then screened for functional receptors^{42,43}. Having identified the initial tachykinin receptor sequence in this way, other receptors have been identified by cross hybridisation strategies, relying on predictions of regions of homology between receptor subtypes and homology between different species. Thus rat NK1⁴⁴, NK2⁴⁵ and NK3⁴⁶ sequences were determined from a rat brain cDNA library.

Human NK1⁴⁷⁻⁴⁹, NK2^{50,51} and NK3⁵² sequences have been reported. There is approximately 95% homology between the rat and human sequences for each receptor and 60-70% homology between the receptor subtype sequences, with the highest degree of homology seen in the transmembrane domain regions⁵². An illustration of the human NK1 receptor is shown in Figure 1-3, adapted from Fong et al⁵³. The receptors consist of an extracellular domain, seven helical membrane domains linked by intracellular and extracellular loops, and an intracellular carboxy terminal domain. The regions responsible for binding to neuropeptides and non-peptide antagonists have been identified by mutational analysis. Interestingly, the NK1 receptor specific antagonist CP 96,345 binds to a different region of the receptor than SP, implying steric hindrance accounts for the antagonist properties⁵³.

The human NK1, NK2 and NK3 tachykinin receptors consist of 407, 398 and 465 amino acids respectively, with molecular weights of 46,269, 44,435 and 52,201. The gene for each consists of five exons, with all four introns located at equivalent positions for each receptor. The sizes of the introns however, vary considerably, such that the gene for NK1 spans 60 kb, NK2 12 kb and NK3 45 kb⁵². The genes for the NK1 and NK2 receptors are located on chromosomes 2 and 10 respectively.

Human NK1 Tachykinin Receptor

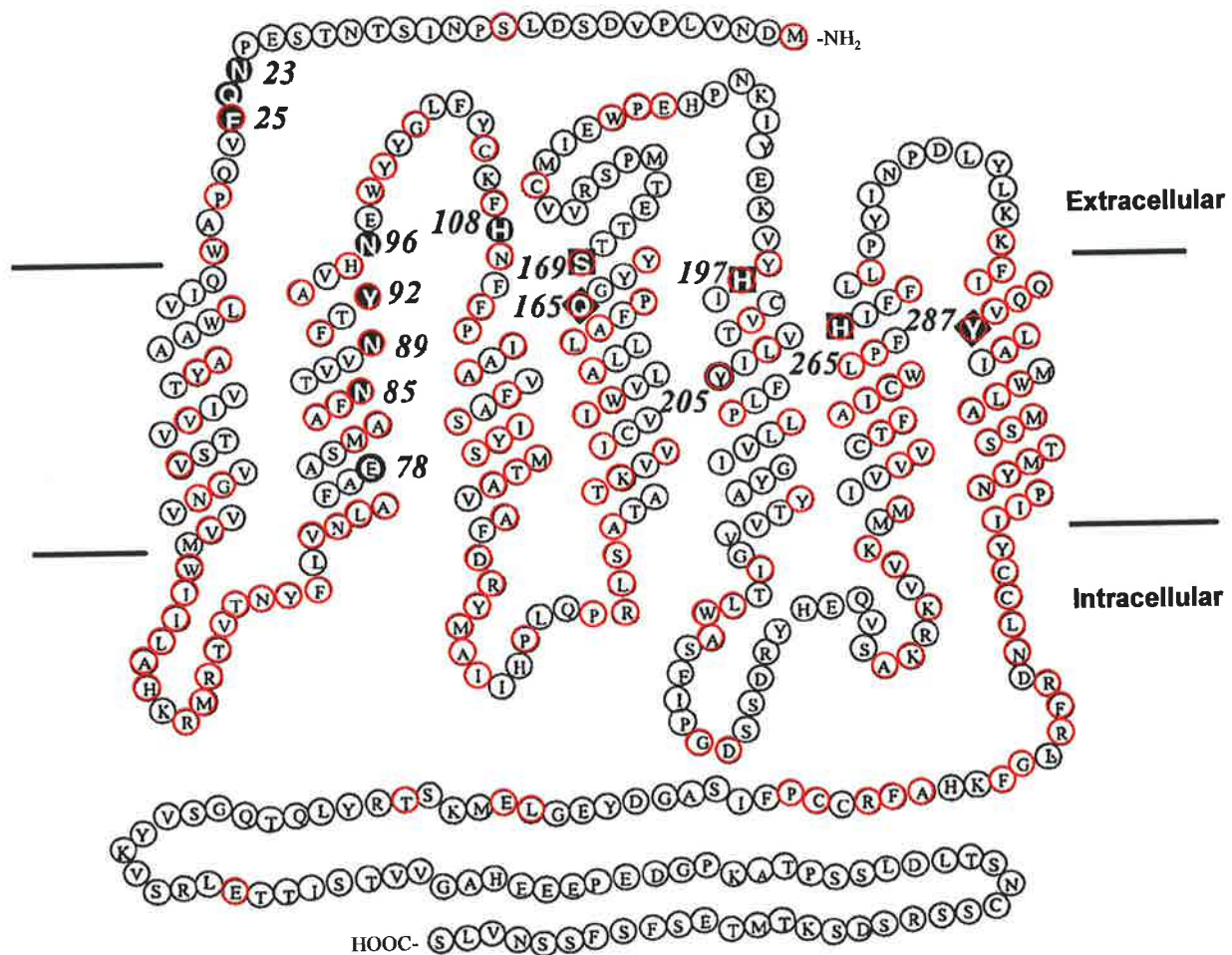


Figure 1-3. Schematic representation of the human NK1 tachykinin receptor. Red circles indicate homology between NK1, NK2 and NK3 receptors. Filled circles indicate amino acids involved in peptide binding. Filled squares indicate amino acids involved in non-peptide antagonist binding. Diamonds indicate both peptide and antagonist binding. Residues 78 and 205 are important for receptor activation. Adapted from Fong et al.

Second messenger responses described include activation of a Gs protein leading to activation of adenylate cyclase, with subsequent conversion of ATP to cAMP and activation of a Gp protein leading to activation of phospholipase C, with the conversion of phosphatidylinositol 4, 5 biphosphates to inositol tri-phosphate and diacyl glycerol (and an increase in intracellular calcium from internal reticular stores via the inositol 1,4,5, triphosphate receptor)³⁸. Using Chinese hamster ovary cells transfected to express either the NK1 or NK2 receptor, Garland et al⁵⁴ identified a biphasic calcium mobilisation response for both of these receptors. In each case an initial rapid response was found to be due to the release of calcium from intracellular stores. This was followed by a second phase of extracellular calcium entry. The initial responses were similar for the two receptors, but the second phase was much more prolonged with NK2 activation.

After receptor activation there is a very rapid desensitisation for the early calcium mobilisation response for both receptors, but the desensitisation to the second phase response is much more marked for NK1 than NK2⁵⁴. In accord with this effect, *in vitro* assays have shown that the response to NK1 activation by SP rapidly diminishes even in the presence of continued high dose SP exposure⁵⁵. Both receptors undergo a rapid internalisation after ligand binding, via clathrin mediated endocytosis. Although it has been postulated that this may have a role in desensitisation⁵⁶, more recent studies show that this may not be the case^{54,57}, as the rapid desensitisation effect following NK1 activation actually precedes internalisation. It has been shown that the C-terminal region of the receptor is critical in the mediation of the desensitisation effect. Chimeric receptors possessing the NK2 sequence, but having the NK1 C-terminal, desensitise in the same manner as NK1 receptors⁵⁷. Phosphorylation of the C-terminal is likely involved (because the presence of phosphatases is critical for *resensitisation*), followed by second messenger uncoupling, but the precise mechanism is unclear⁵⁷. After desensitisation and

internalisation, receptor recycling occurs. This appears to occur via dephosphorylation of the receptor in the endosome, while the bound SP passes to a separate endosome and is degraded. The presence of phosphorylase is much less important for NK2 resensitisation. The recycling of NK1 receptors was shown to take at least several hours in rat blood vessels⁵⁶. These mechanisms of receptor desensitisation and recycling are likely to be key factors in the control of the response to tachykinins in normal and pathological states.

Detailed characterisation of receptor subtype mediation of the different tachykinin responses is important in view of the development of specific receptor antagonists which may have therapeutic potential. Tachykinin receptor distribution in the lungs has been investigated by radio-ligand binding⁵⁸, molecular biology techniques including mRNA in situ hybridisation, polymerase chain reaction and nuclease protection assay⁵⁹ and more recently by immunohistochemistry^{11,60}. Radioligand studies detected binding sites for Bolton-Hunter SP in airway smooth muscle, vascular endothelium, epithelium and submucosal glands⁵⁸. Interestingly, despite the potent effect of NKA in causing the contraction of human airway smooth muscle, radiolabelled NK2 did not bind to smooth muscle but only to arterial vessels²⁹. Significant NKB binding has not been seen in the lung. Bai et al, using a nuclease protection assay, detected NK1 and NK2 mRNA in human membranous airways and subpleural lung⁵⁹. They also found NK1 expression in submucosal glands and airway epithelial cells by in situ mRNA hybridisation, but could not detect NK2 expression with this technique. NK1 immunoreactivity has been detected at the apical border of airway epithelial cells and in the endothelial cells of the rat airway mucosa^{11,60}

Human NK1 receptor - top view

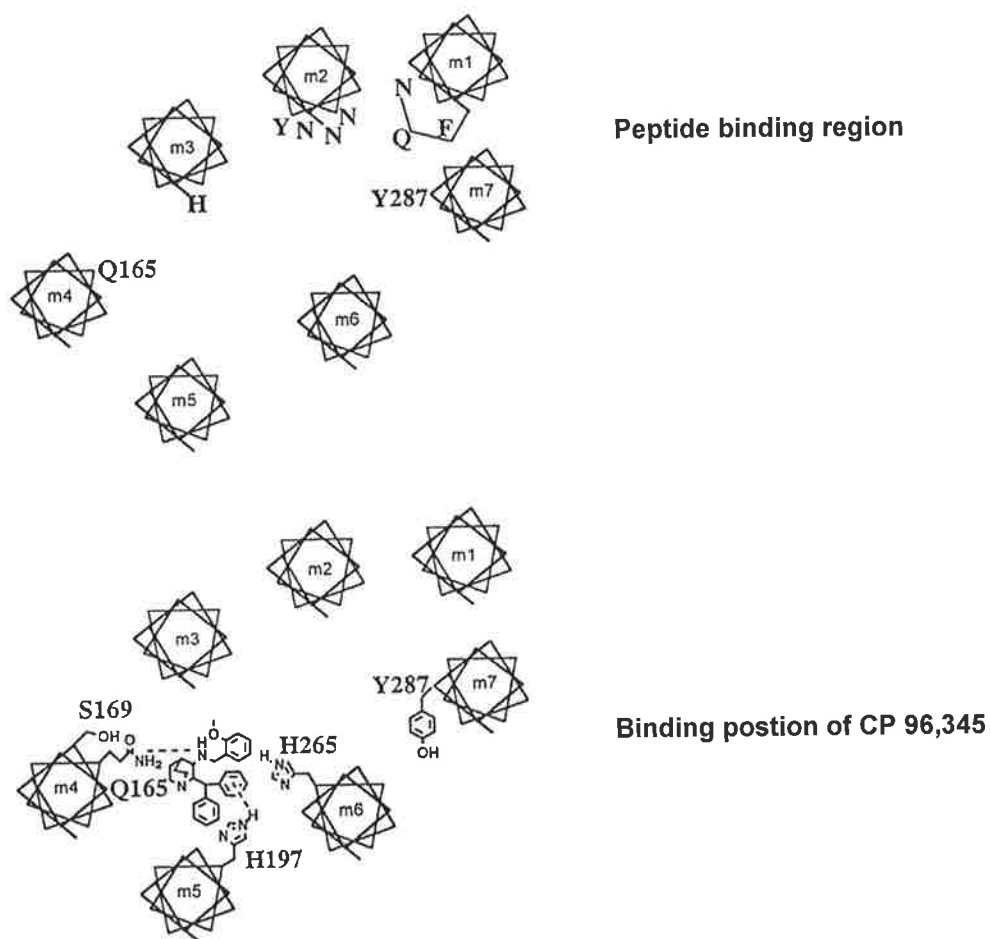


Figure 1-4. The orientation of the transmembrane helices can be appreciated when viewed from above as in the diagram adapted from Fong et al. The binding positions of neuropeptides and the NK1 specific antagonist are shown. It can be seen that SP and CP 96,345 bind to different regions of the molecule, thus antagonism appears to be due to steric hindrance.

Modulation of tachykinin receptor expression.

There is evidence that tachykinin receptor expression is modulated in inflammatory responses. Mantyh et al showed an increase in radiolabelled SP binding in inflammatory bowel disease⁶¹, although the significance of this in Crohn's disease versus ulcerative colitis is unclear. Studies of the level of receptor expression in asthmatic lung have yielded conflicting results. An apparent increase in NK1 receptor mRNA and no change in NK2 mRNA was seen in asthmatic compared to normal lung in one study⁶². However, in a separate report using a larger, more characterised group of subjects, NK1 mRNA was unchanged and NK2 mRNA was increased⁵⁹. In this latter report NK2 mRNA was increased 4-fold in asthmatics. NK1 and NK2 mRNAs were increased two-fold in smokers without airflow obstruction but appeared to fall toward the values of normal subjects in smokers with airflow obstruction. Using radiolabelled NK1 and NK2 receptor specific antagonists (CP96345 and SR48968 respectively) Mak et al⁶³ actually found a decrease in receptor binding in ovalbumin sensitised guinea pigs following repeated antigen challenge. Although these results could be due to a true downregulation of receptor expression, receptor internalisation after ligand binding is also possible⁵⁶ (and hence would reduce binding sites for the radiolabelled ligands – this might explain some of the apparent conflicting results of radioligand studies). Kaltreider et al, in a study using a murine model of pulmonary parenchymal inflammation⁶⁴, showed that allergen challenge lead to an influx of lymphocytes and macrophages, and that these cells had an elevated level of NK1 receptor expression. Use of the NK1 antagonist CP96,345 reduced the cellular influx. On balance, although there is an increase in tachykinin receptors in some inflammatory responses, this is not a generalisable phenomenon, and the underlying biology is complex.

There have been several reports of *in vitro* modulation of NK1 receptor with

glucocorticosteroids. Incubation with steroids led to a dramatic decrease in NK1 mRNA in rat pancreatic acinar cells (AR42J)⁶⁵ and modest decreases in human lymphoblastoma cells (IM9)⁴⁸ and human lung tissue explants⁶² (in the latter two cases approximately 50 and 85% reduction respectively). Correlation between the downregulation of mRNA and reduced number of functional receptors was shown for the IM9 cell response. This effect has been proposed as a mechanism contributing to the anti-inflammatory effect of steroids and fits with the reports of NK1 upregulation at sites of inflammation. However, upregulation of NK1 mRNA in response to steroids has been seen in an astrocytoma cell line. Thus, the mechanism of modulation of NK1 by steroids is incompletely resolved. A typical glucocorticoid response element has not yet been identified in either the rat or human NK1 genes⁵². Downregulation of NK2 receptor expression with the steroid dexamethasone has been demonstrated in bovine smooth muscle⁶⁶. This study provided convincing evidence that downregulation was mediated via steroid receptors and occurred at the level of NK2 receptor gene transcription. Correlation between gene transcription and functional receptor expression was shown using a specific radiolabelled NK2 receptor antagonist. It is tempting to postulate that airway inflammatory diseases tend to lead to an increase in tachykinin receptor levels, and that corticosteroids, which are often an effective therapy for these disorders, act in part by reducing receptor expression. However, further studies are required to confirm this hypothesis.

Tachykinin Effects

Tachykinins have a number of direct effects on tissues which suggest they may have a role in the pathogenesis of airway inflammation. Early studies attempting to attribute particular effects to a particular tachykinin receptor were often hampered by the cross-reactivity of the

native tachykinins for each receptor subtype, but the development of truly receptor specific agonists and antagonists has more recently facilitated a much more precise understanding of tachykinin biology.

Human airway obstruction in vivo

Tachykinin effects include bronchoconstriction which, in humans *in vivo* is mediated predominantly by NKA, indicating an NK2 receptor response. In one study⁶⁷ SP caused no change in specific airway conductance (sGaw) in either asthmatics or normals to a maximal nebulised concentration of 10^{-6} mol/ml (approximate dose delivered = 0.25mg), whereas NKA at half this dose resulted in a mean fall in sGaw of 48% in asthmatics. No haemodynamic changes occurred. Cheung et al⁶⁸ using a dry nebulising technique delivered an SP concentration of 8mg/ml (approximately 4mg delivered) and found a mean 20.8% fall in FEV1 in asthmatics along with an increase in heart rate and a decrease in blood pressure. This latter study also showed an increase in methacholine reactivity 24 hours after SP challenge, but not at 2 hours. This increase in reactivity was relative to a decrease in methacholine reactivity seen with a placebo challenge (believed due to methacholine tolerance), but there was no actual increase above initial baseline reactivity following the SP challenge. The timing of the increase lead the authors to speculate that the increase in reactivity was due to an influx of inflammatory cells. The same authors found a fall in expiratory flow (V_{p40}) of 29.7% in normals challenged with NKA (approximately 0.5mg delivered) which increased to 48.7% following inhibition of NKA digestion with the neutral endopeptidase inhibitor thiorphan. No evidence of NKA induced increase in methacholine reactivity was found in this study⁶⁹. In a separate study⁷⁰ the response to NKA (approximately 0.062mg delivered) in asthmatics was assessed. This dose of NKA

resulted in a mean fall in FEV1 of 27%, increasing to 45% after thiorphan. Corresponding falls in V_{p40} were 75 and 90% respectively. Once again there was no significant change in post-challenge methacholine reactivity. Crimi et al⁷¹ reported the use of a different technique to deliver up to 184 micromoles (239mg) of SP to mild asthmatics and found a dose dependent fall in FEV1 with a PD_{20} of 50.51 micromoles. This reported dose seems at odds with the above studies. Haemodynamic responses were not reported. Following administration of ipratropium bromide there was a shift to the right of the dose response curve with a 2.5 fold increase in PD_{20} . This indicates the contribution of a cholinergic response to the effect of SP administration. The antihistamine astemizole had no effect on SP induced bronchoconstriction.

Although these studies do not constitute a completely comprehensive investigation of the effects of tachykinins in human airways, on balance it is clear that exogenous tachykinins can cause bronchoconstriction, that NKA is more potent in this regard than SP, and that although some response to SP was seen, this was only at high doses and is at least in part mediated via a cholinergic mechanism.

Smooth muscle contraction

In vitro studies with the NK2 specific agonist SR48968 confirm the predominance of the NK2 receptor in the mediation of human bronchoconstriction⁷². Further studies indicate that the amino-terminally extended form of NKA, neuropeptide γ , is the most potent contractile tachykinin in human bronchus *in vitro*⁷³. Interestingly, passive sensitisation of human bronchus with serum from sensitised asthmatics has been shown to enhance contractility to tachykinins⁷⁴, although the mechanism of this response is yet to be determined. However, in contrast to the situation in rodents, and guinea pigs in particular, it is believed that human airways are regulated

mainly by cholinergic mechanisms⁷⁵. Human bronchus responds to electrical field stimulation (EFS) with a fast contraction which is almost completely inhibited by atropine. In comparison, atropine-resistant contractions account for approximately 60% of the contractions seen in response to EFS in guinea pig airways⁷⁶. This has raised doubts about the existence of significant noncholinergic contractions in human airway²⁴. Nevertheless, contractile responses to capsaicin and spontaneous contractions occurring when peptidase inhibitors are added to the organ bath support the existence of functional noncholinergic excitatory nerves in human airway tissues.

There is evidence that tachykinins facilitate cholinergic transmission via receptors on post-ganglionic parasympathetic nerve terminals in some species⁷⁷, including human airways⁷⁸ (albeit in the presence of potassium channel blockade in this case) *in vitro*. A number of authors have commented on the need for more studies in non-rodent mammals, in view of the species differences noted. We have demonstrated that sheep airways *in vitro* contract maximally to NKA, with a pD_2 similar to human tissue, that this contraction is atropine resistant and not subject to tachyphylaxis⁷⁹. Contractions in sheep trachea in response to SP were also atropine resistant, but weak. However, the NK1 receptor specific agonist Sar⁹Met(O₂)¹¹-SP induced sheep tracheal contractions which had quite different properties, being both atropine sensitive and subject to tachyphylaxis, suggesting that the predominant response to SP is actually due to cross-reactivity at NK2 receptors⁷⁹. These results further highlight the similarity between the human and ovine responses, suggesting the sheep as a relevant large animal model for the further investigation of the *in vivo* effects of tachykinins.

Under certain conditions SP has a relaxant effect on bronchial smooth muscle. Rat tracheal strips pre-contracted with serotonin relax in response to 10^{-6} M SP⁸⁰. This effect appears to be mediated by NK1 receptors and is dependent on the presence of epithelium on the tracheal strip. Following contraction and subsequent SP induced relaxation, prostaglandin E₂ (PGE₂)

levels in the organ bath were found to be significantly raised in the epithelium intact preparations. Exogenously added PGE₂ had equal relaxation effects in both epithelium intact and denuded preparations. Clearly this implies SP-induced airway epithelial PGE₂ production has a modulatory effect on SP-induced bronchoconstriction. A similar response has been seen in canine airway preparations⁸¹ but human studies have not been reported. Epithelial dysfunction, damage and cell loss is feature of airway inflammation and asthma in particular and thus the loss of epithelial derived relaxant factors could conceivably potentiate hyperactive bronchoconstrictive responses to noxious stimuli.

Vascular permeability

Tachykinins may mediate bronchial hyper-responsiveness by mechanisms other than stimulation or facilitation of airway smooth muscle contraction. Hyper-responsiveness may occur as a result of structural changes in the airway wall⁸². Resistance to flow through a tube is inversely proportional to the 4th power of the radius, therefore narrowing of the airway lumen by vasodilatation, airway wall oedema, cellular infiltration and mucus secretion will result in a disproportionately greater amount of airway obstruction for any given amount of smooth muscle contraction. It has also been hypothesised that submucosal oedema will facilitate airway narrowing by reducing the normal tractional support of the airway wall⁸².

In the rat, substance P increases vascular permeability by the opening of endothelial gaps at post-capillary venules, an NK1 receptor mediated response¹¹. Vasodilatation and increased blood flow occurs in the trachea and bronchial mucosa, which again appears to be an NK1 mediated response. The NK1 specific antagonist CP99,994 inhibits the vasodilatation response to capsaicin in nasal mucosa^{9,10,83}. The vascular response to histamine and bradykinin is

significantly reduced following capsaicin desensitisation in the rat implying these agents act indirectly via the release of neuropeptides from C-fibre nerves¹⁸. In non-rodent mammals reflex control of bronchial blood flow is mainly mediated by classical central reflex mechanisms. Peripheral sensory stimulation leads to withdrawal of adrenergic sympathetic vasoconstrictor tone and stimulation of parasympathetic cholinergic vasodilatation. There does however, appear to be a small but definite persistent vasodilator response following vagal blockade, which in some experiments is enhanced by the infusion of neutral endopeptidase inhibitor (which inhibits tachykinin degradation) into the bronchial artery¹⁸. These latter findings thus provide some evidence for the release of neuropeptide in the mediation of the vasodilation response. However, Coleridge⁸⁴ makes the point that if axon reflex mechanisms are occurring *in vivo*, they do not occur independently of classical orthodromic central conduction of impulses and stimulation of centrally mediated reflexes, hence the classical sensory afferent function of C-fibres should not be overlooked. In support of a role for tachykinins, Ichinose et al⁸⁵ showed that inhalation of the NK1 selective antagonist FK-888 reduced the time to recovery after exercise-induced asthma, but not the maximal reduction in sGaw. The nature and timing of this effect, in addition to relatively weak effect of NK1 receptors in mediating airway smooth muscle contraction in humans, suggests tachykinin mediated airway wall oedema is a factor in exercise induced asthma.

Mucus secretion

The effect of SP on mucus secretion in human bronchi⁸⁶ and nasal mucosa⁸⁷ has been examined *in vitro*. The effect appeared to be primarily an NK1 mediated response with SP being more potent than NKA or NKB. SP stimulates mucus secretion and goblet cell discharge in

human nasal mucosa, although cholinergic mechanisms are also involved as capsaicin induced nasal secretion is blocked by atropine. SP could be acting in this response by cholinergic facilitation. Ciliary beat frequency is also increased by SP, by direct and cholinergic mechanisms⁸⁸. There is also evidence that ion transport is stimulated by SP. On balance it appears that cholinergic mediation of secretion is more important than C-fibre mediated effects, although the latter may contribute more in disease states⁸.

Inflammatory Cells

SP has effects on inflammatory cells including neutrophil and eosinophil chemotaxis⁸⁹, neutrophil activation^{90,91} and increased immunoglobulin production by lymphocytes⁹². SP leads to mast cell degranulation in human skin but this effect was not seen in human lung mast cells obtained from resected lung specimens⁹³. More recently however, SP-induced degranulation has been reported in human pulmonary mast cells obtained by bronchoalveolar lavage⁹⁴ and has also been demonstrated in these cells using NKA⁹⁵. SP stimulation of mast cell degranulation and histamine release has also been shown in guinea pig lung⁹⁶.

Incubation of human nasal mucosal biopsies with SP has been reported to result in an increase in mRNA for several pro-inflammatory cytokines including IL-1 β , IL-3, IL-5, IL-6, TNF α and IFN γ ⁹⁷. These effects were more pronounced in subjects with allergic rhinitis. SP leads to the release of chemotactic activity for neutrophils from airway epithelial cells⁹⁸, and triggers the adhesion of eosinophils and neutrophils to venules⁹⁹ and neutrophils to epithelium¹⁰⁰. SP also increased gelatinase production by guinea pig alveolar macrophages¹⁰¹.

In view of the elevated levels of SP seen in the setting of allergic responses, some investigators

have proposed that SP may be involved in switching the profile of secreted cytokines to that typically associated with allergic reactions. Allergic responses are typically characterised by the cytokines IL4 and IL5 leading to IgE and eosinophilic responses. Such a response has been referred to as a Th2 – type response, in reference to the T helper lymphocytes responsible for the cytokine production. In contrast a Th1 response is characterised by a non-allergic cell-mediated immunity response chiefly directed by cytokines IL2 and interferon γ . The switching of cytokine profiles from a Th1 to a Th2 type response has received considerable attention in the investigation of inflammatory responses in asthma. Whether tachykinins have an important role in the switching of cytokine profiles is unclear, but it has been shown in vitro that SP can stimulate the release of “Th1-like” cytokines – IL2 and IFN γ - from a Th2 cell line¹⁰². In cells obtained from patients with atopic dermatitis SP stimulated secretion of both IFN γ and IL4¹⁰³ whereas in a murine model SP stimulated IL4 but not IFN γ . Thus, the data available so far has not shown that SP is associated with a clear tendency to either a Th1 or Th2 profile.

In addition to the effects of tachykinins on inflammatory cells it has also been shown that activated eosinophils themselves can stimulate the release of SP from cultured dorsal root ganglia neurons, an effect which may be mediated by major basic protein¹⁰⁴. The complex interaction between neural and immune mechanisms is further illustrated by the demonstration of PPT-A gene expression and SP production by murine eosinophils¹⁰⁵ and rat alveolar macrophages¹⁰⁶. Macrophage PPT-A expression was seen to be upregulated by inflammation and downregulated by corticosteroids.

Trophic effects

Tachykinins also have important trophic effects including the stimulation of proliferation and migration of lung fibroblasts¹⁰⁷ and airway epithelial cells¹⁰⁸, smooth muscle proliferation¹⁰⁹, proliferation of endothelial cells and angiogenesis^{110,111}. In this regard it has been shown that following capsaicin induced C-fibre degeneration, animals develop corneal opacities, skin lesions, delayed fur regrowth, decreased survival of skin flap and an increased susceptibility to develop gastrointestinal ulcer²⁶. Delayed response to tracheal injury has also been noted in adult guinea pigs following capsaicin induced neuropeptide depletion¹³. Tachykinins may play a role in the healing of the airway epithelial damage seen in asthma, but equally could be involved in the development of smooth muscle hyperplasia and subepithelial fibrosis seen in this disease. A protective role for C-fibre afferents has been suggested by several studies. For instance the inflammation and hyper-responsiveness induced by chronic sulfur dioxide (SO₂) exposure is greater in capsaicin desensitised rats¹¹². Similar results have been obtained with endotoxin¹¹³, ozone^{114,115}, acrolein and *Mycoplasma pulmonis* infection¹¹⁶. In tissue culture, SP has been shown to have a protective effect on airway epithelial permeability, an effect largely mediated by NK1 receptors¹¹⁷. Taken together these findings have lead to the suggestion that the increase in SP content in rat airways in response to chronic SO₂ is an adaptive response which could promote tissue repair¹¹⁸. Interestingly, chronic SO₂ exposure did not lead to an increase in PPT-A mRNA in dorsal and nodose ganglia, leading the authors to speculate that post-translational mechanisms were responsible for increased SP. Changes in local production of SP in the airway epithelium itself might also be a possibility, as we have detected PPT-A mRNA in human airway epithelial samples obtained by brush biopsy. PPT-A mRNA has also been detected in rat alveolar macrophages and neutrophils¹⁰⁶. The studies illustrating the trophic and protective effects of tachykinins serve to highlight the balance that exists between the

protective functions of C-fibres and their involvement in pro-inflammatory responses.

Tachykinin detection in airways disease

Studies examining SP content in the lungs of asthmatics have given mixed results. Ollerenshaw et al¹¹⁹ found an increase in the number, length and pattern of SP-containing nerves in the airways of asthmatics compared to normals, although the actual SP content in the tissues was not quantified. The cause of the increased in SP-containing nerves is uncertain, but could be due to constant epithelial nerve stimulation, perhaps indicating an upregulation of nociceptive transmission or a protective response directed toward healing of epithelial damage in view of the trophic effects discussed above. However, studies by Howarth et al^{120,121} in mild asthmatics found no difference in neuropeptide containing nerves between asthmatics and normals and in fact found no positive signal for SP in endobronchial biopsies from either group. Lilly et al¹²² examined SP content in tissue extracts from autopsy specimens of asthmatics and normals using high performance liquid chromatography. They found no difference between the parenchymal SP content of normals and asthmatics and in fact noted a decrease in tracheal content in asthmatics. Nieber et al^{123,124} found an increase in the SP content in the bronchoalveolar and nasal lavage of asthmatics compared to normals, and also a significant increase in SP content in response to allergen challenge only in asthmatics. Similar findings have been reported for nasal lavage in patients with allergic rhinitis¹²⁵. Tomaki et al¹²⁶ found an elevation in SP content in induced sputum in patients with asthma and chronic bronchitis compared to normals, which correlated with sputum eosinophil count and FEV1/FVC ratio. O'Connell et al¹²⁷ examined intraepithelial nerves in bronchial biopsies in patients with idiopathic persistent nonproductive cough. They found no significant difference in total nerve number, SP-immunoreactive nerves, numbers of

inflammatory cells or presence of oedema compared to control subjects, but they did find an increase in CGRP containing nerves. These findings suggesting perhaps an upregulation of sensory afferent function. These patients also had an enhanced cough response to capsaicin.

In a rat model of chronic bronchitis, exposure to SO₂ lead to an increase in SP content in the lung, but PPT-A mRNA in the dorsal root ganglia was not increased, leading the authors to speculate that reduced tachykinin degradation may have contributed to the response¹¹⁸. In another study where the response to hyperoxia was examined in neonatal rats, both an increase in lung SP and an increase in lung PPT-A mRNA was found, although the precise source of the PPT-A mRNA was not identified¹²⁸.

There is some evidence for increased tachykinin receptor expression in airway inflammation. Adcock et al⁶² found an increase in NK1 receptor mRNA in the lungs of asthmatic heart - lung donors compared to normals, whereas Bai et al⁵⁹ found an increase in NK2 receptor mRNA in the membranous airways of asthmatics, but no differences in peripheral lung and no difference in NK1 expression. Smokers had a two-fold increase in both NK1 and NK2 mRNA compared to normals.

From the foregoing it can be seen that there is a degree of apparently conflicting data available for the role of tachykinins in human airways disease, and serves to illustrate the need for further studies. Some of the apparent conflicts may be due to the fact that the principle factors involved in tachykinin biology have typically been evaluated in isolation. In this thesis an attempt is made to investigate the possible role of tachykinins by simultaneously analysing expression of tachykinins, their receptors and the enzyme chiefly responsible for tachykinin degradation. Any nett effect of tachykinins will clearly arise as a result of the balance between at least these factors.

Tachykinin degradation

Once released into the airways, tachykinins are subject to rapid degradation by endopeptidases, the most important being neutral endopeptidase (NEP, E.C. 24 11), a membrane bound protease which has been identified immunohistochemically in several sites in the lung including epithelium, submucosal glands, nerves and airway smooth muscle¹²⁹. Other enzymes including angiotensin converting enzyme (ACE), present on pulmonary endothelium, also have activity against tachykinins but play a less important role, although it has been proposed that the cough induced by ACE inhibitors may in part be mediated via reduced tachykinin degradation. There are three potential cleavage sites for NEP on SP – between Gln⁶-Phe⁷, Phe⁷-Phe⁸ and Gln⁹-Leu^{10 130}. There is some evidence that cleavage fragments of SP have distinct biological activities, for instance the fragment SP(7-11) has been shown to increase prostaglandin production in bovine chondrocytes¹³¹. SP(1-7) has been shown to have specific behavioural effects in rat brain.

Unlike some neurotransmitters, re-uptake of released tachykinins by the nerve terminal does not appear to be an important controlling mechanism for the limitation of tachykinin effects. The limiting effect of receptor desensitisation has already been discussed. Epithelial NEP is also likely to be of particular importance since many of the sensory nerve endings which release tachykinins are located in close proximity to basal epithelial cells. The importance of an intact epithelium in limiting the effects of tachykinins, has been demonstrated in many *in vitro* studies which show an enhancement of tachykinin effects following removal of the epithelium¹³². Loss of NEP is one possible explanation for this. *In vivo* studies^{69,70}, have shown an enhancement of the bronchoconstrictor effect of tachykinins following pretreatment with NEP inhibitors (eg. phosphoramidon or thiorphan). Various insults to the epithelium including respiratory viral infections¹³³, cigarette smoke^{134,135} and toluene diisocyanate¹³⁶ decrease NEP activity and exaggerate neurogenic inflammatory responses¹³⁷ and it has been reported that

glucocorticoids increase airway epithelial cell line NEP production in vitro¹³⁸, although not in all airway cell lines¹³⁹. Airway NEP levels are higher in asthmatics treated with inhaled steroids than those not receiving steroids¹⁴⁰. Recombinant human NEP has been shown to reduce tachykinin induced cough in guinea pigs¹⁴¹. It should be noted that NEP is not specific for TK degradation, but also degrades other potentially proinflammatory peptides including bradykinin. For example, it has been shown that NEP increases the bronchoconstriction response to bradykinin in man¹⁴².

In light of the established importance of NEP in limiting the effects of tachykinins, and of the epithelial damage seen in asthma, it has been proposed that a reduction in airway epithelial NEP may be present in asthma, thereby leading to enhancement of tachykinin effects. The experimental evidence to date does not support this hypothesis however. Patients with asthma respond more to inhaled tachykinins than do normals, but the bronchoconstriction responses seen are enhanced both groups by NEP inhibitors⁷⁰. It was reasoned that if NEP was already significantly depressed in asthmatics at baseline, then further inhibition with phosphoramidon should not have any significant enhancement. This study was conducted using asthmatics of only mild severity however. The lack of any effect of NEP inhibitors on baseline tone in asthmatics has been seen by some²⁴ as evidence that there is no functional increase in tachykinins in the asthmatic airway.

Axon reflex hypothesis

In an effort to combine the various components of the tachykinin axis into a logical theory of the role of tachykinins in asthma, an axon reflex hypothesis has been proposed¹⁴³. The components of this are illustrated in Figure 1-4. However, an anatomical correlate to the

proposed arborised sensory nerves has not been identified in the airway. Part of our investigations have examined the possibility that tachykinins may be produced by the airway epithelium itself, and not only released from sensory nerves.

Axon Reflex Hypothesis

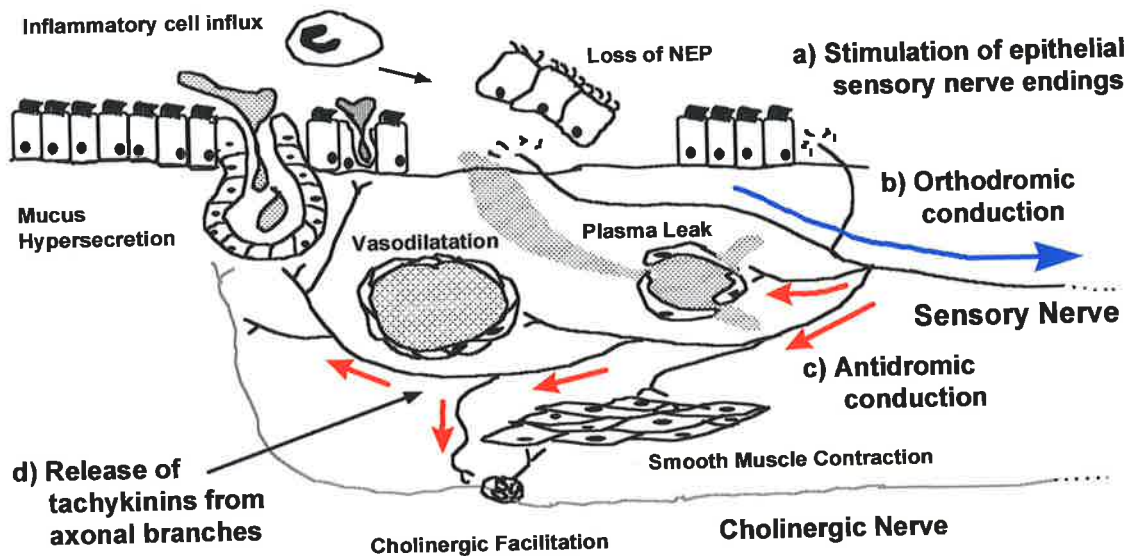


Figure 1-5. The above schema, adapted from Barnes, illustrates a proposed mechanism for the interaction of sensory fibres with airway structures in the pathogenesis of asthma. Stimulation of airway sensory nerves (a) leads not only to the orthodromic conduction of impulses centrally for the mediation of sensory responses (blue arrow, b) but also to antidromic conduction of impulses down arborised axonal branches (red arrows, c) with the resultant release of tachykinins into the local milieu (d). In this way tachykinins may exert a number of local responses including smooth muscle contraction, increased vascular permeability and vasodilation, mucus secretion and stimulation of inflammatory cells. Epithelial loss, as seen in asthma, may further potentiate tachykinin effects due to a loss of NEP and reduced tachykinin degradation. All of these responses are potentially of importance in the pathogenesis of airways inflammation. However, an anatomical correlate to the above proposal has not been identified. Local production of tachykinins by cells resident in the airway wall, including airway epithelium and inflammatory cells may also be possible.

Therapeutic implications

The evidence for proinflammatory effects of tachykinins in the airways has prompted the development of tachykinin receptor antagonists which may have clinical utility. Nonpeptide antagonists have been developed, which are resistant to peptidase degradation and therefore potentially useful as oral agents. Although these agents have been used extensively *in vitro* and in some *in vivo* animal studies, there is little information available regarding their use in human asthma. An NK1 and NK2 receptor antagonist, FK-224, was shown to inhibit the bradykinin induced bronchoconstriction in asthmatics¹⁴⁴ and the selective NK1 antagonist FK-888 was shown to reduce the recovery time after exercise induced asthma⁸⁵. Joos et al¹⁴⁵ reported the use of FK224 in NKA induced bronchoconstriction in asthmatics but found no protective effect with the doses used. It is believed that further trials with FK224, CP96,345 and CP99,994 have been undertaken but are yet to be formally reported¹⁴⁶. Investigation of potentially clinically useful tachykinin receptor antagonists continues to be an area of intense interest. Interestingly, nedocromil sodium has been shown to inhibit the bronchoconstrictor effect of tachykinins in asthmatics¹⁴⁷⁻¹⁵⁰.

Conclusion

In conclusion there is substantial evidence for a role for C-fibre sensory nerves and tachykinins in the pathogenesis of airway hyper-reactivity and inflammation, although there are still many unanswered questions regarding their relevance and mechanisms in human disease. The potential usefulness of tachykinin receptor antagonists as therapeutic agents remains unclear and can only be answered by well conducted trials in patients with airways disease. However, there is a clear need for non-rodent animal models in which responses to tachykinins are relevant to human physiology. Sheep airways have *in vitro* responses to tachykinins that are similar to human responses, and cell trafficking in sheep airways in response to allergen has similarities to human asthma. Thus, in view of this evidence for similarities between human and ovine airway physiology, we wished to evaluate the sheep as a potential *in vivo* model for the evaluation of responses to tachykinins. In this way we hoped to evaluate pharmacological interventions affecting the tachykinin axis which might provide useful information for the development and evaluation of potential human therapies. In addition, although the airway epithelium is of critical importance in the modulation of airway inflammation there is little information about alterations that occur in the tachykinin axis (tachykinins, NEP, tachykinin receptors) at the epithelial level in humans *in vivo*. Although the balance of evidence in the literature suggests an increase in tachykinin activity in airway inflammation, there is conflicting evidence regarding the mechanism by which this may occur. Thus, investigations were performed to evaluate the expression of the principle components of the tachykinin axis at the molecular level in human airways *in vivo*, using a minimally invasive procedure. In this way it was planned to determine the specific receptor subtypes expressed, and to investigate alterations in the balance of expression of tachykinins, NEP and receptors in airway inflammation.

The specific questions I addressed in the following chapters are:

- 1) Are tachykinins involved in acute allergic responses to inhaled allergen in a large animal model relevant to human airways disease?
- 2) What are the mechanisms involved in the acute airway response to SP in this model?
- 3) Which components of the tachykinin “system” are expressed in human airway epithelium *in vivo*?
- 4) Is there evidence for a functional effect of SP on freshly harvested human airway epithelium?
- 5) How are the components of the tachykinin “system” modulated at the epithelial level in human airway inflammation?

Chapter Two

Methods Part 1: Physiological studies using an ovine model

Many of the studies investigating the tachykinin axis *in vivo* utilise rodent models of airway responses. Such studies have yielded important information indicating a role for tachykinins in the mediation of inflammatory airways responses and bronchial hyper-reactivity. However, the relevance of rodent models to the situation in human models has been questioned, and not surprisingly species differences in tachykinin response have been noted. As a means to investigate pulmonary responses *in vivo*, the sheep is an attractive candidate model for a number of reasons. It has previously been shown that sheep which are sensitised to a particular antigen will exhibit airway responses including bronchoconstriction when challenged with the nebulised antigen^{151,152}. These acute responses can be measured in real time and the effect of modulatory influences including pharmacological agents can be assessed. Importantly, in the sheep model, these studies can be conducted in conscious spontaneously breathing animals, thus avoiding any concerns of the confounding influences of anaesthesia. In addition, repeat studies can be performed in the same animals over time, allowing for the investigation of a number of modulatory influences in the same animal without the risk of one intervention impacting on the subsequent responses to other interventions. In addition there is some evidence that sheep develop late responses to challenge and influxes of inflammatory cells which may prove a useful model of chronic human asthma¹⁵³. This chapter describes the basic aspects of the sheep model. Specific details relating to the pharmacological interventions are given in the subsequent results chapters.

Animals

Australian merino ewes aged 12 -18 months were used. They were housed in the Institute of Medical and Veterinary Science (IMVS) animal facility and allowed to roam freely in pastures when not engaged in a series of experiments. When required for physiological studies, the animals were brought to an on-campus facility, housed in specially dedicated pens and fed food and water *ad libitum*. Any health concerns with the animals were attended to promptly by IMVS veterinary staff.

The study was approved by the Animal Research Ethics Committee of the IMVS and Royal Adelaide Hospital.

Allergen sensitisation

Allergen responses in the sheep model are best characterised in the publications of Abraham et al¹⁵⁴ and Kleeberger et al¹⁵⁵. These groups used nebulised extracts of an intestinal parasitic worm, *Ascaris suum*. Unlike sheep in North America, sheep in South Australia rarely encounter *Ascaris suum* naturally, so are not usually sensitised to this parasite. For this reason, a program of active sensitisation was instituted.

Ascaris suum extract was donated by Dr G. Stewart (Institute for Child Health Research, Perth, Western Australia). The lyophilised extract was dissolved in sterile water to a stock concentration of 80,000 protein nitrogen units (PNU)/ml as determined by the Folin-Lowry method, with OD determined using a Beckman DU 640 spectrophotometer (Beckman Instruments Pty. Ltd., Gladesville, NSW, Australia).

Three methods of sensitisation were assessed. Sheep were sensitised with extract using an injection of 5ml of *Ascaris* extract (stock concentration) emulsified in Freund's complete adjuvant (1:4, v/v). Sheep were inoculated via either the subcutaneous, or intraperitoneal route. Intratracheal primary immunisation was also performed by trans-tracheal injection of 10ml of a 1:100 dilution of extract.

Two weeks after primary immunisation, all sheep received trans-tracheal boosters of antigen as for the intratracheal priming. Two weeks later, sheep were tested for skin sensitivity and airways reactivity (defined below). Skin reactions were defined as positive if a wheal > 5mm average diameter developed 30 minutes after intradermal injection of a 1:1000 dilution of stock *Ascaris* extract in normal saline. Based on the results of these initial experiments, SC priming was used in a further 12 sheep.

Vascular access

A number of studies required vascular access for the administration of pharmacological agents.

Venous access for drug delivery was obtained via a permanent indwelling 16 gauge catheter placed in the left jugular vein. In some animals an arterial catheter was placed in the right carotid artery for measurement of blood pressure. Catheters were placed under general anaesthesia (sodium thiopental induction and ethrane inhaled anaesthesia) at least seven days prior to commencement of experiments. The management of the general anaesthetic was performed by IMVS veterinary staff. Venous catheters were flushed daily with heparinised saline. Arterial catheters were connected to a pressurised infusion bag containing 500ml heparinised saline, which was delivered at a rate of 2ml/hr at a pressure of 120 mmHg. Skin was closed

around the catheter with sutures, then the external portion of the catheter was tied or glued to the animal's wool to avoid accidental displacement.

For measurement of blood pressure during challenge studies the arterial line was connected to a disposable pressure transducer (Abbott Critical Care Illinois, USA) and signals were recorded on heat sensitive paper using a polygraph recorder. Mean arterial blood pressure was calculated as $\text{diastolic} + (\text{systolic} - \text{diastolic})/3$.

Measurement of airways resistance

Sheep were fasted overnight prior to experiments.

In the early phase of establishing the model the optimal method for tracheal intubation of the sheep was determined. Initially, sheep were briefly anaesthetised with an injection of sodium thiopental into the jugular vein. Either blind transnasal intubation or intubation with the aid of a fiberoptic bronchoscope was then performed. The animals were watched closely and allowed to awaken. Up to 30 minutes might be required before animals were fully roused and the additional experimental equipment was set up. In later studies it was determined that the use of anaesthesia for intubation was not required and the need for this additional physiological stress on the animals was avoided. In these experiments, unsedated sheep were restrained in a sling in the prone position with their head immobilised. The animals tolerated this procedure remarkably well.

Topical nasal anaesthesia was achieved with a 10%w/v lignocaine spray (Xylocaine, Astra Pharmaceuticals, NSW, Australia) followed by a 2% Xylocaine gel (Astra Pharmaceuticals). The sheep were then intubated transnasally with a cuffed endotracheal tube

(Portex 7.5 mm ID). The tube was placed over a fiberoptic bronchoscope (Olympus 1T10; Olympus Optical Co. Ltd., Tokyo, Japan), then the tube and bronchoscope were advanced gently to just above the vocal cords. Two millilitres of 1% lignocaine (Astra) were then instilled, and shortly thereafter the tube was advanced into the trachea, bronchoscope withdrawn and endotracheal tube cuff inflated. A side hole catheter (2.0mm ID) was introduced through the endotracheal tube and positioned with the hole 5cm distal to the tip of the endotracheal tube. This was used to determine tracheal pressure.

Pleural pressure was estimated using an oesophageal balloon catheter. A 10 cm oesophageal balloon catheter (Sydgate, NSW, Australia) was introduced through one nasal passage and placed in the stomach. Position was determined by monitoring pressure at the tip with a pressure transducer. The catheter was withdrawn slowly so that its final position was in the lower third of the oesophagus, shown by a negative pressure deflection with clearly discernible cardiac oscillations during inspiration. During experiments, the oesophageal balloon catheter and the tracheal catheter were connected to a differential pressure transducer (Gaeltec Ltd, Neomedix Systems, NSW, Australia). Transpulmonary pressure (P_{TP}) was determined as the pressure difference between tracheal pressure and oesophageal pressure as measured by a differential pressure transducer. Flow through the endotracheal tube was measured using a pneumotachograph (Fleisch No.2 Godart/Statham, Holland) heated to 37°C which was in line with a separate differential pressure transducer. While the animals were breathing spontaneously, the signals of flow and pressure were recorded by a 486SX personal computer (Protech Equipment, Adelaide, Australia) for breath by breath analysis using software written specifically for the project (Mr K. Tiivas, Department of Thoracic Medicine, Royal Adelaide Hospital). Immediately prior to each experiment the system was calibrated using a 1.5 litre calibration syringe at various flow rates. The computer programme allowed the on-line display of lung

resistance (R_L), dynamic compliance (C_{DYN}), tidal volume and breath frequency. Tidal volume was obtained by integration of the flow signal received from the pneumotachograph and C_{DYN} from the volume and pleural pressure measurements. R_L ($\text{cmH}_2\text{O} \cdot \text{L}^{-1} \cdot \text{sec}^{-1}$) was calculated as Pressure (P)/ Flow, where $P = P_{TP} - 1 / C_{DYN} \times \text{Volume}$ using the mid tidal volume technique^{156,157}. Total lung volume was not determined. A schematic diagram of the circuit set-up is shown in Figure 2-1, and photographs of the actual set-up are shown in Figures 2-2 and 2-3.

Reported values of R_L were calculated by taking the mean value of at least 5 consecutive breaths. The software programme contained a number of algorithms to identify artefacts caused by swallowing or animal movement. Breaths were deemed acceptable if both tidal volume and breath frequency were within 20% of the values for the previous breath, P_{TP} was positive and the start and end of the breath were clearly delineated. Colour coding of the breath signals on the computer screen allowed for immediate real time monitoring of the quality of the data being collected, and the pattern of signals could be monitored to determine immediately if there were technical problems such as blockage of catheters with mucus.

Aerosol Delivery

Ascaris extract and certain pharmacological agents were administered by inhalation. Aerosol was delivered using a ventilator and nebuliser (Bird™ Mark 14, CA, USA). The ventilator was set to deliver a tidal volume of 350ml at a rate of 20 breaths per minute using a 3% CO_2 in air mixture. Preliminary studies demonstrated that nebulisation with air alone resulted in a period of apnoea (due to hypocapnoea). The nebulising jet was delivered via a custom made dosimeter, which nebulised for one second per breath and delivered $7.2\mu\text{L}$ per breath

(Biomedical Engineering, Royal Adelaide Hospital, Australia). In this way a precise dose of agent could be delivered into the airway.

Sheep Model - Circuit Schematic

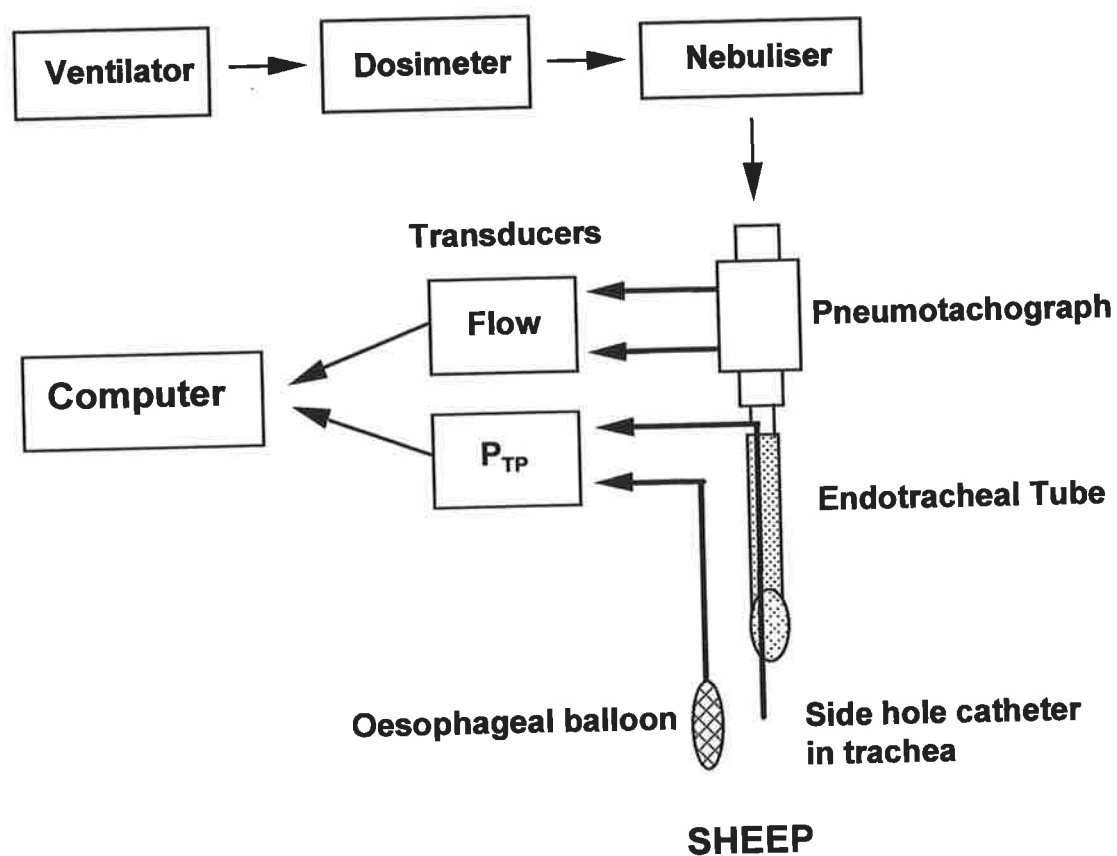


Figure 2-1. Schematic diagram showing arrangement of apparatus for measuring lung resistance (R_L) in sheep.

Endotracheal Tube Placement



Catheter and Transducer Placement

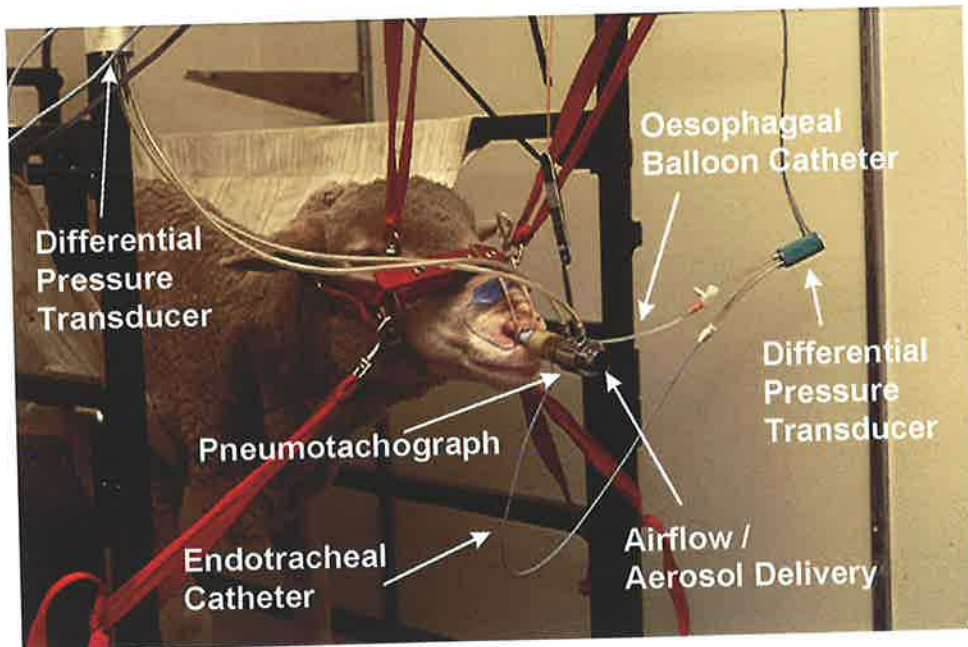
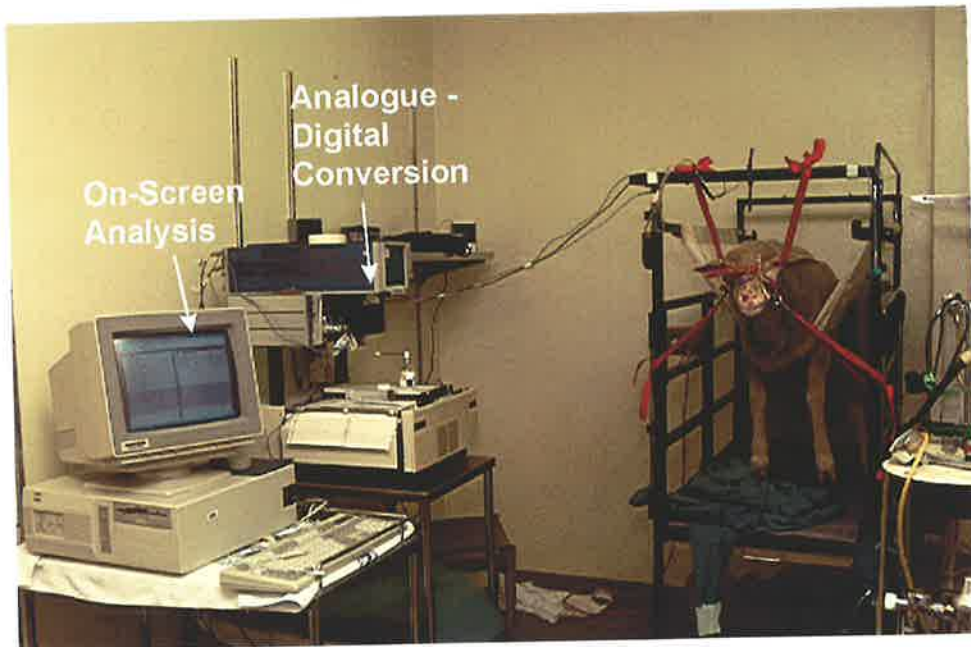


Figure 2-2. Measurement of airways resistance in sheep model. The arrangement of apparatus is shown.

Sheep Model Set - Up



On-line breath by breath analysis

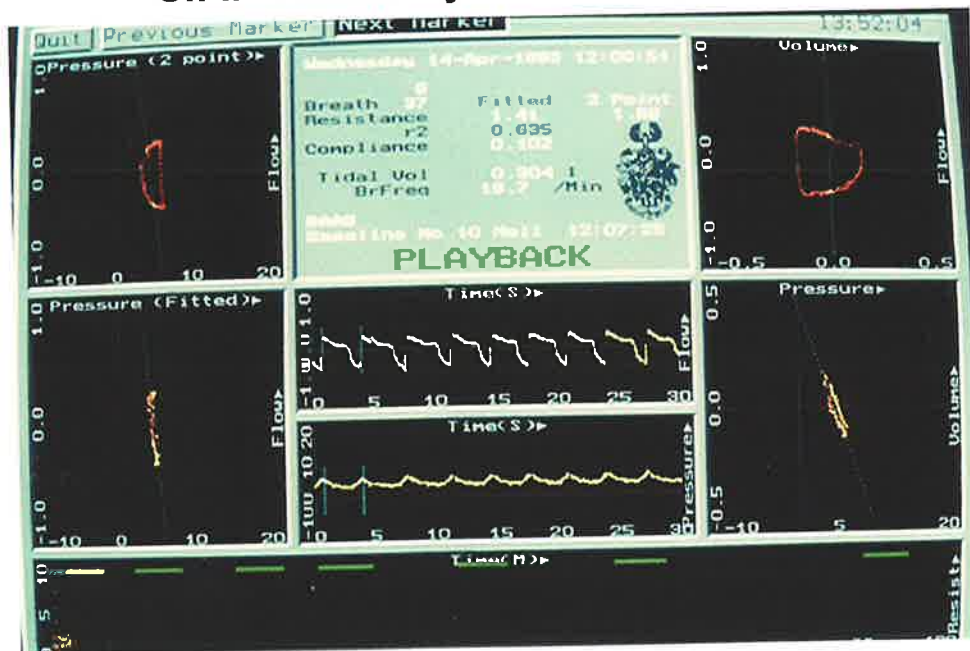


Figure 2-3. Measurement of airways resistance in sheep model. a) Connection of apparatus to computer. b) Computer screen.

Methods Part 2: Molecular and cellular analysis of tachykinin receptors, preprotachykinin A and neutral endopeptidase in human airway epithelium

All laboratory chemicals were obtained from Merck Pty. Ltd., Kilsyth, Vic., Australia unless otherwise specified.

Human airway epithelial samples

Samples of human airway epithelium were obtained directly from human subjects for the various aspects of the study. The rationale and aims of the individual studies are discussed further in the results chapters.

Subject Selection

All potentially suitable patients undergoing routine fiberoptic bronchoscopy for clinically indicated reasons were asked to participate in this study (see exclusions below) and informed consent was obtained. At the time of the clinical consultation where the patients were first advised of the need for bronchoscopy, potential subjects were informed about the existence of the study and the procedure involved, and supplied with a written information sheet about the study. These patients typically had several days to consider whether they wished to participate, then if they wished to do so, the procedure was again explained and written consent was obtained. The brushing procedure typically constituted only a few minutes extension of their diagnostic bronchoscopy procedure (which itself often included the use of brush biopsy for diagnostic purposes). For part of the study, bronchoalveolar (BAL) lavage was also performed, and if so this

procedure was specifically included in the informed consent. Clinical judgement was used to assess the suitability of patients in each case and those considered high risk based on the nature of their underlying disorder were not considered for the study. These included elderly patients with limited pulmonary reserve, immunocompromised patients and those with any coagulopathy. Patients with evidence of an active infectious process, including tuberculosis (on clinical and/or radiological grounds), or who were known or suspected carriers of human immunodeficiency or hepatitis viruses were excluded. Regardless of underlying condition or prior consent, no attempt to obtain research material was made in patients who were tolerating the bronchoscopy procedure poorly. The diagnostic nature of the procedure always maintained prime importance and was not compromised by the research study. The patients' indication for bronchoscopy, smoking history and medication history were recorded. Further details regarding the characteristics of the patients recruited are given in the relevant results chapters. The study protocol was approved by the Research Ethics Committee of the Royal Adelaide Hospital following the guidelines of the declaration of Helsinki. Every subject was supplied with contact information for the principle researchers (Drs. Reynolds, Holmes and Scicchitano as well as the chairperson of the ethics committee), so that any questions or concerns that arose after the study could be addressed. Patient subjects were not financially compensated for involvement in the study. Patients who were known or suspected carriers of HIV or hepatitis were not included in the study.

In a later phase of the study, normal volunteers (nonsmokers who had no history of any pulmonary disease) were also asked to participate. These subjects were provided the same information as the patients, and written consent was obtained. For these subjects financial compensation of \$100 was paid.

Bronchoscopy procedure

The patients were premedicated with 0.6mg of intramuscular atropine (Astra Pharmaceuticals, Sydney, NSW, Australia.) then sedated with titrated doses of intravenous midazolam (Roche Products Pty Ltd., Sydney, NSW, Australia.) and fentanyl (Astra). Supplemental oxygen was provided via nasal cannulae placed on the lower lip. Flow rate was 2 litres / minute minimum and increased if necessary. Oxygen saturation was monitored throughout the procedure by pulse oximetry and maintained above 90%. Transnasal fibreoptic bronchoscopy was performed using either an Olympus 1T10 or XT20 bronchoscope (Olympus Optical Co. Ltd., Tokyo, Japan). Topical 2% viscous lignocaine (Astra) was applied to the nostrils, then 0.5% via the bronchoscope to the pharynx, larynx and airways. A minimal amount of lignocaine (0.5%, 1-2mls) was used in the airways in view of the reported adverse effect lignocaine has on the viability of epithelial cells obtained by brush biopsy¹⁵⁸. In this latter report, the authors reported using no lignocaine at all distal to the vocal cords, however we found this was usually not well tolerated. The 0.5% concentration we used was just at the threshold of toxicity reported (ie approximately 80% viability when cells were incubated in this solution *in vitro* for 15 minutes at 37°C. All specimens were gathered from areas of the lung away from any obvious radiological or endobronchially visible pathology. This was to avoid any contamination of sample with cells from possible malignant or previously undiagnosed infectious processes. Brushing specimens were obtained by positioning the tip of the bronchoscope in a subsegmental airway, then gently advancing a standard cytology brush (Fuginon Inc., Wayne, NJ, USA) into four to six distal airways. Epithelial cells were obtained with several gentle passages of the brush into each airway so as to avoid bleeding. Cells were collected by washing the brush in 5ml of RPMI 1640 culture media (Gibco Laboratories, Grand Island, New York, USA) in a 10ml sterile conical tube and kept on ice. Bronchoalveolar lavage was performed by wedging the tip of the bronchoscope into a

subsegmental airway. A 50ml aliquot of sterile normal saline (at room temperature) was instilled into the airway with a syringe, then this was aspirated using low suction into a 50ml plastic suction trap. Two further aliquots of saline were instilled and aspirated in this manner into two further traps. To avoid contamination with airway mucus, the first aliquot was subsequently processed separately. Aspirated specimens were immediately transferred to 50ml polypropylene tubes (to avoid attachment of cells to the polystyrene plastic trap) and kept on ice. For analysis of SP content the polypropylene tube contained an inhibitor of NEP (phosphoramidon (Sigma-Aldrich Pty., Ltd. Castle Hill, NSW, Australia), final concentration 10^{-5}M) to inhibit SP degradation.

Characterisation of cells

Epithelial Cells

An aliquot of the epithelial cell suspension was examined directly in a 20mm diameter cell culture dish by phase contrast microscopy. Viability of the cells was assessed by the observation of ciliary beating and trypan blue exclusion. For this assessment, cells were resuspended in 0.2% (final concentration) trypan blue stain (Gibco), allowed to stand for five minutes, then the proportion of blue (non-viable) cells and total cells was counted. Rhythmic ciliary beating was seen on greater than 70% of the ciliated cells. Both trypan blue exclusion and ciliary beating could be assessed simultaneously in the one microscope field, and was seen to closely correlate - ie no beating was seen on blue stained cells. The cells were often in small strips or clumps as described previously¹⁵⁹. An aliquot of the epithelial cell suspension (50µl) was sedimented onto a microscope slide using a Shandon Cytospin 2 (Shandon Southern Products Ltd., Astmore, Cheshire, UK) for 5 min at 500rpm, then alcohol fixed, stained with a standard Papanicolou stain and examined under light microscopy to assess the morphological features of the cells. This procedure was performed by the Clinical Cytology laboratory of the IMVS, and the slides were evaluated by a cytologist experienced in assessing such samples. This technique showed that 95 - 97% of the cells were of epithelial type based on morphology. The cells demonstrated well preserved nuclear and cytoplasmic architecture. Because of the clumped nature of the specimens precise quantitation of cell number was often not possible by direct counting, however an estimate was made by resuspending the cells as homogeneously as possible then placing a small aliquot onto a standard haemocytometer (Sigma) and counting cells under phase contrast microscopy. For this assessment, cells within the eight 4 x 4 grids of the haemocytometer were counted, then the average of the eight grids taken. This number multiplied by 10^4 is the

concentration of cells per millilitre. Cells obtained by BAL were processed for Papanicolou staining as above, then cells were counted by an experienced cytologist.

Cytokeratin Staining

The epithelial nature of the cells obtained by brushing was further confirmed by immunohistochemical staining for cytokeratin. Cell suspensions were centrifuged at 200g for 10 min, then the media removed, cells washed in 5ml phosphate buffered saline (PBS - 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4 , 0.24g KH_2PO_4 in 1 litre H_2O , pH 7.4), then applied to aminopropyltriethoxysilane (APT; Sigma-Aldrich, Castle Hill, NSW, Australia) coated slides using a cytopspin as above. Cells were then allowed to air dry overnight. Cells were fixed in 5% acetone in methanol, then washed in PBS (3 x 5 min). Slides were then blocked with 10% normal horse serum (NHS) for 30 min. Slides were then incubated overnight with mouse anti-human anti-cytokeratin antibody (CAM 5.2, Becton Dickinson, San Jose, CA, USA) diluted 1/500 in 10% NHS. Negative controls consisted of slides incubated with no primary antibody, and cells incubated with a matched (IgG_2) isotype antibody. Next day slides were washed 3 x 5 min in PBS then incubated with a biotinylated rabbit anti-mouse secondary antibody (DAKO, Glostrup, Denmark) diluted 1/40 in 1% NHS for 1 hour. Slides were again washed 3x5 min in PBS, then endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in PBS for 20 min. Slides were again washed in PBS 3x5 min, then incubated with avidin-horse radish peroxidase conjugate (HRP; Pierce, Rockford IL, USA) in 1% NHS for 1 hour. Slides were then washed again in PBS 3x5 min then incubated for 10 min with NiDAB solution (made by adding 10ml of 5mM Tris/0.015M NaCl + 0.5ml nickel chloride + 50 μ l hydrogen peroxide + 100 μ l diaminobenzidine solution (Sigma)). This reaction was stopped by washing 3x in PBS, then slides were

rinsed in tap water. Counterstaining was performed with either 0.5% Methyl green in 0.1M acetate buffer or haematoxylin. Slides were then dehydrated in 100% alcohol, cleared in HistoClear and mounted using DEPEX (BDH, Poole, England). Positive staining was indicated by a brown precipitate within cellular cytoplasm when viewed by light microscopy.

Analysis of peripheral blood

Although care was taken to avoid blood contamination of the epithelial specimens, to check for the effect of minor blood contamination on the gene expression results, whole blood and leukocytes from three normal volunteers were collected and analysed for gene expression along with the epithelial specimens. Ten millilitres of blood was collected from each volunteer and placed in heparinised tubes. One millilitre of this was taken, the cells pelleted by centrifugation (400g, 10 min) and the serum discarded. RNA was extracted from the total cell pellet as detailed below. Buffy coat leukocytes were obtained from the remaining 9ml by centrifugation as above, and cells were washed twice in 0.15M NaCl. Residual erythrocytes were lysed by the addition of 40ml of lysis buffer (0.15M NH_4Cl /0.1mM Na_2EDTA /10mM NaHCO_3) at 37° C for 5 min. Cells were recovered by centrifugation, washed once in lysis buffer, then once in 0.15M NaCl, then RNA was extracted from the cell pellet as detailed below.

Cell culture

Some experiments were conducted using immortalised airway epithelial cell lines and primary cultures. Immortalised cell lines used were 1HAEo-¹⁶⁰ and 16HBE14o-¹⁶¹, both of which are SV40 transformed normal human airway epithelial lines (gift of Dr. D. Greunert, University

of California at San Francisco). These lines were propagated in RPMI media (Gibco) containing 10% foetal bovine serum (FBS; Gibco), gentamicin (50µg/ml) and penicillin G (100U/ml) and grown in plastic flasks (Corning - Costar, New York, USA). Cells were passaged when confluent. To passage cells, the monolayer was washed three times with sterile calcium-magnesium free PBS, then incubated with 2ml of 0.05% trypsin / 0.5% EDTA at 37°C until cells had rounded and loosened (usually 5 minutes). Cells were released from the flask with a sharp tap, then trypsin was inhibited by adding 10ml of complete medium. Cells were centrifuged at 200g for five minutes then resuspended in an appropriate volume of either medium or PBS depending on subsequent application. For simple passage cells were split 1:5 and replated in complete medium. When cells were to be used for experiments they were counted using a haemocytometer and viability was assessed by trypan blue exclusion. Typically > 95% of cells were viable by this assessment. The required number of viable cells was then plated into the wells of a plastic tissue culture plate (eg 6 or 24 well plate depending on the application) in complete RPMI medium.

Primary cultures were established from fresh brushing samples using a modification of the technique of Lechner and La Veck^{162,163}. Brushing specimens were collected in RPMI as before, centrifuged at 200g for 10 min, then resuspended in 1ml of LHC9 medium (Biofluids, Inc., Rockville, Marland, USA) containing gentamicin (50µg/ml) and amphotericin B (2.5µg/ml) and placed in 35 mm culture dishes that had been coated (to facilitate attachment of epithelial cells) with 2% gelatin / 0.1% bovine fibronectin / 0.01% bovine serum albumin (Sigma). To coat plates, 250µl of coating solution was incubated on the dish for at least 2 hours, with the dish being sealed with parafilm to prevent the solution drying. Just prior to plating of the cells, any excess solution was aspirated. Dishes containing cells were incubated at 37°C in 5% CO₂ and left undisturbed for 4 days. At this time media was then changed to fresh LHC9 (without

amphotericin) and second daily thereafter. After 2 - 3 weeks cells had become 80% confluent, and had the typical 'cobble - stone' appearance reported for airway epithelial cells¹⁶². Cells were passaged by removing media, washing with sterile calcium-magnesium free phosphate buffered saline (PBS), then incubating 3-5 min with 0.5ml 0.05% trypsin / 1% polyvinylpyrrolidone / 0.05% EDTA at 37°C. Once cells had loosened from the dish, 0.5ml of a 1mg/ml solution of soybean trypsin inhibitor (Sigma) was added to stop the digestion. Cells were collected and centrifuged at 200g for 5 min, then resuspended in LHC9 media and plated onto coated plates.

RNA Extraction

RNA is susceptible to rapid degradation by RNases. These enzymes are widespread on surfaces, hands of laboratory personnel and in the general laboratory environment, thus specific precautions must be taken to avoid RNases when working with RNA. All RNA extraction procedures involving human tissues were conducted in a biohazard level 2 laminar flow cabinet, both to avoid exposure to potential human pathogens and to reduce possible exposure of samples to RNases. Before and after extraction procedures the interior of the cabinet was sterilised by exposure to ultraviolet light. Latex gloves and clean gowns were worn at all times. Autoclaved polypropylene microcentrifuge tubes and pipette tips were used. All stock solutions used for RNA extraction and analysis (except alcohols and chloroform) were treated with diethyl pyrocarbonate (DEPC; Sigma) 1ml per litre for at least 12 hours at 37°C to inhibit RNases, then autoclaved at 120°C for 30 minutes. Water used in the reactions is ultrapure (Milli - Q processed; Millipore Australia Pty Ltd, North Ryde, NSW, Australia). All procedures with samples were performed in a separate room to the subsequent analysis of PCR products, to avoid potential contamination of samples with previously amplified cDNA.

Total cellular RNA was initially extracted using the method of Chomczynski¹⁶⁴. Cells were lysed in 4M guanidinium thiocyanate (Sigma) /25mM sodium citrate, pH 7.0/0.5% sarcosyl / 0.1M β -mercaptoethanol (BDH Chemicals, Poole, England). The RNA was extracted with phenol - chloroform, precipitated with isopropanol using yeast tRNA (Boehringer Mannheim Australia Pty. Ltd., Rose Park, SA, Australia.) as a carrier, then resuspended in DEPC - treated water. Later studies used a modification of the Chomczynski technique by using a commercially available RNA extraction solution, RNazol B (Bresatec, Adelaide, South Australia). This technique is less time consuming and gave more consistent results. Brushing samples (collected in 5ml RPMI in a 10ml polypropylene tube) were centrifuged at 200g for 10 min, supernatant removed, then the cells were lysed in 1ml RNazol B. Lysate was transferred to a 1.5ml microcentrifuge tube then 150 μ l chloroform / isoamyl alcohol (24:1) was added. The tube was shaken for 15 seconds, then stood on ice for 5 min. The tube was then centrifuged at 13000 rpm in a bench-top microfuge for 15 min at 4°C. The clear aqueous upper layer (500 μ l) was carefully removed in aliquots using a 100 μ l pipette tip, taking care not to disturb the white interface or blue lower organic layer, and placed in a new microcentrifuge tube. Re-extraction of this aqueous layer with an equal volume of chloroform / isoamyl alcohol was found to be necessary to remove residual traces of phenol. To achieve this, 500 μ l of chloroform / isoamyl alcohol was added to the tube, which was then shaken for 15 seconds, and centrifuged at 13000 rpm for 1 min. The upper layer (400 μ l) was then removed and placed into a new microcentrifuge tube. Because of the relatively small number of cells and because initial experiments yielded very small and inconsistent amounts of RNA, 10 μ g of yeast tRNA was added to each tube to co-precipitate with the cellular RNA. This step substantially improved the quality of yields¹⁶⁵. The tube was then vortexed and 400 μ l of pre-cooled (-20°C) isopropanol was added to precipitate the RNA. Tubes were left at -20°C overnight to maximise precipitation. Tubes were then centrifuged at 13000 rpm

in a microfuge at 4°C for 30 min. The supernatant was carefully tipped off, then the pellet washed in 1ml of 70% ethanol / DEPC treated water. Tubes were again centrifuged for 5 min, then the supernatant was totally removed using a glass pasteur pipette, which had been drawn out to a fine point over a Bunsen burner flame. The RNA pellet was air dried in the laminar flow cabinet for 5 min, then 22µl of DEPC treated water was added. Tubes were then heated to 65°C for 10 min to aid the solubility of the RNA. Tubes were then vortexed for 30 seconds and briefly spun down. To quantify the RNA and assess purity, 2µl was taken from each tube and added to 48µl of DEPC treated water. The absorbences of these dilutions at 260 and 280nm (OD_{260} and OD_{280}) were determined using a spectrophotometer (Beckman DU 640, Beckman Instruments Pty. Ltd., Gladesville, NSW, Australia.). Initially 50µl of DEPC treated water was placed in a quartz cuvette and read to establish a 'blank' control. The RNA specimens were then sequentially placed in the cuvette and the OD_{260} and OD_{280} readings were obtained. Plots of the absorbences over the range 200 - 300nm were obtained. A smooth curve with a definite peak at 260nm indicated a satisfactory specimen. The ratio of OD_{260} to OD_{280} was used as an index of protein contamination, values of 1.8 - 2.0 were optimal, with values lower than this indicating protein contamination. A distinct peak at 270nm was occasionally seen in the earlier experiments, which was found to be due to residual phenol contamination at the time of the first extraction. This problem was abolished with the routine introduction of the second chloroform / isoamyl alcohol extraction step. The OD_{260} was used to quantitate the RNA, based on the fact that an OD_{260} of 1.0 corresponds to an RNA concentration in the cuvette of 40µg/ml. For example an OD_{260} of 0.25 gives a concentration of $40 \times 0.25 = 10\mu\text{g/ml}$. Multiply by 25 to take account of the 2 : 48 dilution factor in the cuvette gives 250µg/ml, or 0.25µg/µl for the concentration of the RNA sample, and $0.25\mu\text{g}/\mu\text{l} \times 20\mu\text{l} = 5\mu\text{g}$ RNA total in the sample tube. RNA was stored at -70°C until required. The extraction technique used for cultured cells was essentially the same, typically 1ml

of RNazol was used to directly lyse cells in a confluent 35 mm dish. However, consistently good RNA yields were obtained without the use of yeast tRNA as a co-precipitant, because of the higher number of cells available, therefore this step was omitted.

Although the RNazol B technique results in the extraction of good quality RNA, which in the manufacturer's specifications is meant to be free of DNA, we found contamination with small amounts of genomic DNA was occasionally revealed by the high sensitivity of RT-PCR. Several attempts to totally eliminate genomic DNA by treating the RNA samples with DNase (Boehringer) were made. However, despite subsequent heating steps to inactivate DNase (as recommended by the manufacturer), residual DNase activity remained and greatly compromised subsequent reverse transcription and PCR. Further processing of samples by phenol-chloroform extraction and ethanol precipitation resulted in significant net losses of sample RNA without consistently improving subsequent PCR results. For this reason discrimination between genomic DNA and cDNA reverse transcribed from mRNA was primarily achieved by the position of the amplification primers (see below).

Each RNA extraction was accompanied by a concurrent "sham" extraction using all reagents but no cells to be used as a negative control for the PCR analysis. The inclusion of this control ensured that results were not confounded by contamination of reagents with small amounts of amplified cDNA product from previous PCR reactions.

The yield of RNA ranged from 5 to 10µg per epithelial brushing specimen.

Reverse Transcription Polymerase Chain Reaction

Design of PCR primers

DNA sequences for the genes of interest were obtained from the Genbank database on CD-ROM. Information regarding the position of introns was obtained either from this database or from the original journal articles in which the sequence was published. Each gene sequence was obtained on computer disc from the CD-ROM, then loaded onto a Macintosh SE-30 computer. A computer program (Oligo 4, National Biosciences Inc. Plymouth, MN, USA)¹⁶⁶ was used to help choose optimal primers for PCR amplification. In most cases this required the editing out of intron sequences from the original DNA sequence as obtained from Genbank. This left a cDNA (and therefore RNA) sequence. This could then be opened by the Oligo program. The position of the various exons expressed in the cDNA was noted and it was ensured that 3' and 5' PCR amplification primers were chosen from different exons. This was to ensure that PCR amplification of genomic DNA (which may contaminate the RNA extracted from the cells) could be distinguished from the products resulting from the amplification of reverse transcribed RNA. Genomic DNA products would contain intron sequences and thus be larger than products derived from RNA. Once suitable regions of the gene were identified, the nucleotide positions corresponding to these ranges were entered into the computer program and primer options were identified. These options were given by the program based on certain parameters which could be manually set. These parameters included primer length (typically 18 - 21 base pairs), uniqueness of 3' ends, 3' terminal stability (reduced annealing stability at the 3' terminal helps to enhance specificity by reducing nonspecific annealing), absence of hairpin loops in the primers and absence of primer dimer formation (particularly at the 3' terminal, where dimer formation can markedly reduce target amplification efficiency because primers are annealed to each other and

effectively unavailable for annealing to the intended target). From the list of options given and by selectively manipulating the parameter settings an optimal primer set for each gene of interest was chosen. Amplified products of between 200 and 500 base pairs were preferred. Optimal annealing temperatures for amplification, based on the melt temperatures (the temperature at which an annealed primer or probe detaches from the cDNA template) of the two primers and the PCR product, were calculated by the computer program.

Oligonucleotide probe sequences were then chosen, designed to hybridise to a region of the amplified product internal to the amplification primers. The chosen primer sequences were then checked for specificity against the Genbank database. This combination of a specific primer pair and specific probe ensured optimal sensitivity and specificity. Oligonucleotide primers and probes were synthesised by the Department of Haematology, IMVS using a DNA synthesiser 391 (Applied Biosystems, Scaresby, Vic., Australia.). These oligonucleotides were supplied on a synthesis column, thus for use they were washed from the column with ammonia (3 x 1ml washes, 20 min per wash). This was followed with an incubation at 55°C overnight ("de-protection" step) then drying in a SpeedVac. The oligonucleotides were then dissolved in autoclaved ultrapure (Milli-Q) water, quantitated by spectrophotometry (using OD₂₆₀ of 1 = 20µg/ml for oligonucleotides) and made to a working stock concentration of 20µM and stored at -70°C.

The specific primer and probe sequences, along with the reference for the sequence obtained from Genebank^{49,51,52,167-171} are given in table 1.

Table 1. Oligonucleotides used for RT-PCR.

Target gene	Oligonucleotide sequence	Annealing temp. (°C)	Product size (bp)	Reference
NK1	sense - CCC AAA CAT CTC CAC TAA CA antisense - GAT CAT GCA CAC GAC TCT G probe - CCT ATG CTG TCC ACA ACG AAT	55	511	Hopkins, 1991
NK2	sense - GCC CTG AGA GCA ACA CCA C antisense - GCC AGG CCA CCA CGC ACT T probe - ATC ACA GCC ATG TTT GTC AGC	59	513	Graham, 1991
NK3	sense- ATC CCT TGA AAC CCA GAC T antisense - AAA GGA CAC CAG CGA AAT G probe - ATC CCT TGA AAC CCA GAC T	52	562	Takahashi, 1992
NEP	sense - CAT ATT GAC CAA CCT CGA C antisense - GCT GAG GCTGCT TAC AAG probe - GCA CTG GAA TCT ATA AAG AG	55	500	Shipp, 1988
PPT-A	sense - ATT ACT GGT CCG ACT GGT antisense - CTT CTT TCA TAA GCC ACA GA probe - CAG ATC AAG GAG GAA CTG CCG	52	274	Harmar, 1986
IL8	sense - TTG GCA GCC TTC CTG ATT TCT antisense - CAG CCC TCT TCA AAA ACT TCT probe - TAC TCC AAA CCT TTC CAC CCC	55	262	Mukaida, 1989
ICAM-1	sense - CAA GTT GTT GGG CAT AGA GAC antisense - GCA GCG TAG GGT AAG GTT C probe - GAA CTG GCA CCC CTC CCC TCT	55	241	Voraberger, 1991
Actin	sense - CAC CGC CGC ATC CTC CTC T antisense -CCT GCT TGC TAA TCC ACA TCT G probe - TCC TGG CCT CAC TGT CCA CCT	59	398	Erba, 1988

Because of the high degree of homology between the three receptor subtypes all primer pairs were not only checked for specificity by computer analysis but also functionally assessed by testing amplification specificity against a panel of tachykinin receptor cDNAs (gift from Dr A. Graham, Biotechnology Dept., ICI Pharmaceuticals, Cheshire, UK). No cross-reactivity between any specific primer pair and the other receptor cDNAs was seen (detailed results given in Chapter 5).

Reverse transcription polymerase chain reaction was performed using an RT-PCR kit

(Perkin Elmer - Cetus, Scoresby, Vic., Australia.) according to the manufacturer's instructions. A Perkin Elmer DNA Thermal Cycler 480 was used for RT and PCR reactions. RNA was reverse transcribed to cDNA using random hexamers (2.5 μ M), deoxyribonucleotide triphosphates (dATP, dCTP, dTTP, dGTP, 1mM each), PCR Buffer II (final concentration. 50mM KCl, 10mM Tris-HCl pH 8.3), 5mM MgCl₂ and Murine Leukemia Virus reverse transcriptase (2.5U/ μ l). Total reaction volume (per 1 μ g of RNA) was made to 20 μ l with DEPC treated water. Reaction proceeded for 10 min at room temperature to allow for initial annealing and extension of hexamers, then for 15 min at 42°C, then 99 °C for 5 min to destroy and remove the reverse transcriptase. This latter step is essential to prevent subsequent inhibition of PCR amplification. The resultant cDNA was then amplified (in a total volume of 100 μ l per 20 μ l cDNA) with AmpliTaq DNA polymerase (2.5 U / 100 μ l), PCR Buffer II, MgCl₂ (2mM) and specific oligonucleotide primers (0.2 μ M). To reduce pipetting errors and to improve handling efficiency master mixes of reagents and primers were first prepared (in slight excess), then these were aliquotted to the required number of reaction tubes (500 μ l GeneAmp PCR tubes, Perkin-Elmer). The appropriate cDNA was then added, then the reaction mixture was overlayed with 80 μ l of sterile paraffin oil, to prevent evaporation. Aerosol resistant pipette tips (Integrated Sciences) were used throughout, to prevent contamination of master mix reagents. Preliminary studies evaluating variable concentration of primers showed the above concentrations to be optimal.

All PCR reactions were prepared in a laminar flow biohazard cabinet in a separate room from that used to analyse amplified products, to avoid any contamination from previous analyses. All reagents were kept on ice and master mixes were prepared on ice and kept on ice until transferred to the PCR cycler, which was pre - heated to 95°C before reaction tubes were inserted for the initial denaturation step. This method was adopted in an effort to avoid any false priming,

because nonspecific annealing of primers to DNA and low level Taq polymerase activity can occur at room temperature. Investigation of optimal magnesium concentrations for the use of these reagents across a concentration range of 0.8 to 5mM showed the concentrations recommended by the manufacturer (as above) to be satisfactory. An example of the effect of variable magnesium concentration on PCR is shown later in Figure 2-6, along with a discussion about amplifying two genes in the one reaction.

The cycling program used for amplification of all targets was similar except for the annealing temperature (table 1) and in later semi-quantitative experiments, the total cycle number. The basic program consisted of 95°C for 5minutes, to ensure complete denaturing of the cDNA, followed by repeat cycles of the specific annealing temp (eg 55°C) for 1 min then denaturing (95°C) for one minute. This protocol allows for both annealing and extension during the 1 min at the annealing temperature. This "two - step" cycling procedure was based on the manufacturer's instructions as being optimal for the reagents. An alternative approach often cited in the literature uses a "three-step" cycle - eg denaturing at 95°C for 1 min, 1 min at the specific annealing temperature, then an additional extension step of 1 min at 72°C before the next denaturation at 95°C¹⁵⁹. Instead of multiple 72°C steps, the 'two - step' protocol simply has one single 7 min step at 72°C at the end to ensure complete extension of all amplified fragments. For the gene targets we wished to analyse we found no advantage in the three step protocol in preliminary analyses, so we continued with the recommended two-step cycling protocol. Negative controls consisting of the "sham" RNA extractions were used with each primer set and amplified alongside the patient samples. Because of the massive amplification of target cDNA possible with PCR, these controls were essential to ensure that no contamination of reagents had occurred with previously amplified products. In certain experiments (indicated in Chapter 5) an additionally stringent control of performing amplification of RNA samples without reverse transcription was

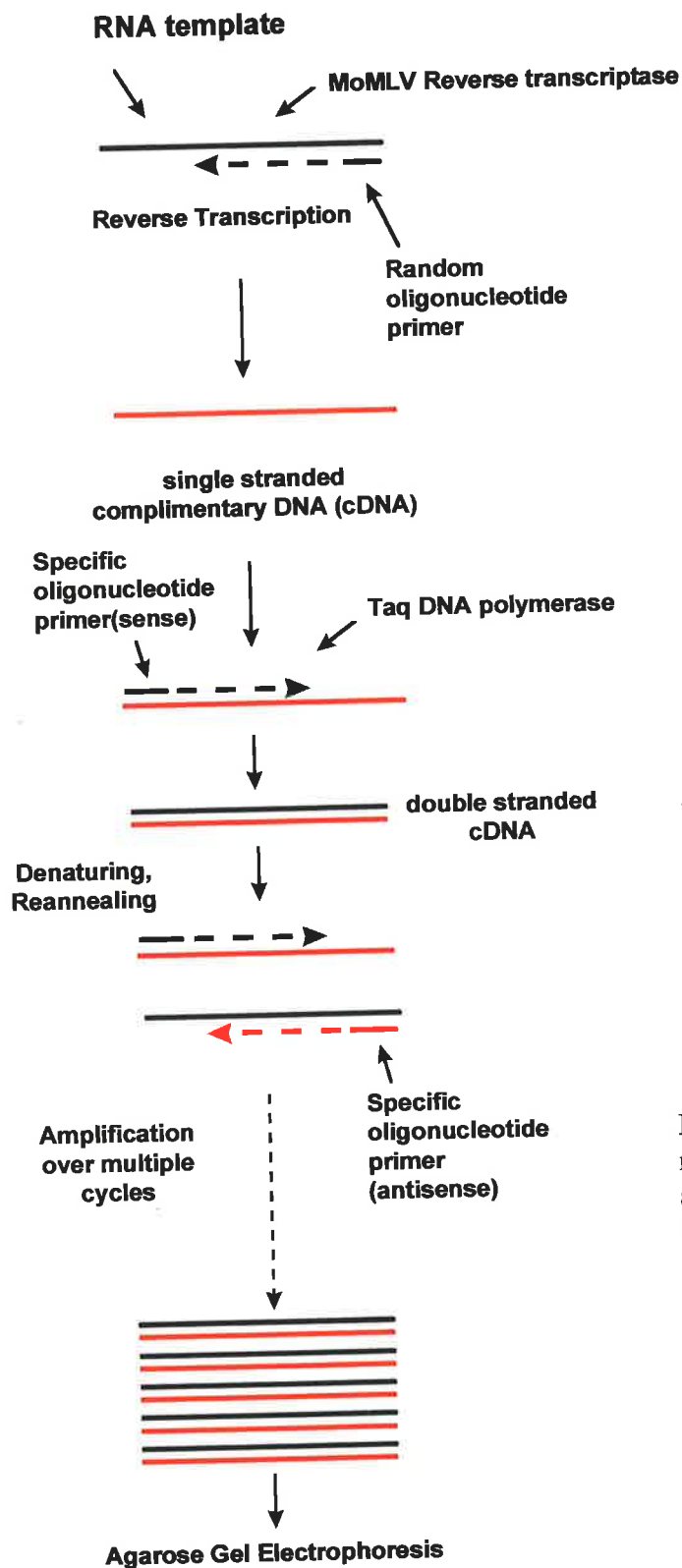
also included. After amplification, PCR products were either analysed immediately or stored at -20°C. A diagram illustrating the PCR and subsequent processing is shown in Figure 2-4.

Analysis of PCR Products

Agarose Gel Electrophoresis

Agarose gels (1%) were prepared by adding 1 gram of agarose (Sigma) to 100ml 1 x TAE buffer (50 x TAE buffer is 242g Tris / 57.1ml glacial acetic acid / 37.2g EDTA in 1 litre water). Agarose was dissolved by boiling in a microwave oven for 3 min, cooling to 65°C, then adding 5µl ethidium bromide stock solution (Sigma; 10mg/ml in water, stored at 4°C) and pouring the solution into a 10cm x 15cm gel mould (Bio-Rad Laboratories Pty., Ltd. Regents Park, NSW, Australia) and allowing the gel to set for 30 min. The solution containing the amplified PCR products was carefully removed from under the paraffin oil by passing a pipette tip through the oil layer, expelling air and any collected oil, then gently aspirating the products. This solution was transferred to a 1.5ml microcentrifuge tube, spun briefly at 13000 rpm to ensure any residual oil moved to the top and side of the tube. The volume to be analysed (typically 15µl) was pipetted from the microcentrifuge tube and placed in a new microcentrifuge tube. This ensured no oil remained in the analysed sample. Three microlitres of loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xyaline cyanol in H₂O) was added to the sample, vortexed, spun briefly, then 15µl was withdrawn and loaded into a lane of a 1% agarose gel. Pipette tips were changed between samples. DNA size markers (Puc 19 digested with Hpa II or SPP-1 digested with Eco R1 (both from Bresatec, Adelaide, SA, Australia)) were loaded into one of the lanes. Gels were electrophoresed in 1x TAE buffer in a 1 litre capacity gel tank (Biorad) at 70 volts (Biorad power source) for 1-2 hours.

Reverse Transcription - Polymerase Chain Reaction



Ethidium Bromide stained agarose gel, viewed by ultraviolet transillumination

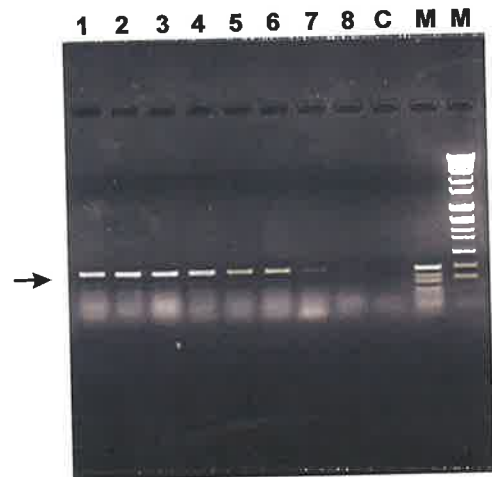


Figure 2-4. Amplification of specific mRNA sequences by RT-PCR. Identity of amplified band (arrow) confirmed by size on agarose gel electrophoresis.

Once the gel had run sufficiently, it was photographed using ultraviolet transillumination (Stratagene transilluminator, Stratagene, CA, USA) and a Polaroid Land Camera with Polaroid 667 film (Polaroid Australia Pty. Ltd. North Ryde, NSW, Australia.). A ruler was included in the photograph alongside the gel to provide a scale for later analysis of positive radioactive signal position after Southern blotting and radiolabelled probing.

Southern Blotting

After photography, gels were rinsed for 20 min x 2 in 500ml denaturing solution (1.5M NaCl / 0.5M NaOH), then 20 min x 2 in 500ml neutralising solution (1.5M NaCl / 0.5M Tris, pH 7.0). A metal tray was part filled with 20xSSC solution (3M NaCl / 0.3M trisodium citrate.2H₂O, pH 7.0), then overlayed with a glass bridge. A 20cm wide blotting paper wick (Whatman paper, Whatman Inc., Kent, UK) was soaked in 20xSSC then laid across the bridge so that each end dipped into the 20xSSC solution. The gel was then carefully laid on the wick ensuring there were no bubbles underneath. Hybond N+ membrane (Amersham, Uppsala, Sweden) was cut to the size of the gel, wetted in water then soaked for 10 min in 20xSSC. The membrane was carefully laid on top of the gel, ensuring no bubbles, then three layers of Whatman paper cut to the size of the gel were laid on top. A 5cm stack of absorbent paper towels was placed on top of this, then a 1 kg weight. The blot was covered in plastic wrap to reduce evaporation, then the transfer of amplified products by capillary action was left to proceed overnight. Next day the blot was dismantled, the position of the gel wells was carefully marked on the membrane and the membrane was briefly rinsed in 2xSSC. The membrane was dried for 10 min at 80°C in an oven. The DNA was then crosslinked to the membrane using a UV crosslinker (Stratagene) set at 240nm wavelength for 3 min. The membrane was then either stored at room temperature protected from light and dust, or probed immediately.

After the early experiments, processing of Southern blots was improved with the use of a vacuum blotter (Hoefer Scientific Instruments, San Francisco, CA, USA) (Figure 2-5). Following photography, the blotter was set up by placing 2 19 x 23cm layers of Whatman paper (soaked in 20xSSC) on top of the mesh plate inside the apparatus. This was overlaid with a 14 x 19cm piece of Hybond N+ membrane (pre - wetted and soaked in 20xSSC), then a latex rubber gasket which had a rectangular hole in the centre 1cm smaller in each dimension than the dimensions of the gel. The rubber gasket was securely clipped into place. The gel was laid squarely over the hole in the gasket, making direct contact with the membrane below and ensuring there were no bubbles between gel and membrane. A vacuum of 10cmH₂O was applied via a connection beneath the mesh layer, using a vacuum pump (AMRAD Pharmacia Biotech, Sydney, NSW, Australia.). Denaturation was performed by overlaying the gel with denaturing solution and allowing this to soak through for 15 min. Residual denaturing solution was then removed and replaced with neutralising solution, again for 15 min. This was then removed and the gel was covered with 20xSSC solution for 1 hour. At this time the apparatus was dismantled and the membrane briefly rinsed in 2xSSC. Direct comparison of this technique with standard capillary blotting showed that the vacuum technique gave a more efficient transfer as well as being substantially faster. The blotter could also be set up to transfer two 10x15cm gels at once. If it was ever necessary to compare samples run in different gels (due to a high total number of samples), then each gel had a standard lane in which a fixed amount of positive control (previously amplified cDNA) was run, to detect any subtle variation in transfer efficiency between the two gels (this proved to be minimal). After transfer the position of the gel wells was marked on the membrane, then the membrane was dried and the DNA crosslinked as above. A schematic illustration of the Southern blot procedure is shown in Figure 2-5.

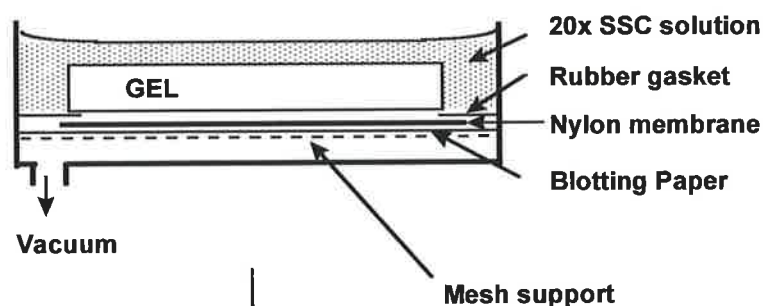
Radiolabelled Oligonucleotide probing

All radioactive materials were handled behind perspex shields using latex gloves and wearing protective gowns. Radioactive waste was disposed of according to regulations.

Membranes were pre-hybridised for 2 hours at 59°C in 5xSSPE (20xSSPE is 3.6M sodium chloride, 0.2M sodium phosphate, 0.02M EDTA, pH 7.4), 5 x Denhardt's reagent (0.04% Ficoll, type 400 (AMRAD Pharmacia), 0.04% polyvinyl pyrrolidone (Sigma), 0.04% bovine serum albumin (Sigma)), 0.5mg denatured salmon testis DNA (Sigma), 0.5% sodium dodecyl sulphate (SDS)). The relevant oligonucleotide probe was end labelled with $\gamma^{32}\text{P}$ -ATP (Bresatec, Adelaide, SA, Australia.). The labelling reaction was performed in a 1.5ml microcentrifuge tube by combining 1 μl probe (0.2 $\mu\text{g}/\text{ml}$ stock concentration), 2 μl H_2O , 1 μl One-Phor-All Buffer (AMRAD Pharmacia), 5 μl $\gamma^{32}\text{P}$ -ATP and 1ml T4 polynucleotide kinase (AMRAD Pharmacia), then incubating at 37°C for 1 hour. The tube was then heated at 65°C for 10 min to inactivate the enzyme. Labelled probe was added into the prehybridisation solution in the hybridisation bag, or in later experiments in a hybridisation roller bottle in a dedicated hybridisation oven. Depending on the oligonucleotide probe sequence, slightly differing hybridisation temperatures were recommended on theoretical grounds (based on estimated melt temperature) by the Oligo computer program. In practice it was found that a temperature of 59°C gave sensitive and specific results for all probes. Hybridisation proceeded overnight. The membranes were then washed in 1xSSPE, 0.1% SDS for 30 min x 2 at room temperature then in 5xSSPE / 0.1% SDS for 15 min at the hybridisation temperature. Membranes were lightly blotted with a paper towel, wrapped in plastic film (Glad Wrap) then exposed to xray film (X O-MAT AR film, Eastman Kodak Company, Rochester, NY, USA) in cassettes at -70°C (to reduce background radiation) for various periods from 6 to 72 hours, then the films were developed using an automated film

processor (Kodak). The position of the well markings on the membrane were carefully transcribed onto the autoradiograph. Positive signals were cross checked with the photograph of the corresponding ethidium bromide stained gel to confirm the size of the amplified product to which the probe had hybridised.

Southern Blotting



UV crosslinking
 ↓
 Prehybridisation
 (blocks background)
 ↓
 Hybridisation to internal
 oligonucleotide probe



Wash
 ↓
 Labelled membrane
 exposed to X-ray film
 (autoradiography) or
 phosphorimager
 screen for scanning

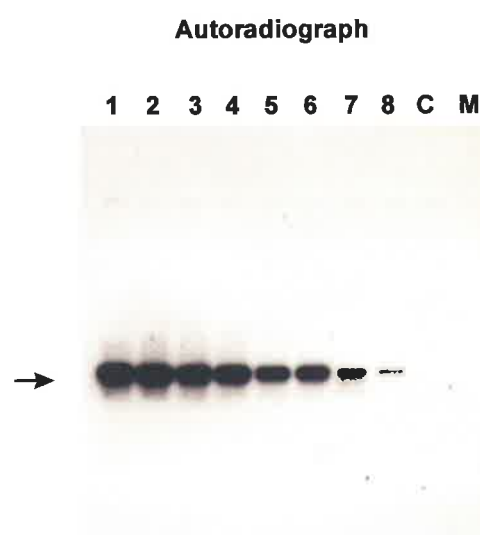


Figure 2-5. Further confirmation of specific mRNA sequences by using Southern blotting. Amplified DNA fragments are denatured and transferred to nylon membrane (vacuum transfer shown). Membrane is incubated with specific sequence probe allowing identity of amplified band (arrow) to be confirmed by hybridisation. Autoradiograph cross-referenced to markers on scaled photograph of ethidium bromide stained gel to confirm size of band. Phosphorimager detection of intensity of radioactive signal can be used for quantitation.

Semi-Quantitative PCR

A semi-quantitative PCR technique was developed to enable comparisons of gene expression in airway epithelial brushing specimens between subjects and between different pharmacological challenges in cells from the same subject. A PCR based technique was used primarily because of the small amount of RNA available for analysis and the low levels of expression of some of the target genes we investigated. The aim of the technique was to demonstrate relative differences between samples rather than absolute quantitation. To achieve this gene expression can be expressed as the level of signal intensity for the gene of interest, relative to a denominator to correct for variables such as differences in sample size and quality. The simplest such denominator is cell number, but in the case of brushing specimens the cells are harvested in sheets and clumps which makes accurate counting impossible. In addition, this form of normalisation would not take into account differences in RNA yield or quality between samples, factors which are essential to control for because of the highly labile nature of RNA. Quantity of total RNA itself can be used, but this again does not take account of RNA degradation and is limited by the sensitivity of spectrophotometer quantitation, particularly at the low levels of RNA available. Another important variable is the efficiency of the reverse transcription reaction, which may be compromised by low levels of impurities in samples. The commonest approach used that takes account of all of these factors is to use the level of expression of a control gene as the denominator. The RNA for the control gene will undergo the same extraction and reverse transcription process as the gene of interest. Relative quantitation between two samples can then be expressed as the ratio of the signal for the gene of interest to the control gene in each sample¹⁷². It is important to choose a control gene which is ubiquitously expressed at a constant level. Such genes are usually referred to as 'housekeeping genes' and commonly used genes include the gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

and actin^{159,165,173}. The use of random hexamers (as opposed to specific 3' primers) to prime reverse transcription facilitates this approach by effectively generating a "library" of cDNAs because reverse transcription of all species of RNA occurs in the one tube in the one reaction. In this way a single reverse transcription reaction allows the subsequent PCR analysis of several different target genes in the one sample, along with a housekeeping control gene. When samples are to be compared, a single master mix of reverse transcription reagents is prepared, then aliquotted between samples. After reverse transcription, controlled (limited cycle number) amplification by the polymerase chain reaction is performed¹⁷⁴⁻¹⁷⁷. A controlled reaction is required because the amplification is initially linear, but then plateaus as the Taq polymerase activity decreases as cycle number and amount of product increases¹⁶⁵. The cycle number at which this plateau is reached depends on the individual kinetics of the amplification reactions for each target gene and the starting level of expression of the gene. The higher the initial level, the earlier the plateau is reached. For this reason the linear range of amplification must be determined individually for each gene and each tissue. Once the amplifications for either the control gene or the gene of interest have plateaued, it is not possible to elicit quantitative information.

It is possible to co-amplify for both the gene of interest and a control gene in the one reaction tube, by using both sets of amplification primers in the reaction mix¹⁷⁸. This "internal standard" approach is said to avoid tube to tube variation which may occur, for example, because of subtle differences in heating and cooling cycle rates in different parts of the thermocycler. In fact however, tube to tube variability (standard error of the mean) has been reported to be only 4 - 15%¹⁶⁵. The Perkin - Elmer Thermocycler 480, which was used in these studies is manufactured using stringent controls to ensure uniformity of temperature across the heating block, thus minimising the potential for tube to tube variability. The variability from tube to tube in our studies was determined by parallel amplification of identical aliquots of cDNA and also by

analysis of serial dilutions within the quantitative cycle limit. The average variability within any one run (average of all targets) was 10.2% (range 4.6 - 14.8%). Thus, we found our results were in keeping with reported values and tube to tube variability was not considered to be a major concern. Furthermore, the internal standard approach has several potential problems. Because a control gene (eg actin) may be expressed at levels higher than genes of interest, as is the case for the tachykinin receptors, the control gene will reach an amplification plateau sooner. It may not be possible to determine a cycle number at which the gene of interest is reliably detected before the control gene amplification plateaus. In addition, it has also been reported that the competing kinetics of having two different amplifications occurring in the same tube can significantly affect the overall amplification of results^{165,179}. A specific example of this relevant to the current study is shown in Figure 2-6, where co-amplification for actin and NK1 receptor led to obvious suppression of NK1 amplification.

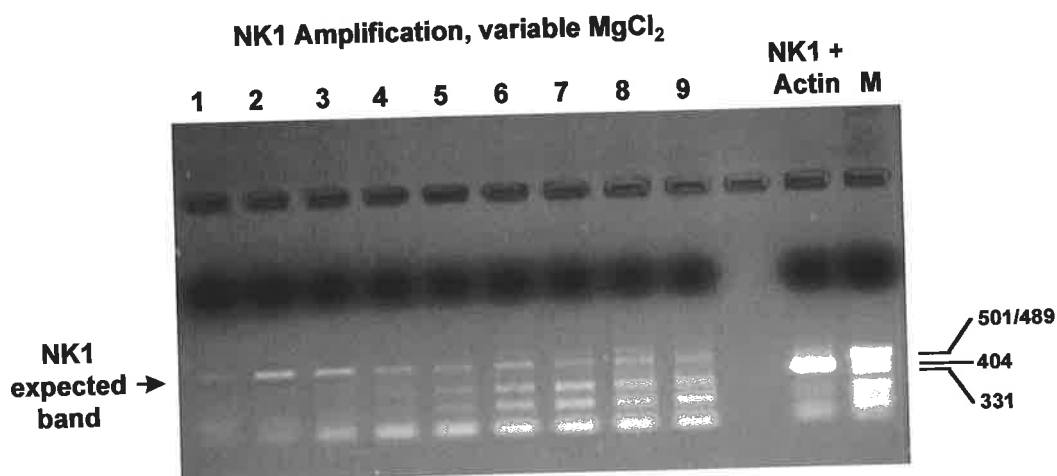


Figure 2-6. Ethidium bromide stained gel. Lanes 1 - 9 show the effect of varying magnesium concentration by 0.5 mM (final) on the specificity of PCR amplification for NK1 receptor cDNA (35 cycles). Optimal performance is seen in lane 2. Also shown is the effect of co-amplification for actin and NK1 (35 cycles) in the same tube, using same $MgCl_2$ concentration as lane 2. The much greater abundance of actin suppresses amplification of NK1, thus co-amplification of NK1 with actin as an internal control is not feasible.

Other internal control strategies include the use of artificial cRNA or cDNA standards. These approaches add a degree of complexity to the analysis, but do offer the potential for absolute quantification of transcript copy number. In competitive PCR strategies for instance, serially diluted standards of known absolute amount can be added into replicate sample tubes and co-amplified along with the endogenous target gene¹⁸⁰. To ensure comparable amplification kinetics, standards are constructed so that they will amplify with the same primers as the target gene. Separation of standard from target after amplification is usually done on the basis of size - ie the standard is slightly larger or smaller than the target, and is distinguished by gel electrophoresis. Because of the competition for amplification between the standard and the endogenous target gene there will be a point at which the standard and target reach equilibrium and thus have equivalent intensity bands on subsequent analysis. The amount of standard that achieves this equilibrium point then equates to the amount of endogenous target gene in the sample. Even with such approaches however, the use of a "housekeeping" gene control is still required to normalise samples for variability in quality and amount of total RNA. Accurate quantitation and stability of standards in storage must also be assured.

In our studies, we were interested in relative differences in gene expression rather than absolute levels. For the reasons discussed, we therefore chose to use a relatively simply approach to quantitation which used serial dilution of input cDNA to determine appropriate cycle number, along with an external tube housekeeping gene control. The control gene used for these analyses was actin¹⁵⁹, which consistently gave quantitative results when amplified for 18 cycles. Presence of a linear range of amplification for all genes investigated was determined by serially diluting reverse transcribed cDNA in a mixture of buffer, MgCl₂, and nucleoside triphosphates to keep the concentrations of these reagents constant. Concurrent PCR reactions were then performed on these dilutions, then the products were analysed by Southern blotting. As with the qualitative

analyses, amplified products were detected by probing with internal oligonucleotide probes. This ensured the specificity of the amplified products, as well as enhancing the sensitivity of the technique. A schematic of the semi-quantitative PCR strategy is shown in Figure 2-7.

For semi-quantitative experiments, agarose gel electrophoresis, Southern blotting and probing was performed as previously discussed above, but after hybridisation to labelled probes washing and wrapping, membranes were exposed to a phosphorimager storage screen (Molecular Dynamics Sunnyvale, CA, USA). The screen was then read by a phosphorimager (Molecular Dynamics) which gave an image on computer screen analogous to an autoradiograph. Hybridisation signal (expressed as pixel number) was quantitated by ImageQuant software (Molecular Dynamics) run by an IBM personal computer. When quantifying positive signals, the entire signal band of interest was outlined with a rectangle tool, background calculated, then positive signal within the rectangle calculated. This system gives linear results over a much broader range than is possible using autoradiographs and laser densitometry. For printing of images data was downloaded via a network connection to an Apple PowerMacintosh and imported into Adobe Photoshop in Tiff format.

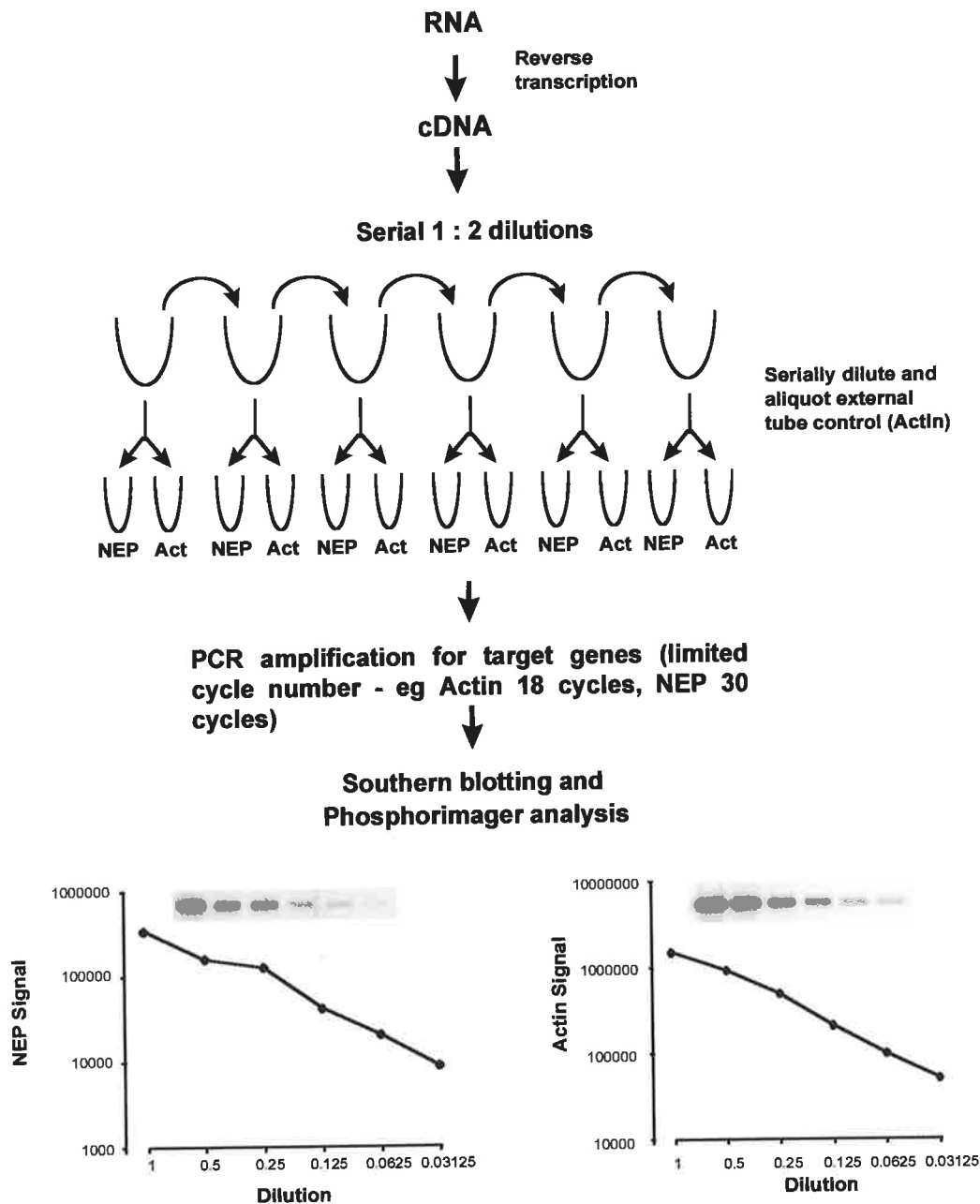


Figure 2-7. Schematic illustration of the approach to semi-quantitative PCR. Serial dilutions prior to amplification illustrate amplification in the quantitative range on subsequent analysis. In this example ratio of NEP signal normalised to Actin signal (to correct for any sample to sample variation in amount of RNA, RNA quality or efficiency of reverse transcription) could then be compared to NEP/Actin ratio of other samples amplified in the same run.

Hybridisation signals (as represented by pixel numbers) were analysed using Microsoft Excel software. Graphs were constructed by plotting serial dilution factor on the x axis and signal intensity on the y axis, using logarithmic scales. A linear relationship between dilution factor and signal intensity could then be identified, indicating amplification in the quantitative range. Signal intensity of data points within this quantitative range was determined for both the target gene of interest ("A") and the control or housekeeping gene ("B"). When analysing relative differences between two samples the ratio A/B for sample one was compared to the ratio A/B for sample two.

RNA Slot Blot

RNA slot blot analysis was used when available RNA was plentiful, as with the cultured cell lines. Total cellular RNA was extracted from cells and dissolved in DEPC treated water as above. To this RNA solution (typically 20µg RNA in 20µl H₂O) was added three volumes (eg 60µl) of a denaturing solution (deionised formamide 1ml : formaldehyde 324µl : 10x MOPPS 200µl, reagents from Sigma) in a microcentrifuge tube. This mixture was heated for 10 min at 65°C in a dry heating block, then rapidly cooled in ice. A slot blot apparatus (Bio-Rad) was cleaned with 70% ethanol and rinsed several times in DEPC treated water. A nylon membrane (Hybond) was cut to size, wet in DEPC treated water and soaked in DEPC treated 20xSSC for 10 min. The base of the apparatus was overlaid with Whatmann blotting paper (cut to size and wet in 20xSSC). The membrane was laid on top of this, then the upper part of the apparatus was screwed firmly in to place on top. A vacuum was applied to a port below the blotting paper. The individual slots were loaded with 500µl 20xSSC which was allowed to be drawn through. This rinse was repeated once with 20xSSC then once with 10xSSC. A denatured RNA sample was brought to a total volume of 1ml with ice cold 20xSSC, mixed thoroughly with the pipette, then

500µl was loaded into slot one. 500µl of fresh ice cold 20xSSC was added back into the microcentrifuge tube and again mixed thoroughly, effectively resulting in a 1 : 2 dilution. 500µl of this was then loaded in slot two. This procedure was repeated until the required number of dilutions were loaded (4-8), then the next RNA specimen was loaded in a similar fashion. This technique was adopted specifically to prevent any renaturing of the RNA during the loading process and to reduce RNA degradation. Once all specimens were loaded each slot was rinsed through twice with 10x SSC then air was allowed to pass through for five minutes. The apparatus was then dismantled and the membrane was dried for ten minutes at 80°C. The RNA was then crosslinked to the membrane using a UV crosslinker set at 240nm wavelength for 3 min. The membrane was then either stored at room temperature protected from light and dust, or probed immediately.

Radiolabelled cDNA probing

To enhance sensitivity, cDNA probes rather than oligonucleotide probes were used to probe the total RNA on the slot blots.

Plasmids containing tachykinin receptor cDNAs were obtained from Dr A. Graham, Biotechnology Dept., Zeneca Pharmaceuticals, Cheshire, UK :-

Human NK1 receptor - 1278 base pair full length cDNA in pMAM *neo Blue*, between the Eco RV and Xba1 restriction sites of the polylinker region.

Human NK2 receptor - 1441 base pair full length cDNA in pMAM *neo Blue*, between the Eco RV and Xba1 restriction sites of the polylinker region.

Human NK3 receptor - 1492 base pair full length cDNA in pBluescript KS (+) between the Hind III and SalI restriction sites of the polylinker region.

A plasmid containing the human interleukin 8 (IL8) sequence was obtained from Prof. M. Vadas¹⁸¹, Dept. of Immunology, Hanson Center for Cancer Research, Adelaide, Australia -

Human IL8 - full length sequence in pUC19. An 800 base pair PstI digestion fragment was used as a cDNA probe.

Preparation of Plasmids

Small quantities of plasmids were obtained, therefore cloning and growth in competent bacteria was required to generate adequate stocks for subsequent experiments. All plasmids used contained genes for ampicillin resistance. All manipulations involving bacteria were conducted in a laminar flow cabinet in a specially designated 'PC2' class room in accordance with regulations. Preparation of plasmid stocks was achieved by a sequence of steps consisting of transformation, isolation of transformed clones, small scale plasmid expansion ("mini-prep"), confirmation of correct plasmid expansion (restriction digest analysis), then large scale plasmid preparation ("maxi-prep").

Preparation of competent cells

Bacteria were prepared in a way that would allow subsequent transformation by the "heat-shock" method. E. coli bacterial cells (strain MC106, Gibco) were prepared from a glycerol stock that had been stored for several months at -70°C. Five µl of this stock was inoculated into 2ml of

L-broth (Tryptone 10g, yeast 5g, NaCl 10g in 1 litre H₂O, pH 7.5, autoclaved) in a 10ml sterile tube and incubated at 37°C in a shaking incubator (225 rpm) overnight. Next day 1ml of this culture was inoculated into 50ml L-broth in a sterile conical flask, and returned to the incubator. Rate of growth was periodically checked by analysing an aliquot of the culture suspension with a spectrophotometer (Beckman). Using a glass 1ml cuvette, the instrument was blanked with L-broth. 1ml aliquots of the culture were periodically removed (in a laminar flow cabinet) and the absorbance at 600nm (OD₆₀₀) was read. When this had reached 0.35-0.45, corresponding to the logarithmic growth phase of the bacteria (after approximately 1 hour incubation), the flask was cooled on ice for 30 min. The culture suspension was then transferred to a pre-cooled, sterile 50ml polypropylene centrifuge tube and centrifuged at 6000g (Beckman, JA-10 rotor) for 15 min at 4°C. The supernatant was gently tipped off, decontaminated with Biogram disinfectant and discarded. The cell pellet was gently resuspended in 17ml of pre-cooled (4°C) simple frozen storage buffer ("FB": KCl 7.4g, CaCl₂·2H₂O 7.5g, glycerol 100g, KAcetate 10ml of a 1M stock, in 1 litre distilled H₂O, pH adjusted to 7.5, then autoclaved) using a pipette, then kept on ice for 30 min. This buffer facilitates subsequent transformation (plasmid incorporation). The suspension was centrifuged again for 15mins, 3000rpm, 4°C. Supernatant was discarded as before, then the cell pellet resuspended in 4mls FB.

Transformation of bacteria

The cell suspension was divided among several pre-cooled, sterile 10ml polypropylene tubes, 200µl per tube, (enough tubes to have three tubes per plasmid being prepared) plus an extra tube to act as a negative control. For each plasmid the three tubes were prepared by adding 10, 20 or 50ng of plasmid stock. This dose range was used to help ensure that following subsequent

growth on agar plates, at least one of the concentrations would yield a plate from which a single colony could easily be picked (as opposed to no growth or a total overgrowth). The negative control tube had no plasmid added. Following instillation of plasmids, the tubes were kept on ice for 30 min. The cells were then heat-shocked by partially immersing the tubes into a water bath at 42°C for 75 seconds, then returning the tubes to ice. This step enables entry of the plasmid into the bacterial cell. 800µl of L-broth was then added to each tube, and the tubes were incubated, shaking, for 30 min at 37°C. Ampicillin agar plates had been prepared by adding tryptone 10g, yeast 5g, NaCl 10g and agar 15g in 1 litre H₂O, pH 7.5, autoclaving, cooling to 60°C, then adding 1ml ampicillin (from a stock of 100mg/ml). The solution was then poured into 100mm sterile petri dishes in a laminar flow cabinet, and allowed to set. 200µl of bacterial culture from each 10ml tube was plated onto an ampicillin agar plate, then the plates were incubated overnight at 37°C. Next day all plates containing transformed bacteria showed good growth whereas the untransformed negative control bacteria (ie the “no plasmid” control) failed to grow. This indicated successful transformation by plasmids as evidenced by the imparting of ampicillin resistance to the transformed cells.

Small scale plasmid preparation

A 'mini-prep' plasmid purification to confirm the identity of the transforming plasmids was performed. Single colonies of transformed cells were picked with a sterile wire loop and inoculated into 10ml polypropylene tubes containing 2ml L-broth, then incubated at 37°C, shaking (225 rpm), overnight. For each plasmid, 4 colonies were picked and each put into a separate tube. Next day 1.6mls was transferred from each tube to a microcentrifuge tube. Glycerol stocks were made with the remaining 400µl (by adding an equal volume of sterile glycerol

(Gibco)) and stored at -70°C in 1.8ml sterile Nunc cryotubes (Nalge Nunc International, Rochester, NY, USA). The 1.6mls was spun at 13,000 rpm (bench top microfuge, Eppendorf) briefly to pellet cells and the supernatant removed. Cells were resuspended by vortexing in 50 μl TES buffer (25mM Tris, 10mM EDTA, 15% sucrose). Following this, 200 μl 0.2M NaOH/1%SDS was added and mixed by gentle inversion. Then, 150 μl sodium acetate (pH 4.6) was added to the lysate, mixed by gentle inversion and the tubes stood at -20°C for 1 hour. Tubes were then centrifuged at 13000 rpm for 15 min (thereby sedimenting bacterial chromosomal DNA) then the supernatant, containing the plasmids, was transferred to a new tube. The plasmid DNA was recovered from the supernatant by extraction with phenol : chloroform : isoamyl alcohol (25:24:1, v/v/v) then ethanol precipitation as follows. An equal volume of TE-equilibrated-phenol : chloroform : isoamyl alcohol was added to the tube, vortexed, centrifuged 5 min at 13000, then the upper, aqueous phase removed to a new tube. 2.5 volumes of cold (-20°C) 100% ethanol was added and the plasmid DNA precipitated at -20°C for 5min. The DNA was pelleted by centrifuging at 13000rpm for 15 min, then the pellet washed in 70% ethanol, air dried briefly then resuspended in 20 μl TE/RNase buffer (25mM Tris, 10mM EDTA plus 1mg/ml RNase A (Sigma)). The RNase is included to remove any contaminating bacterial RNA. Confirmation of the presence of the appropriate plasmid was made by performing a digestion reaction designed to separate the cDNA insert from the plasmid backbone, then performing agarose gel electrophoresis to determine the size of the cDNA fragment. A fragment of the appropriate size, cut by the appropriate enzymes, was taken as sufficient evidence of correct plasmid incorporation. A typical digestion reaction (using enzymes purchased from AMRAD Pharmacia) for the NK1 receptor plasmid consisted of 1 μl plasmid DNA, 2 μl One-Phor-All Buffer, 1 μl Eco RV, 1 μl Xba 1, 15 μl H₂O added together in a microcentrifuge tube, mixed gently, then incubated for 1 hour at 37°C . Five μl loading buffer was then added and the reaction

products run in a 1% agarose gel. Once the plasmid identity had been confirmed, the glycerol stocks made earlier from the 'mini-prep' transformed bacterial cultures were used for a large scale plasmid purification ('maxi-prep').

Large scale plasmid preparation

For large scale plasmid preparation, T-broth was used, prepared as follows - 12g bacto tryptone (Gibco), 24g bacto yeast (Gibco) and 4ml glycerol (Gibco) was added to 900ml distilled water. In a separate bottle, 2.31g KH_2PO_4 and 12.54g K_2HPO_4 was added to 90ml distilled water. The solutions were autoclaved, cooled to 60°C, then mixed together (in a laminar flow cabinet). 20 μl of transformed bacteria (from appropriate glycerol stock) was added to 250mls T-broth with 100 $\mu\text{g}/\text{ml}$ ampicillin (final concentration.) in a 1 litre conical pyrex glass flask and incubated overnight, shaking (225 rpm) at 37°C.

Next morning, cultures were transferred to 500ml polypropylene centrifuge tubes and centrifuged at 6000g for 15 min at 4°C (Beckman rotor). Supernatant was decanted and cells resuspended in 10ml of TE / SDS buffer (25mM Tris, 10mM EDTA, 1% SDS). To the homogenous suspension was added 20ml 0.2M NaOH/1% SDS, followed by gentle mixing by inversion, then 15ml sodium acetate pH 4.6, again followed by gentle mixing. Tubes were cooled at -20°C for 15 min, then centrifuged at 6000g for 15 min at 4°C. The cleared supernatant was then carefully decanted through gauze into a pre-weighed 50ml polypropylene tube. The majority of the bacterial chromosomal DNA was thus either left as a loose pellet in the first tube or trapped in the gauze. An equal volume of room temperature isopropanol was then added to precipitate the plasmid DNA, and the tubes were allowed to stand at room temperature for 15 min. The tubes were then centrifuged at 6000g for 15 min at 24°C. Supernatant was discarded, pellet washed

once in 70% ethanol, air dried then resuspended in TE buffer (2.5ml). Ethidium bromide was added to each tube (200 μ l of a 10mg/ml solution). The weight of the tube contents was then determined by weighing the tube and subtracting the original weight of the tube when empty (determined earlier). An equal weight of caesium chloride powder was then added, plus an additional 100mg of powder. This was dissolved by vortexing, then the solution was loaded into Beckman ultracentrifuge tubes (g-MAX), ensuring tubes were carefully balanced by weight. The tubes were then sealed with a heating iron and adaptor, then centrifuged at 400,000g at 20°C overnight in a Beckman Optima Max-E ultracentrifuge (MLA-80 rotor). Next morning, a discreet pink band consisting of the plasmid DNA was present in the mid-portion of the tube. This band was carefully extracted with a syringe and an 18 gauge hypodermic needle inserted through the side wall of the tube. The extracted band (in a volume of approximately 1ml) was placed in a 10ml polypropylene tube, and brought to a total volume of 2ml by adding TE buffer. 2ml of isoamyl alcohol was added and the tube was vortexed vigorously. In this way the pink ethidium bromide was seen to move to the upper phase which was removed and discarded. This extraction procedure was repeated until all traces of pink discolouration were removed (a total of 5 extractions). Following extractions, plasmid DNA was precipitated by adding 5ml of cold 100% ethanol and standing at -20°C for 30 min. Tubes were centrifuged at 10,000g for 30 min, then supernatant discarded and pellet briefly dried. Pellet was dissolved in 250 μ l TE buffer and transferred to a 1.5ml microcentrifuge tube. DNA was again precipitated by adding 50 μ l of 3M sodium acetate pH 4.6 then 1ml of 100% ethanol, mixing then standing at -20°C for 30 min, followed by centrifugation at 13,000 rpm for 15 min. Supernatant was removed, pellet washed twice in 70% ethanol, dried and then dissolved in 500 μ l H₂O and DNA concentration determined by spectrophotometry as previously described.

Isolation of cDNA Sequence

For use as a template for generating radiolabelled probes, the cDNA sequence was separated from the plasmid backbone. Restriction enzymes were used to cut the plasmid DNA in the polylinker regions at either end of the cDNA. The restriction sites for each cDNA are indicated above. A typical digestion reaction consisted of combining reagents in the following proportions - 100µg plasmid, 10µl restriction enzyme 1, 10µl restriction enzyme 2, in One - Phor -All buffer (made to a final concentration of 1x by adding an appropriate amount of sterile water). Reaction was performed in a microcentrifuge tube incubated at 37°C for 4 hours, then heated to inactivate the restriction enzymes (65 or 85°C for 10 - 30 min, depending on the enzymes used). A small aliquot was run in a 1% agarose gel to check for completeness of digestion. If satisfactory, the entire reaction volume was loaded in a 1% gel (using a preparative comb) and run sufficiently to clearly separate cDNA band from plasmid band. The cDNA band was cut from the gel using a razor blade and the cDNA recovered from the gel by electroelution as follows. The gel slice was placed in a strip of 1.5cm diameter dialysis tubing (molecular weight cut-off 10,000 daltons), with a small amount of 1x TAE running buffer, then the tube was clamped at either end and placed transversely in a gel running tank filled with running buffer. Current was applied for sufficient time to ensure cDNA passage out of the gel, then the current direction was reversed briefly (30 secs). The running buffer containing the cDNA was aspirated from the dialysis tubing and concentrated by spinning through a Centricon 30 microconcentrator tube. The tube was topped up and re-spun twice with sterile water to remove salts, then the retentate collected into a microcentrifuge tube and the cDNA quantitated by spectrophotometry (using OD₂₆₀ of 1 = 50µg/ml for DNA). A small aliquot was also run in a 1% agarose gel to check the integrity of the cDNA.

Radiolabelling of cDNA Probes

Radiolabelled probes were made using a Giga-prime random primer labelling kit (Bresatec). cDNA template (25 - 100ng in 6µl water) was denatured by heating to 95°C for 10 min in a microcentrifuge tube, then cooling on ice. To this was added 6µl decanucleotide solution, 6µl nucleotide buffer (containing dATP, dGTP and dTTP), 5µl α ³²P dCTP and 1µl enzyme (Klenow fragment of DNA polymerase). The reaction was incubated for 15 min at 37°C, then heated to 65°C for 10 min to inactivate the enzyme. Unincorporated nucleotides were removed by spinning the reaction volume through a G50 Sephadex column. Sephadex (Sigma) was initially prepared by expanding beads in TEN buffer (0.1M NaCl, 10mM Tris, pH 8.0, 1mM EDTA, pH 8.0) and autoclaving. To make a column a 1ml syringe (plunger removed) was plugged at the tip with siliconised glass wool. The syringe was placed in a sterile 10ml centrifuge tube, then filled with the Sephadex bead slurry. The tube was spun at 1000 rpm for 3 min, then the syringe refilled and respun as necessary to give a 1ml column. The column was then equilibrated with 10mM Tris / 1mM EDTA / 0.1% SDS / 5mM β -mercaptoethanol by spinning 3 x 200µl aliquots through the column. The labelling reaction products were made up to 100µl with the Tris/EDTA/SDS/ β -mercaptoethanol, then spun through the column. Unincorporated nucleotides became entrapped in the Sephadex, whereas the larger labelled probes passed through. This fraction was collected and a 1µl aliquot was combined with 1ml of scintillation fluid for the radioactivity to be read in a scintillation counter (1900TR liquid scintillation analyzer. Packard Instrument Co., Inc. CT. USA.). Typically, the reaction yielded $>10^9$ counts per min per µg DNA and probing was performed using at least 10^8 cpm total.

Probing membranes with cDNA labelled probes was essentially similar to that described for oligonucleotide probes, except pre-hybridisation and hybridisation steps were performed at

65°C. Standard wash steps post-hybridisation were 2xSSPE/0.1%SDS twice for 10 min at room temperature, 1xSSPE/0.1%SDS once at 65°C for 15 min, then 0.1xSSPE / 0.1%SDS once at 65°C for 10 min.

Immunofluorescent Staining for NK1 Receptor

A rabbit anti-NK1 receptor antibody, raised against the C-terminal amino acids (376-407) of the rat NK1 receptor was supplied by Dr. J Krause (Department of Anatomy and Neurobiology, University of Washington in St. Louis, MO, USA). This antisera has been previously characterised¹⁸² and is known to react to human NK1 receptor in CNS tissues (J. E. Krause, personal communication), and does not cross-react with rat or human NK2 or NK3 receptors¹⁸². Fresh 4% paraformaldehyde was prepared on the day of the experiment by dissolving 4g paraformaldehyde in 100mls PBS in a pyrex bottle sitting in a boiling water bath. Once dissolved the solution was cooled to 4°C on ice, then the pH adjusted to 7.4. Epithelial cells obtained by brushing were washed once in PBS, resuspended in PBS, then cytopun onto APT coated slides. Immediately after removing the slides from the cytopun they were immersed in 4% paraformaldehyde at 4°C for fifteen minutes, then washed 3 x 5min in PBS. 16HBE14o- and 1HAEo- cells, grown in RPMI/10% foetal calf serum on gelatin/fibronectin/BSA as above, were cultured 24 - 48 hours prior to staining. This time point was chosen because a serum effect had been noted in synovial cell cultures (J. Krause, personal communication) which resulted in an increase in NK1 receptors at this time. Cells were harvested by trypsinisation with 0.05% trypsin/0.5%EDTA for 5min at 37°C, then the trypsin inactivated by adding an equal volume of RPMI / 10%FBS, cells washed 3x in PBS, resuspended to a concentration of 1×10^6 /ml in PBS, then cytopun, fixed and washed as above. Cells were then permeablised by immersing slides in

0.1% Triton X-100 (Sigma) for 10 min, washed again 3 x 5 min in PBS, then immersed in 5% dry milk powder in PBS for 2 hours to block background. Slides were removed and a circle drawn around the cytospot with a wax pen. Dilutions of primary antibody, prepared by diluting sensitised sera 1/50 -1/500 in 1% normal sheep serum, were then spotted onto the cells, 30µl per slide, and the slides were incubated at either 4°C or room temperature for between 30min and overnight in a humid box. Best results were seen when using a 1/200 dilution of primary antibody at 4°C overnight. Negative controls consisted of normal rabbit serum, to the same dilution as primary antibody, and normal sheep serum with no primary antibody. In subsequent detection steps we obtained best results using fluorescent-conjugated rather than HRP-conjugated secondary antibody. After the incubation period slides were washed 3x in PBS, then secondary antibody (FITC conjugated sheep anti-rabbit; Jackson ImmunoResearch West Grove, PA USA), diluted 1/40 in normal sheep serum, was then spotted on to the cells, 30ml per slide, then the slides were incubated in the dark for 20 min at room temperature. Slides were protected from light during all subsequent steps. Slides were then washed 3x in PBS, and mounted with coverslips using Vectashied mounting medium (Vector Laboratories Inc., Burlingame CA, USA). Slides were then viewed using a fluorescent microscope. Staining results are shown in chapter five (Figure 5-10).

Immunohistochemical staining for NEP

Neutral endopeptidase was detected with a mouse J5 monoclonal antibody (Coulter Immunology, Hialeah, FL USA.) directed against the human Common Acute Lymphoblastic Leukemia Antigen (CALLA) CD 10, which is identical to NEP. Cells were cytospun as above then fixed in 4% paraformaldehyde. Primary antibody was applied overnight at 1/100 dilution at

room temperature. Secondary biotinylated rabbit anti mouse (DAKO), diluted to 1/500 was applied for 30 min., followed by streptavidin - HRP at 1/1000 for 30 min and then detection with DAB for five minutes. Specificity of the staining methods was determined by omitting primary antisera or replacement with comparable isotype sera. Staining results are shown in chapter five (Figure 5-11).

Immunohistochemical staining for SP

Rabbit anti SP antibody, directed against the C-terminus of SP was purchased from AUSPEP (Melbourne, Australia). We attempted immunohistochemical detection of SP using this antibody but were unable to detect specific staining in the fresh epithelial cell samples. These results may have been compromised due to technical difficulties with high background, although staining in a positive control (sheep jejunum), could be seen. Several attempts were made to achieve positive signal for SP and many variables were adjusted. Different fixatives (including paraformaldehyde, Zamboni's fixative, glutaraldehyde, alcohol) and the analysis of frozen sections was tried. Detection by both fluorescent and HRP conjugated secondary antibodies was tried. Pre-absorption of primary antibody with rat liver powder in an effort to reduce background was attempted without much success. An attempt was also made at antigen retrieval by microwave irradiation but this also did not help. In view of this we used a highly sensitive ELISA to examine extracts from the brushing samples. This ELISA was also based on the use of the Auspep polyclonal anti-SP antibody.

Measurement of Substance P

Substance P is present in tissues at low concentrations and is prone to rapid enzymatic degradation, therefore a technique was required to extract non-degraded SP from tissues, then concentrate it to enable subsequent quantitation by ELISA. Tissues were processed according to the protocol of Scicchitano et al¹⁸³.

SP Extraction

Cells were pelleted by centrifugation at 200g for 10 min at 4°C, then washed three times in PBS and resuspended in 1ml PBS. The suspension was then boiled in 10 volumes of 1M acetic acid / 10mM β mercaptoethanol for 10 min. For analysis of SP in BAL fluid, cells were first removed as above, then a one tenth volume of 10M acetic acid containing 100mM β -mercaptoethanol was added and the mixture was boiled for 10 min. The fluid was then cooled on ice and stored at -70°C.

The suspension was centrifuged then the supernatant passed through Sep-Pak C18 cartridges (Millipore Australia Pty. Ltd.) to bind the SP. Cartridges were pre-wet with 2ml of methanol, washed with 5ml distilled water followed by 0.1% (v/v) trifluoroacetic acid (TFA) in distilled water. Flow rates did not exceed 5ml/min. Supernatants or cell pellet extracts were applied to the cartridge then the cartridge was washed with 20ml 0.1% TFA. SP was then eluted from the column in 5ml of methanol which was collected and cooled to -70°C. This SP containing fraction was then lyophilised in a freeze drier before analysis by ELISA. Recovery rate of SP from the cartridge was >85% as assessed by using a known quantity of commercially available SP peptide (Auspep, Melbourne, Australia).

SP Enzyme Linked Immunosorbent Assay (ELISA)

Soluble SP was detected in cell pellet extracts and BAL fluid using the rabbit anti-SP antibody purchased from Auspep in a competitive inhibition ELISA. SP peptide was conjugated to bovine serum albumin (BSA) according to the method of Scicchitano et al¹⁸³ to enable binding of the SP to the ELISA tray. In the following procedure "0.1M PBS" is 80g NaCl / 2g KH₂PO₄ / 11.5g Na₂HPO₄ in 1 litre H₂O, pH 7.4. Samples and standards (consisting of known concentrations of Auspep SP) were solubilised in 2 parts primary antibody diluent (0.1M PBS pH 7.4/0.05% Tween 20/ 1% horse serum / 0.05% sodium azide) and 1 part methanol/0.1M HCl (1:1 v/v). Nunc Immuno Plate Maxisorp flat bottom ELISA trays were sensitised with 100µl of 0.5µg/ml SP-BSA conjugate for 4 hours. Wells were washed with 0.01M PBS pH 7.4 containing 0.05% Tween 20 between steps. Non-specific binding sites were blocked by adding 200µl 1% horse serum in 0.01M PBS pH 7.4 containing 0.05% sodium azide for a minimum of 30 min. Wells were washed, then loaded with 50µl of primary antibody diluent. 50µl of either sample or standard (to generate a standard curve) was added into the first well of a line, then serial 1:2 dilutions performed down the tray. 50µl of Auspep rabbit anti-SP (1/16,000) was then added to each well (thus final concentration is 1/32,000). Quality controls of known concentration (consisting of stocks of previously analysed material) were included in each tray as well as negative controls. The trays were left overnight at 4°C, during which time the sample SP competes with the SP-BSA conjugate for binding sites on the SP antibody - the higher the SP in the sample, the less antibody binds to SP-BSA attached to the tray, therefore the less antibody remains after washing and ultimately the less signal in the well. Next day trays were washed and 100µl of secondary antibody (horseradish peroxidase conjugated swine anti-rabbit (DAKO)) was added at 1/1500 dilution and incubated for 2 hours at room temperature, then wells were washed. For detection, O-phenylenediamine-HCl (OPD)tablets (Sigma) were used in substrate buffer

containing 40mM citrate/2.7mM phosphate pH 5. To prepare the substrate solution one 10mg tablet was added to 10ml substrate buffer 5 minutes before use, then 7 μ l of 30% H₂O₂ was added to this immediately prior to use. 100 μ l of this substrate solution is then added to each well. Trays were incubated for 30 min in the dark. Reaction was then inhibited by the addition of 100 μ l 2.5 M sulphuric acid to each well. OD was read at 490nm, blanked on air with reference at 630nm using an MR 7000 EIA-CALC Dynateck ELISA reader (Dynateck, USA). A curve was constructed by plotting OD₄₉₀ vs standard concentration (Figure 2-8). The sigmoid program used for curve fitting is based on the Rodbarbs Four Parameter equation. This fitted curve routinely generated an r^2 value of 0.99. OD readings for samples were compared to those of the standards for quantitation, provided that the OD values obtained for samples fell within the linear range of the curve fit. The sensitivity of the ELISA was five femtomoles per well.

Measurement of Interleukin 8 (IL8)

A technique to evaluate the effect of SP on IL8 production in cultured cells was established. To measure IL8 levels a new competitive inhibition ELISA was developed using the same principles as the SP ELISA, with modifications. Recombinant human IL8 (Amgen, Thousand Oaks, CA, USA) was used to sensitise Nunc Maxisorb flat bottomed ELISA trays. It was not necessary to conjugate IL8 to BSA, plates could be sensitised with diluted stock IL8. Nor was it required to extract or concentrate the IL8 from samples (in this case culture supernatants). 100 μ l of IL8 (15ng/ml in 0.01M PBS) was pipetted into the ELISA wells and incubated for 4 hours at room temperature, then the wells were washed. Non-specific binding sites were blocked as above, then primary antibody diluent, samples and standards were loaded as above and serial dilutions performed. Primary antibody (goat anti-human IL8, R&D Systems, Mineapolis, MN,

USA) was added, final concentration 1/ 6000. The combination of 15ng/ml for sensitisation and primary antibody dilution of 1/6000 was determined by prior optimisation studies. In these studies a checkerboard array of combinations was tested. On one axis of a 96 well plate serial 1:2 dilutions of recombinant IL8 from 4mg/ml to 7ng/ml were made. On the other axis, dilutions of primary antibody 1/500, 1/1000, 1/2000, 1/3000, 1/6000 were made. The combination of reagents which gave an OD₄₉₀ of 1 upon subsequent detection was considered optimal. This would effectively equate to a sample IL8 concentration of zero in subsequent studies. Owing to the competitive nature of the ELISA, any IL8 in samples would drive the OD reading down, into the most quantitative range of the ELISA reader instrument. Plates were incubated with primary antibody overnight at 4°C, then signal was detected in a manner similar to above, except in this case the secondary antibody was a biotinylated rabbit anti - goat antibody, used at 1/40,000 final concentration and incubated for 1 hour at 37°C, followed by washing and then incubation with streptavidin - horse radish peroxidase (1/2000 for 1 hour at 37°C). This was followed by washing and detection with OPD as above. The ELISA has a sensitivity of 0.1ng/well. Standard curve is shown in Figure 2-8.

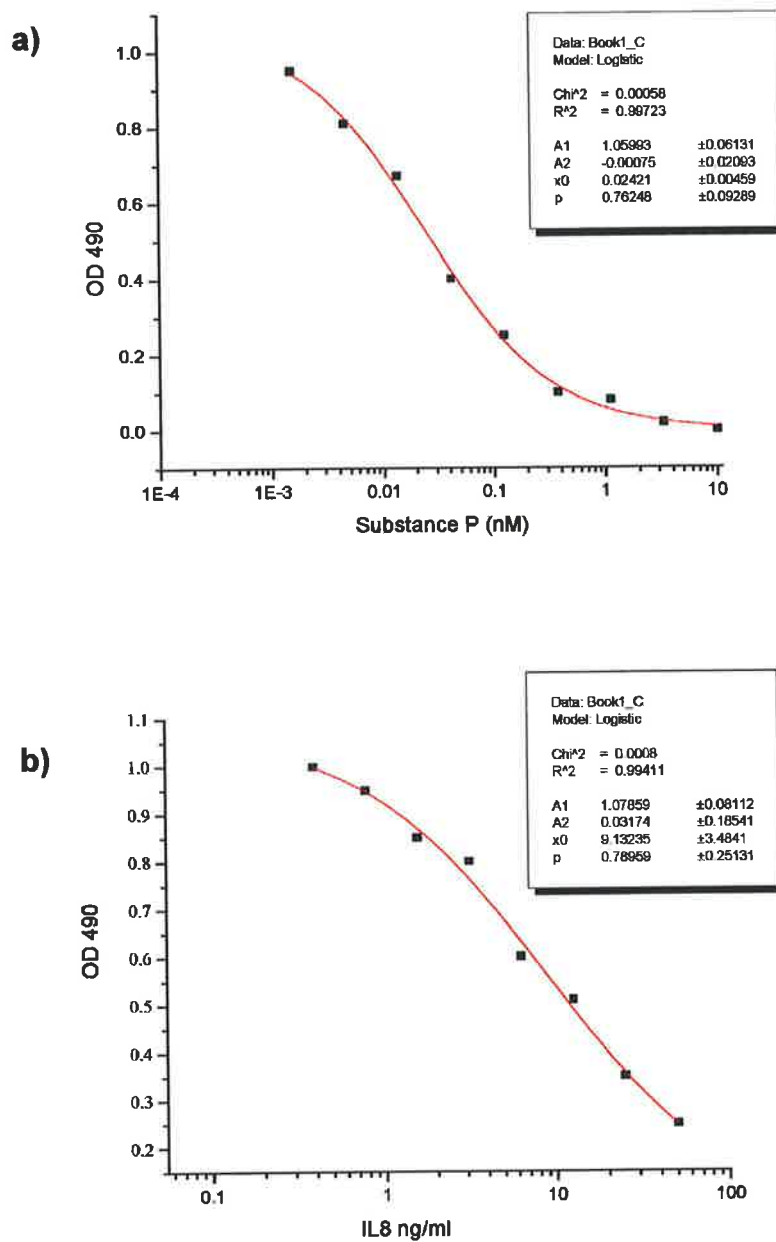


Figure 2-8. Representative standard curves obtained using Substance P (a) and IL8 (b) ELISAs.

Chapter Three

Tachykinins are involved in the acute allergic response to aerosolised allergen.

The first application of the sheep model undertaken was an assessment of the ability to generate reproducible allergic responses in the animals, and once that had been achieved, to seek evidence for a role for tachykinins in the acute allergic response.

As discussed in chapter one, several studies, particularly in the guinea pig and the rat, have demonstrated an important role for tachykinins in the mediation of airways responses. However, the importance of tachykinins in non-rodent mammals is less clear. The studies described in this chapter were designed to test the hypothesis that tachykinins are involved in acute allergic responses in the ovine model. This hypothesis was tested in three ways. Firstly, the neutral endopeptidase (NEP) inhibitor, phosphoramidon, was used to reduce tachykinin degradation, which would therefore enhance responses to endogenous tachykinins if they play a role in the allergic response. Next, the NK1 specific receptor antagonist CP-96,345 was used, which was hypothesised to reduce allergen induced responses by blocking receptor activation and finally, the neurotoxin capsaicin was used to deplete C-fibre nerves of their neuropeptide content, thereby reducing the response to allergen by precluding the release of endogenous tachykinins.

Materials and Methods

Details of the apparatus used for determining airways responses in the sheep model and for the aerosol delivery of agents can be found in the Methods section, chapter 2.

Experimental protocol

Chemical reagents

Ascaris suum extract was donated by Dr G. Stewart and prepared as a stock concentration of 80,000 protein nitrogen units (PNU)/mL as discussed in the Methods chapter. Phosphoramidon (Sigma) was dissolved in sterile water to a stock concentration of 1 mg/mL then aliquotted and stored at -20 °C. CP-96,345 (donated by Pfizer Pharmaceuticals, New York, USA) was made to a stock concentration of 100 mg/mL in sterile phosphate buffered saline (PBS, pH 7.4), stored at -20°C, then diluted to a dose of 0.1 mg/kg in 10 ml of normal saline on the day of the experiment. Capsaicin (Sigma) was dissolved in sterile water to a stock concentration of 30 mg/mL and stored at -20°C. Working dilutions were made with sterile water on the day of the experiments.

Ascaris sensitisation

Ascaris suum extract was used as the antigen in our sheep model based on the publications of Abraham et al¹⁸⁴ and Kleeberger et al¹⁵⁵. In a group of 12 sheep, primary immunisation was performed using an injection of 5 ml of ascaris extract (stock concentration) emulsified in Freund's complete adjuvant (1:4, v/v). Sheep were inoculated via either the

subcutaneous (SC, n=4), or intraperitoneal (IP, n=4) route. Intratracheal primary immunisation (IT, n=4) was also performed by transtracheal injection of 10 mls of a 1:100 dilution of ascaris extract. Two weeks later all sheep received transtracheal boosters of antigen as for the intratracheal priming. Two weeks later, sheep were tested for skin sensitivity and airways reactivity (defined below). Skin reactions were defined as positive if a wheal > 5mm average diameter developed 30 minutes after intradermal injection of a 1:1000 dilution of stock ascaris extract in normal saline. Based on the results of these initial experiments (see below), SC priming was used in a further 12 sheep.

Inhalational challenge

Challenges with inhaled allergen were performed in a total of 24 sheep. In each experiment, a baseline (control) challenge with nebulised normal saline (20 breaths) was initially given. Allergen was then given in a dose response fashion, with the initial dose being 20 breaths. Doubling doses were then given to a maximal total cumulative dose of 620 breaths. R_L after each dose was monitored on a breath-by-breath basis. Once the increase in R_L had reached a plateau (typically within 5 mins of inhalation) the next doubling dose was given. Sheep were considered to be airway responders if there was a 100% increase in R_L above baseline. The degree of airway reactivity to ascaris was expressed as the PC_{100} , defined as the total cumulative number of breath units (BU) required to induce a 100% increase in resistance over baseline (calculated as $(R_{L \text{ experimental}} - R_{L \text{ baseline}}) / R_{L \text{ baseline}}$), where 1 breath unit (BU) equalled 1 breath (7.2 μ l) of an 80,000 PNU/mL solution of ascaris extract. The PC_{100} value was determined by interpolation from cumulative dose-response curves of BU vs change in R_L , using DeltaGraph software (DeltaPoint Inc. CA, USA). To reduce animal distress, the allergen challenge was deliberately stopped once a

PC₁₀₀ was achieved. Animals that failed to achieve a significant RL response after 620 breaths of ascaris were considered nonresponders.

Reproducibility studies

The reproducibility of the airway response to allergen was assessed in all allergic sheep. PC₁₀₀ values were determined at fortnightly intervals over 6 weeks. Only sheep which displayed a reproducible PC₁₀₀ value (defined as a coefficient of variation of less than 10%) over the 6 week period were used for the subsequent intervention studies. The mean PC₁₀₀ value for each sheep obtained over these 3 studies was used as the baseline airway response for comparison to the effects of subsequent drug interventions.

Pharmacological interventions

Drugs were given 20 minutes prior to ascaris challenge. Phosphoramidon (40 breaths of 1 mg/mL, equivalent to 288 μ L) was delivered as an aerosol. CP-96,345 (0.1 mg/kg) was given intravenously as a bolus injection into the right jugular vein. The dose of phosphoramidon was based on an initial extrapolation from previous reports¹⁵² followed by preliminary dose range evaluation. Similarly, a preliminary dose-response evaluation of CP-96,345 was conducted. As a guide, Constantine et al¹⁸⁵ reported that CP-96,345 at a dose of 0.05 - 1.0 mg/kg inhibited SP induced hypotension in dogs. We found 1mg/kg induced a 300% increase in airways resistance, so the dose was titrated down until no change in R_L occurred. A dose of 0.1 mg/kg was subsequently used. Following drug administration, an inhalational challenge with ascaris was conducted as above. A control ascaris challenge (ie. with no active drug administration prior to ascaris) was performed in between the two drug intervention studies to ensure no change in

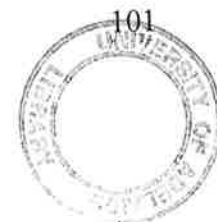
baseline reactivity had occurred. The phosphoramidon, intervening control and CP-96,345 studies were conducted at 2 weekly intervals over a 6 week period.

Capsaicin desensitisation

In a separate set of experiments capsaicin desensitisation was performed aiming to deplete sensory nerves of neuropeptides. Following confirmation of the reproducibility of baseline airway responsiveness, the allergic sheep received 4 increasing doses of nebulised capsaicin as follows - day 1: 10 breaths of 0.3 mg/mL; day 2 : 10 breaths of 3 mg/mL; day 4: 10 breaths of 30 mg/mL and day 5: 20 breaths of 30 mg/mL. The response to allergen following capsaicin desensitisation was measured on day 8.

Statistical analysis

All values shown are means $\pm$ SEM. Differences in PC₁₀₀ values between control and drug interventions were analysed using repeated measures ANOVA. Significance between group means was found using Fisher protected least significant difference analysis. Student's t-Test was used to determine significance between pre and post capsaicin desensitisation. Analyses were performed using Statview II software (Abacus Concepts Inc. CA, USA) Significance was accepted at $p < 0.05$.



Results

Allergic response to ascaris

Allergic airways responses ($> 100\%$ increase in R_L) were successfully induced in 7 of 24 immunised sheep (6 SC primed, 1 IP primed). There was no correlation between skin reactivity and airways responses. The magnitude of individual responses varied widely between sheep and bore no relation to priming technique. Four of the responding sheep gave reproducible responses. These four sheep were used in the subsequent studies. There was no significant difference in baseline R_L between responder ($n=7$) and non-responder ($n=17$) sheep (Mean R_L 1.46 ± 0.47 vs 1.61 ± 0.63 $\text{cmH}_2\text{O} \cdot \text{L}^{-1} \cdot \text{sec}^{-1}$ respectively). After allergen challenge, R_L was monitored continuously and peaked at approximately five minutes post-inhalation. Following induction of an increase in R_L of 100% above baseline, the time for R_L to return to baseline was 30 - 60 mins. All responder sheep were screened for the presence of a late response increase in R_L at 6 - 10 hours post-inhalation. No late responses were detected.

The effect of phosphoramidon and C- 96,345

Prior to allergen, there was no significant change in R_L following either phosphoramidon or CP-96,345 administration (Table 3-1). In comparison to the control challenges, phosphoramidon significantly potentiated the response to allergen in all of the sheep, as reflected by the much lower dose of allergen required to reach PC_{100} . Interestingly, although the baseline responsiveness to allergen was quite variable between the sheep, there was much less variability in the effect of phosphoramidon when measured as a % of the baseline response (Table 3-2 & Figure 3-1)

Table 3-1. Effect of intervention on baseline R_L (cmH₂O.L⁻¹.sec⁻¹, means $\pm$ SEM)

Sheep Number	Control	Phosphoramidon	CP96,345
#1	1.12	1.04	1.16
#2	1.64	1.54	1.80
#3	1.98	1.09	1.63
#4	1.65	1.15	1.76
Mean $\pm$ SEM	1.60 $\pm$ 0.18	1.20 $\pm$ 0.11	1.59 $\pm$ 0.15

Table 3-2. Effect of Phosphoramidon and CP96,345 on allergen-induced bronchconstriction in allergic sheep.

Sheep	Control	Phosphoramidon		Intervening Control		CP96,345	
	PC ₁₀₀	PC ₁₀₀	% of Control	PC ₁₀₀	% of Control	PC ₁₀₀	% of Control
#1	44 $\pm$ 2	18	41	55	125	138	290
#2	510 $\pm$ 15	110	22	600	117	NA (>620)	NA
#3	9 $\pm$ 0.5	4	44	7	72	19	211
#4	48 $\pm$ 4	8	16	60	125	170	354
Mean $\pm$ SEM			31 $\pm$ 7 *		110 $\pm$ 13		285 $\pm$ 41 *

PC₁₀₀ - values are breath units of *Ascaris Suum*

NA - PC₁₀₀ was not achieved

Control values are means $\pm$ SEM of 3 reproducibility studies

* p<0.05 vs Control

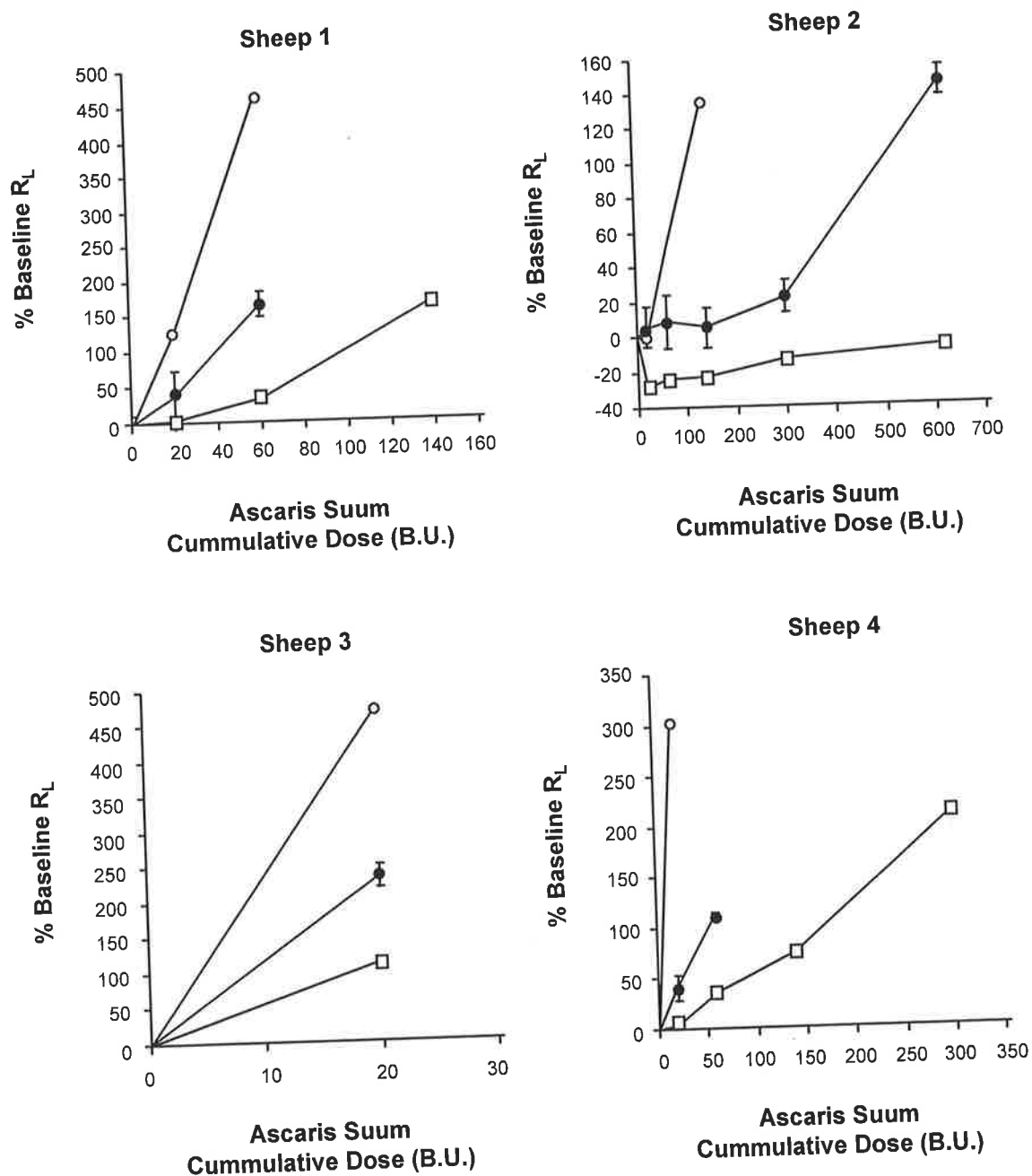


Figure 3-1. Graphs show the response of individual sheep to ascaris alone (●), or after pre-treatment with phosphoramidon (○) or CP 96,345 (□).

CP96,345 markedly attenuated airway responses to allergen in all sheep. The effect was comparable in three of the sheep when considered as a % of the baseline, and in one sheep (the one least responsive in the baseline allergen challenges), PC₁₀₀ was not reached by the conclusion of the experiment at 620 cumulative breaths. Over the course of the experimental protocol there was no significant change in underlying airways responsiveness to allergen as demonstrated by the intervening control study, thus validating the use of the initial control values as a reference against which to measure the effects of the pharmacological interventions.

Capsaicin desensitisation

The capsaicin desensitisation study was conducted 12 months after the initial intervention study. It was noted that the allergen responsiveness had changed from the earlier study, but the responses were reproducible in the period immediately prior to capsaicin administration. Capsaicin caused constant coughing and mucus secretion during the administration although these effects could not be precisely quantified. It was not technically possible to monitor R_L during administration due to cough artefact. Capsaicin desensitisation significantly attenuated the response to allergen as shown by the substantial increase in the PC₁₀₀ dose (Table 3-3).

The effect of allergen sensitisation on non-specific bronchial responsiveness (eg response to inhaled histamine), was not systematically studied in these investigations.

Table 3-3. Effect of capsaicin desensitisation on allergen-induced bronchoconstriction in allergic sheep.

Sheep	Control	Capsaicin	% of Control
	PC ₁₀₀	PC ₁₀₀	
#1	280	NA (>620)	NA
#2	30	210	700
#3	6	23	383
#4	24	140	583
Mean±SEM			555 ± 93 †

PC₁₀₀ - values are breaths units of *Ascaris Suum*

NA - PC₁₀₀ was not achieved

† p=0.04

Discussion

This study demonstrates that reproducible airway responses to allergen in conscious sheep actively sensitised to *Ascaris suum* can be achieved, and identifies a role for tachykinin-containing nerves in the mediation of the acute bronchoconstrictor response to allergen. To our knowledge these interventions have not previously been described in sheep apart from the effect of capsaicin in one preliminary report¹⁵¹.

Previous reports of the sheep model of experimental asthma used animals naturally sensitive to *Ascaris*. We found very few of our sheep were naturally sensitised according to skin test reactivity and found no positive airways responses in naive animals. Hence we instituted an immunisation program which was successful in inducing positive airways responses in 29% of animals. We have some preliminary data suggesting immunisation with aluminium hydroxide as an adjuvant, as has been reported in pigs¹⁸⁶, may yield a higher proportion of responders. A novel aspect of our model is the real time breath by breath computer analysis of R_L and the algorithms used to identify spurious breaths. This approach greatly simplified resistance calculations, enabled immediate recognition of the plateau in resistance during inhalational challenges and eliminated the potential for operator bias.

One of the major advantages of the sheep model of experimental asthma in comparison to rodent models is that the technique for measuring airways resistance is relatively non-invasive and therefore allows sequential studies in the same animal. In this regard evaluation of the baseline reproducibility of allergic responses for the individual animals is therefore important. Abraham has previously reported reproducible airways responses to allergen in individual sheep over time¹⁸⁴ However, Kleeberger¹⁵⁵ reported a significant reduction in response to allergen following biweekly challenges over 10 weeks. In our study we used only responder animals

which had reproducible airways responses in the period immediately before the interventional studies.

Inhibition of the enzyme NEP significantly enhanced the airway response to allergen. This effect has been reported previously in the guinea pig¹⁸⁷ and implies that NEP degradable bronchoconstrictor substances, including tachykinins, are released in response to allergen challenge. In support of this concept, Nieber et. al¹²⁴ demonstrated an increase in the SP content of bronchoalveolar lavage from asthmatic patients following local endobronchial allergen challenge. Several studies, including some in humans⁷⁰ and sheep¹⁵² have shown that NEP inhibitors increase the airway responses to exogenously administered tachykinins. It should be noted however, that NEP is not specific for tachykinin degradation but also degrades bradykinin, endothelin and other peptides, thus we cannot state categorically that the increase in PC₁₀₀ in our model is due to inhibition of tachykinin degradation. For example, phosphoramidon has been shown to enhance the airway response to bradykinin in humans¹⁴². Further, although phosphoramidon is widely used as an NEP inhibitor, it does have inhibitory effects on other enzymes including endothelin converting enzyme¹⁸⁸, angiotensin converting enzyme (which also degrades SP)¹⁸⁹ and elastase¹⁹⁰. Nevertheless we consider the principle relevant action in the airway to be NEP inhibition. Our findings suggest that the sheep model may be suitable to further investigate the modulation of allergic airway responses by NEP, an issue of potential importance in human asthma¹⁴⁰.

The non-peptide specific NK1 receptor antagonist CP-96,345 inhibits the hypotensive effect of SP in dogs¹⁸⁵ and hyperpnoea-induced bronchoconstriction in guinea pigs¹⁹¹, but its effect in a large animal model of experimental asthma has not previously been reported. We have shown that this antagonist markedly reduced the acute bronchoconstrictor response to allergen, suggesting that endogenous tachykinins are released and contribute to allergen-induced

bronchoconstriction. Possible mechanisms for a tachykinin effect include direct NK1-mediated smooth muscle contraction, enhancement of cholinergic contraction, stimulation of vasodilatation, increased vascular permeability, and stimulation of histamine release. Corcoran et al¹⁹² found that intravenous tachykinins increased R_L in sheep, with SP being more potent than NKA, implying an NK1 receptor-mediated response. This response was shown to be primarily indirect as it was inhibited by atropine and was thought to be due to modulation of cholinergic contraction. Evidence for myelinated nerve reflex mediation of the airway response to intravenous SP was also demonstrated in studies using vagotomy and vagus nerve cooling¹⁹³. Responses in humans have been reported by Crimi et al⁷¹, who found that ipratropium bromide reduced the airway response to inhaled SP in asthmatic humans, thus also indicating that SP was acting at least in part via cholinergic mechanisms. Also in agreement with these studies, we have *in vitro* data indicating that NK1 mediated contraction of sheep tracheal smooth muscle is atropine sensitive⁷⁹. However, others have reported that the *in vivo* airway response to allergen in sheep is not reduced by atropine and therefore not mediated by a cholinergic reflex mechanism¹⁸⁴. Thus, although under certain circumstances CP-96,345 may be acting *in vivo* via the modulation of cholinergic responses, a more direct effect in the context of allergen induced responses cannot be ruled out.

Apart from effects on smooth muscle, there is evidence that NK1 receptors may contribute to airways obstruction by effects on vessels. Ichinose et al⁸⁵ found that the NK1 receptor antagonist FK-888 reduced the recovery time after exercise-induced asthma. The nature and timing of this effect lead the authors to suggest that FK-888 was antagonising NK1 receptor-mediated increases in vascular permeability. Bowden et al¹¹ have shown in the rat that tachykinin induced increases in vascular permeability are mediated by the opening of gaps in post-capillary venules, and that this is an NK1-mediated response. Delaunois et al¹⁹⁴ found that CP-96,345

completely inhibited the SP-induced increase in capillary filtration coefficient in a rabbit model of pulmonary oedema. Our findings in the sheep may therefore be partly explained by antagonism of tachykinin induced effects on airway vessels following allergen challenge.

Another possible mechanism of action of CP-96,345 in allergen-induced bronchoconstriction may be an effect on histamine release from pulmonary mast cells. Abraham et al¹⁸⁴ has shown that the acute response to allergen in sheep is associated with an increase in plasma histamine levels. SP also increases histamine release from both human⁹⁴ and guinea pig⁹⁶ pulmonary mast cells and the release of histamine from guinea pig lung can be blocked by specific NK1 and NK2 receptor antagonists. However, Crimi et al⁷¹ has shown that the acute bronchoconstriction response to inhaled SP in humans was not blocked by the antihistamine astemizole. Whether endogenous tachykinins stimulate significant histamine release in allergic responses is still uncertain. Our *in vitro* studies of sheep trachea showed that the contractile effect of SP, NKA or the NK1 specific agonist Sar⁹ Met(O₂)¹¹-SP could not be blocked by the antihistamine pyrilamine⁷⁹.

In addition to NK1 antagonism, CP-96,345 has been shown to have calcium channel blocking activity leading to hypotension and bradycardia. This effect has been seen at a dose of 3mg/kg in guinea pigs¹⁹¹ and 0.5-1mg/kg in dogs¹⁹⁵. We considered the possibility that a calcium channel blocking effect may have confounded our results with this agent, but do not believe this to be the case. Firstly, the dose used here is lower than that seen to cause significant cardiovascular responses in other species. In the guinea pig study, no non-specific inhibition of bronchoconstriction was seen at 3mg/kg, as evidenced by the fact that CP-96,345 did not inhibit the contractile response to the NK2 specific agonist Nle¹⁰-NKA(4-10). In fact, it has been shown that diminished airway blood flow (as might occur if hypotension and bradycardia were induced) actually prolongs the bronchoconstriction response to histamine, perhaps due to reduced

mediator clearance¹⁹⁶. This suggests that any confounding effect might actually enhance the bronchoconstriction response to allergen, rather than reduce the response as we show here. Furthermore, when CP-96,345 was administered at the dose of 1mg/kg we in fact found that this caused bronchoconstriction, and the dose was subsequently titrated down until no R_L response was seen (Table 3-1, and further documentation of this is given in chapter four). Although we do not believe that calcium blocking activity confounded our interpretation of NK1 antagonism, the cardiodepressive effects of CP-96,345 suggest it will not be suitable for human therapy. A phase I trial of this agent was in fact concluded early due to side effects (R. Scicchitano, personal communication). Newer agents such as CP-99,994⁸³, which lack calcium channel blocking activity, may prove more useful.

Capsaicin desensitisation is an established technique for examining the role of C-fibre afferents and their neuropeptides in the mediation of physiological responses¹⁷. Capsaicin desensitisation resulted in a decrease in the response to allergen in our sheep model. A similar effect has been reported for guinea pigs sensitised to ovalbumin^{21,22} and in pigs sensitised to ascaris¹⁹⁷. In contrast, Abraham et al¹⁵¹ examined the effect of capsaicin in sheep and found that the acute and delayed responses to allergen, 48 hrs after capsaicin desensitisation were not reduced compared to the pre-capsaicin response. However, the control sheep in their study developed increased responsiveness to a second allergen challenge 7 days later, whereas in the capsaicin treated animals no such enhancement was seen. This lack of enhancement in the capsaicin treated animals was interpreted as evidence that capsaicin desensitisation had prevented specific bronchial hyper-responsiveness. Depletion of tachykinins from sensory nerves will prevent the subsequent release of these neuropeptides in response to stimuli such as allergen challenge, whether this release occurs directly or via axon reflex mechanisms¹⁷. However, neuropeptide depletion will also affect the sensory afferent function of C-fibre nerves and their

role in the mediation of classical centrally mediated reflex arcs including efferent cholinergic vasodilatation and smooth muscle contraction⁸⁴. The immediate effects we noted during capsaicin administration (cough, mucus secretion) are consistent with those reported for other species including man^{12,23}. Unfortunately, it was technically impossible to obtain reliable R_L data during or immediately after capsaicin administration, therefore we were unable to confirm an immediate bronchoconstriction, which we postulated may occur as a result of the release of endogenous tachykinins. The reduced response to allergen we noted following capsaicin desensitisation provides further evidence of a role for tachykinins in the mediation of the allergic response.

Conclusion

The three approaches taken in this study, namely modulation of tachykinin degradation, specific receptor antagonism and neuropeptide depletion, when taken together provide *prima facie* evidence for a role for tachykinins in the mediation of the acute airway response to allergen in non-rodent mammals. The sheep model described herein will be useful to evaluate newer tachykinin antagonists which may have therapeutic potential in human disease.

Chapter Four

The response to intravenous tachykinins in sheep is mediated by NK1 receptors, is atropine sensitive and exhibits marked tachyphylaxis.

In the previous chapter, a role for tachykinins C-fibre afferents and tachykinins in the mediation of the acute response to allergen was identified¹⁹⁸. The current chapter describes further studies undertaken to more completely characterise the mechanisms involved in tachykinin mediated ovine airways responses.

Although *in vitro* studies have elucidated many key neurologically mediated airway responses, more complete characterisation of the role of the nervous system in airways disease requires relevant *in vivo* model systems. In the field of tachykinin research, as has previously been mentioned, rodent models have been a popular tool and several studies, particularly in the guinea pig and the rat, have demonstrated an important role for tachykinins in the mediation of airways responses. However, the importance of tachykinins in non-rodent mammals and the validity of extrapolating rodent data to the human situation has been questioned²⁴. *In vitro* and *in vivo* evidence suggests that ovine airway responses may more closely correlate to human physiology than do rodent or pig responses^{79,199,153}. In this chapter, the mechanism of tachykinin-induced airway responses was further characterised by using exogenous tachykinins. In distinction to previous reports, the key finding of a marked tachyphylaxis to the effects of intravenous SP was identified. Having identified this effect, the actions of various pharmacological agents on the lung resistance (R_L) response to SP were then examined, taking deliberate steps to ensure the interpretation was not confounded by the presence of tachyphylaxis. These studies help to expand

the understanding of tachykinin physiology *in vivo* and provide new insights into the use of the ovine model in tachykinin research.

Materials and Methods

Animals

Australian merino ewes aged 12 -18 months were used for these studies. These sheep had not been sensitised to allergen. The details of the measurement of airways resistance is given in the Methods chapter.

Peptides and chemicals

SP (Auspep, Melbourne, Australia) was dissolved in 10% ethanol / 0.1% BSA in sterile saline (Abraham, 1991) to a concentration of 1mg/ml and stored at -70°C. Phosphoramidon (Sigma Chemical Co., St Louis, USA) was dissolved in sterile water to a stock concentration of 1mg/ml then aliquotted and stored at -20 °C. CP-96,345 (Pfizer Pharmaceuticals, New York, USA) was made to a stock concentration of 100mg/ml in sterile phosphate buffered saline (PBS, pH 7.4), stored at -20°C, then diluted to a dose of 0.1mg/kg in 10ml of normal saline on the day of the experiment. Atropine, (Astra Pharmaceuticals) was diluted in 10ml 0.9% NaCl to a dose of 0.1mg/kg and delivered intravenously by a 10 min infusion. Sodium cromoglycate (10mg/ml solution; Fisons, UK) was used as supplied, 4ml being delivered by inhalation. Indomethacin (Sigma) was dissolved in 0.8% NaHCO₃ to a working concentration of 10mg/ml and administered at a dose of 2mg/kg intravenously. Histamine acid phosphate (Sigma) was dissolved in 0.9% NaCl to various working concentrations on the day of the experiment and administered intravenously. Pyrilamine (Sigma) was dissolved in 10ml sterile water to a total dose of 2mg/kg body weight on the day of the experiment. NKA (Auspep) was dissolved in 10% ethanol / 0.1% BSA in sterile saline to a stock concentration of 1mg/ml and stored at -70°C. Doses of 5×10^{-6} and

10^{-5} mol/kg were made up in 10% ethanol / 0.1% BSA in sterile saline on the day of the experiment.

Aerosol Delivery

SP (in preliminary experiments), phosphoramidon and sodium cromoglycate were administered by inhalation. Aerosol was delivered using a ventilator and nebuliser (Bird™ Mark 14, Palm Springs, California, USA). During aerosolisation the ventilator was set to deliver a tidal volume of 350ml using a 3% CO₂ in air mixture, at a rate of 20 breaths per minute. The nebulising jet was delivered via a custom made dosimeter, which nebulised for one second per breath, delivering 7.2μL per breath (Biomedical Engineering, Royal Adelaide Hospital, Australia).

Tachykinin administration

Three methods of tachykinin administration were evaluated in preliminary studies - inhalation, bolus intravenous injection and intravenous infusion. SP administered by inhalation (to a maximal dose of 144μg, 20 breaths of a 1mg/ml solution) did not result in any significant increase in R_L , even after phosphoramidon (288 μg inhaled) administration. Subsequently, SP was given either by bolus injections (5×10^{-6} mol/kg) or by a short infusion (0.01mg/kg total dose made up in 2mls diluent, at a rate of $1 \text{ ml} \cdot \text{min}^{-1}$ over 2 minutes) using a syringe driver pump. NKA was also given by bolus injection (5×10^{-6} - 10^{-5} mol/kg). In all cases intravenous lines were first primed with diluent (10% ethanol / 0.1% BSA in normal saline) to reduce the adherence of neuropeptides to the plastic tubing.

Study design

Time course of blood pressure and airway response to SP.

The airways response to a bolus dose of SP (5×10^{-6} mol/kg i.v.) and short infusion of SP (0.01 mg/kg over 2 min) was assessed in 6 sheep. R_L was monitored at baseline and continuously for 15 min from the onset of SP administration. In 5 sheep the blood pressure response was measured concurrently with the airway response.

Assessment of tachyphylaxis

The effect of repeated SP infusions given on the same day was examined in 4 sheep. Following an initial infusion (time=0), repeat infusions were given at 120, 180 and 210 min, thus the effects of SP infusions separated by 30, 60 and 120 mins were assessed. R_L was measured at baseline (5 min) and for 15 min from the onset of the infusion.

Assessment of day to day reproducibility

Reproducibility of the response to a single daily SP infusion was determined over 3 consecutive days in 6 sheep. R_L was measured as for the tachyphylaxis studies.

Modulation of SP effect with drugs

The effect of five drug interventions were assessed in the same 6 sheep used for the reproducibility studies. The drugs given were: the NEP inhibitor phosphoramidon (244 μ g inhaled), the NK-1 receptor antagonist CP-96,345 (0.1mg/kg i.v.), atropine (0.1mg/kg i.v.), indomethacin (2mg/kg i.v.) and sodium cromoglycate (40mg inhaled). The drug studies were performed in a random order at weekly intervals over a 5 week period. Each drug study consisted of two SP challenges on subsequent days. One of the two challenges was designed as a control, whereby SP administration was preceded by the administration of the relevant diluent for the active drug under investigation. For the control and active drug challenges, R_L was measured initially for 5 mins to establish a stable baseline. Active drug or diluent was then administered and R_L was measured 20 mins later to ensure there was no significant shift in R_L from the original baseline. SP was then administered by infusion while R_L was monitored continuously for 15 mins. The peak R_L was determined, and expressed as a percentage of the R_L measurement taken immediately prior to the commencement of the SP infusion.

Effect of antihistamine on SP-induced increases in R_L

In a separate set of experiments, the effect of the anti-histamine pyrilamine on SP-induced increase in R_L was assessed in 3 sheep. The ability of pyrilamine to block the bronchoconstrictor response to histamine was first assessed. Histamine was administered intravenously in a dose response fashion to a maximum cumulative dose of 18.75 μ g/kg. Two days later, the anti-histamine pyrilamine was administered intravenously (2mg/kg) 20 minutes before histamine administration. One week later the R_L response to a bolus injection of SP (5×10^{-6} mol/kg) was measured, then, two days after this, pyrilamine (2mg/kg i.v.) was given 20 mins prior to SP. In

each case R_L was monitored at baseline for 5 mins and continuously for 15 mins from the administration of each of the agents used.

Comparison between the R_L response to SP and NKA

The R_L response to equimolar bolus doses of SP and NKA (5×10^{-6} mol/kg) were compared in four sheep. SP and NKA responses were measured on separate days to avoid confounding by tachyphylaxis. In addition, the effect of atropine on NKA-induced increases in R_L was assessed in two sheep. NKA was delivered as an intravenous bolus injection (10^{-5} mol/kg) and the R_L response was measured. Two days later, atropine (0.1 mg/kg i.v.) was administered 20 min prior to NKA, then the R_L response was again measured.

Data Analysis

Data are presented as means $\pm$ SEM expressed as % of baseline R_L . All statistical analyses were performed using a repeated measures ANOVA. Significant differences between group means was determined using Fisher's Protected Least Significant Difference analysis. The significance level was set at $P < 0.05$ for all analyses.

Results

Change in R_L in response to SP.

In preliminary analyses, SP was administered by inhalation up to a maximal concentration of 1mg/ml (total dose 144 μ g) in 4 sheep. No significant effect on R_L was seen with this dose of SP. Intravenous administration of SP was then evaluated. Bolus intravenous administration of SP (5×10^{-6} mol/kg) lead to a transient increase in R_L , lasting approximately 5 breaths (data not shown). In an effort to induce a more prolonged effect, we assessed the response to a 2 min infusion of SP (0.01mg/kg total dose over 2 min) in 6 sheep. There was minimal prolongation of the R_L response however. An important finding was that R_L began to return to baseline even while the infusion was still running and had returned to baseline by the time the infusion was complete (Figure 4-1). No significant change in mean arterial blood pressure was seen either during the airway response or subsequently.

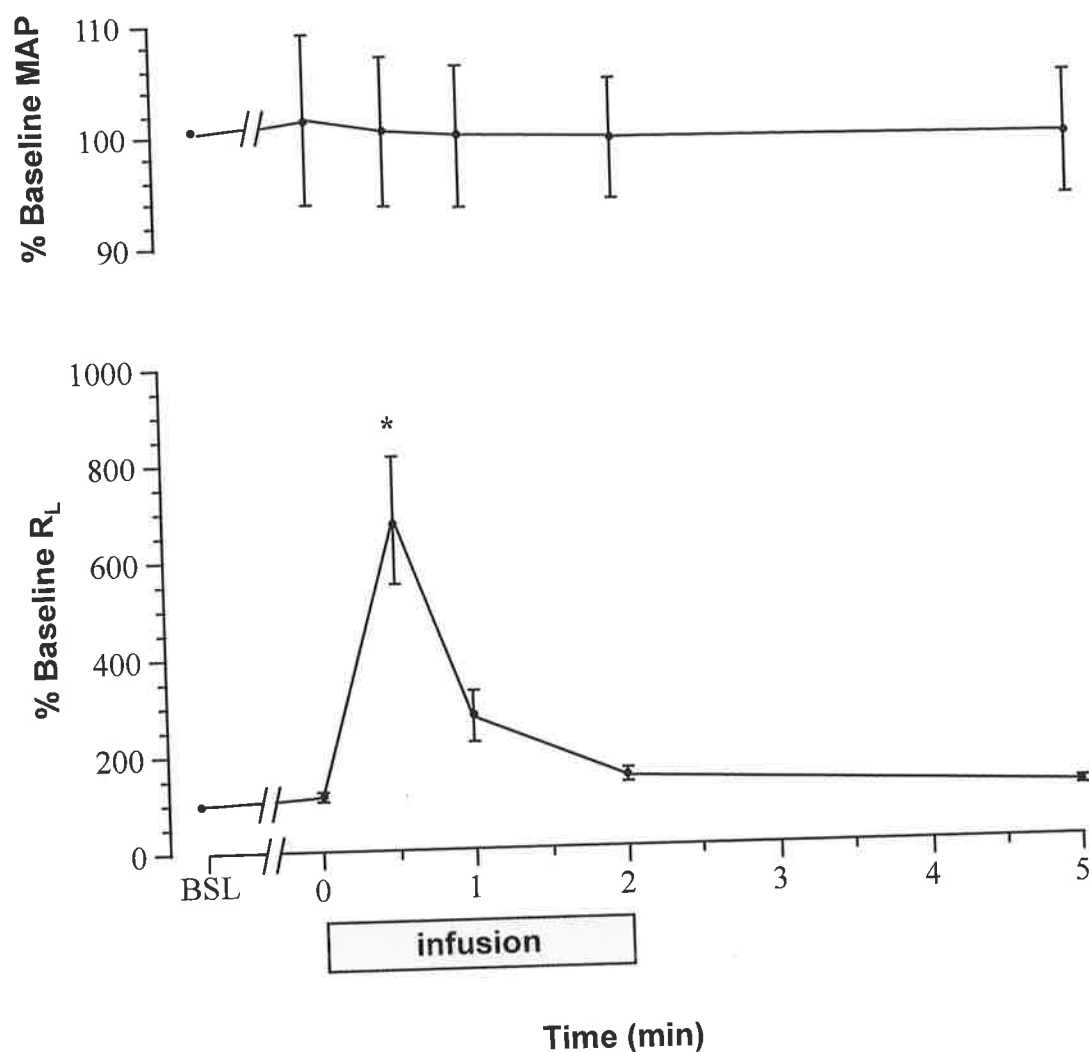


Figure 4-1. R_L and Mean arterial BP (MAP) response to intravenous SP. SP was administered by infusion (0.1 mg/kg over 2 min) into the jugular vein. Continuous measurements of R_L were made using a tracheal catheter and oesophageal balloon connected to a differential pressure transducer as described in the text ($n = 6$ sheep). MAP was measured concurrently using a carotid artery catheter in 5 of the sheep. Data is presented as a percentage of the pre-infusion baseline values and the mean $\pm$ SEM of the group is shown for five minutes from the onset of the infusion. * $p < 0.05$ vs baseline.

Tachyphylaxis

The finding that the R_L response to infused SP returned to baseline even while SP was still being infused suggested the presence of tachyphylaxis. To investigate this further SP was administered by repeat infusions over short time intervals in 4 sheep. The initial SP infusion ($t=0$) and the second infusion 2 hours later ($t=120$) both produced increases in R_L above baseline (Figure 4-2). However, in comparison to the initial SP infusion, the responses at $t=120$ were significantly attenuated. Furthermore, SP infusions at $t=180$ and $t=210$ (ie 60 and 30 min interval between successive infusions respectively) resulted in even less increase in R_L . In fact, the R_L in these latter responses did not significantly rise above pre-infusion baseline levels. During the course of this study we also noted whether SP was having any medium term effect on R_L by comparing the immediate pre-SP infusion baseline R_L values at each infusion time point over the $3\frac{1}{2}$ hour duration of the study. No change in baseline values was seen (Figure 4-2).

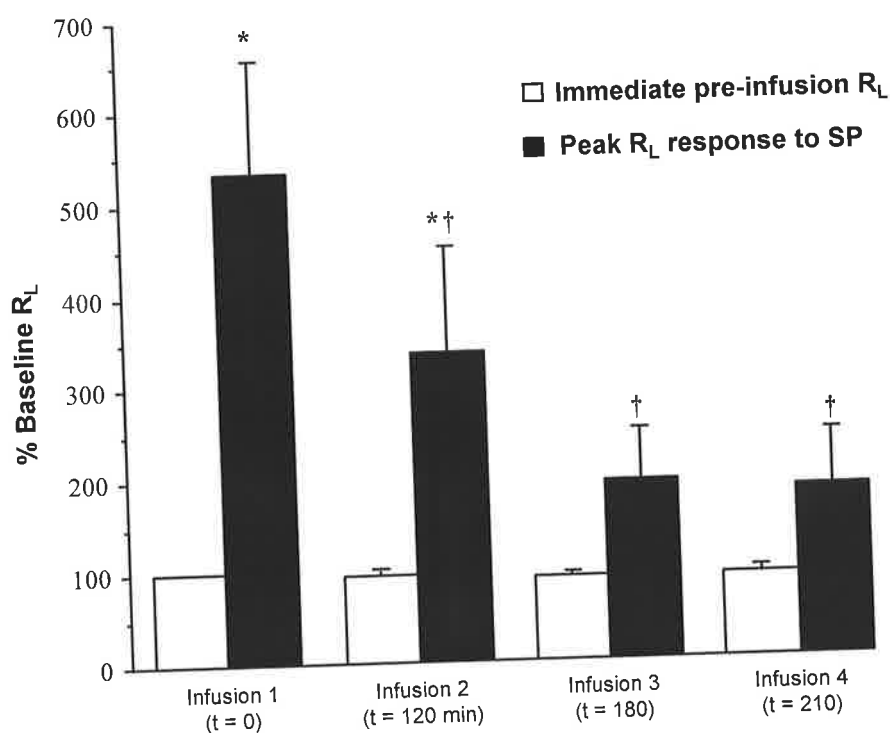


Figure 4-2. Repeat SP infusions show the presence of tachyphylaxis. SP infusions were administered four times during a total 3.5 hour period to 4 sheep. After the initial infusion ($t=0$), repeat infusions were separated by 120 min, 60 min and 30 min ($t=120$, 180 and 210 respectively). Immediate pre-infusion R_L and the peak R_L increase after SP are shown and presented as a % of baseline R_L (mean $\pm$ SEM for the group). * $p<0.05$ vs baseline, † $p<0.05$ vs peak R_L at $t=0$.

Day to day reproducibility

To enable proper design of studies aimed to evaluate pharmacological modulation of the effect of SP, we first evaluated the reproducibility of responses to SP over time in individual sheep. We also wanted to be sure that these studies were not confounded by tachyphylaxis. We thus evaluated responses at 24 hour intervals over a three day period. Although there was significant inter-sheep variability in the response to infused SP, there was no significant differences in the responses of individual sheep to SP infusions administered at 24 hr intervals over a 3 day period. (Figure 4-3). There was also no significant change in the absolute values of baseline R_L for individual sheep over the course of the 3 day reproducibility study (data not shown). These findings indicated that drug intervention studies conducted at 24 hour intervals would be unlikely to be confounded by either spontaneous variation in the response of individual sheep, tachyphylaxis due to the previous SP dose, or shifts in baseline R_L (either spontaneous or due to SP).

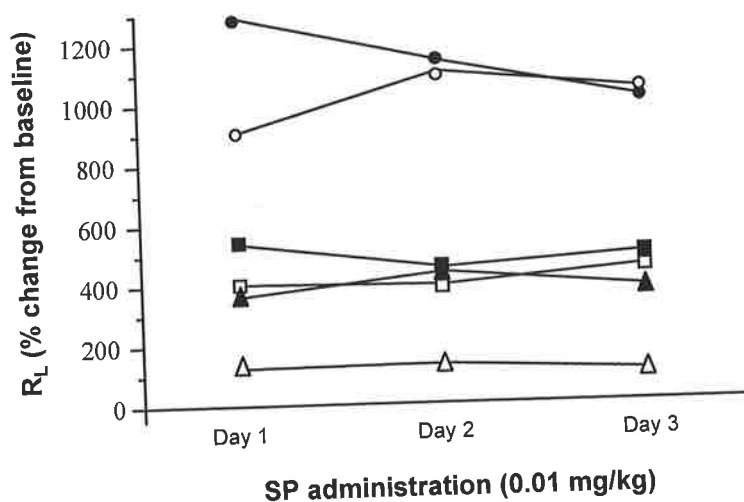


Figure 4-3. Reproducibility of R_L response to SP over a three day period. The R_L response to SP was measured at 24 hour intervals over three days in 6 sheep. The responses for each of the individual 6 sheep are shown, expressed as % change from pre-SP baseline values.

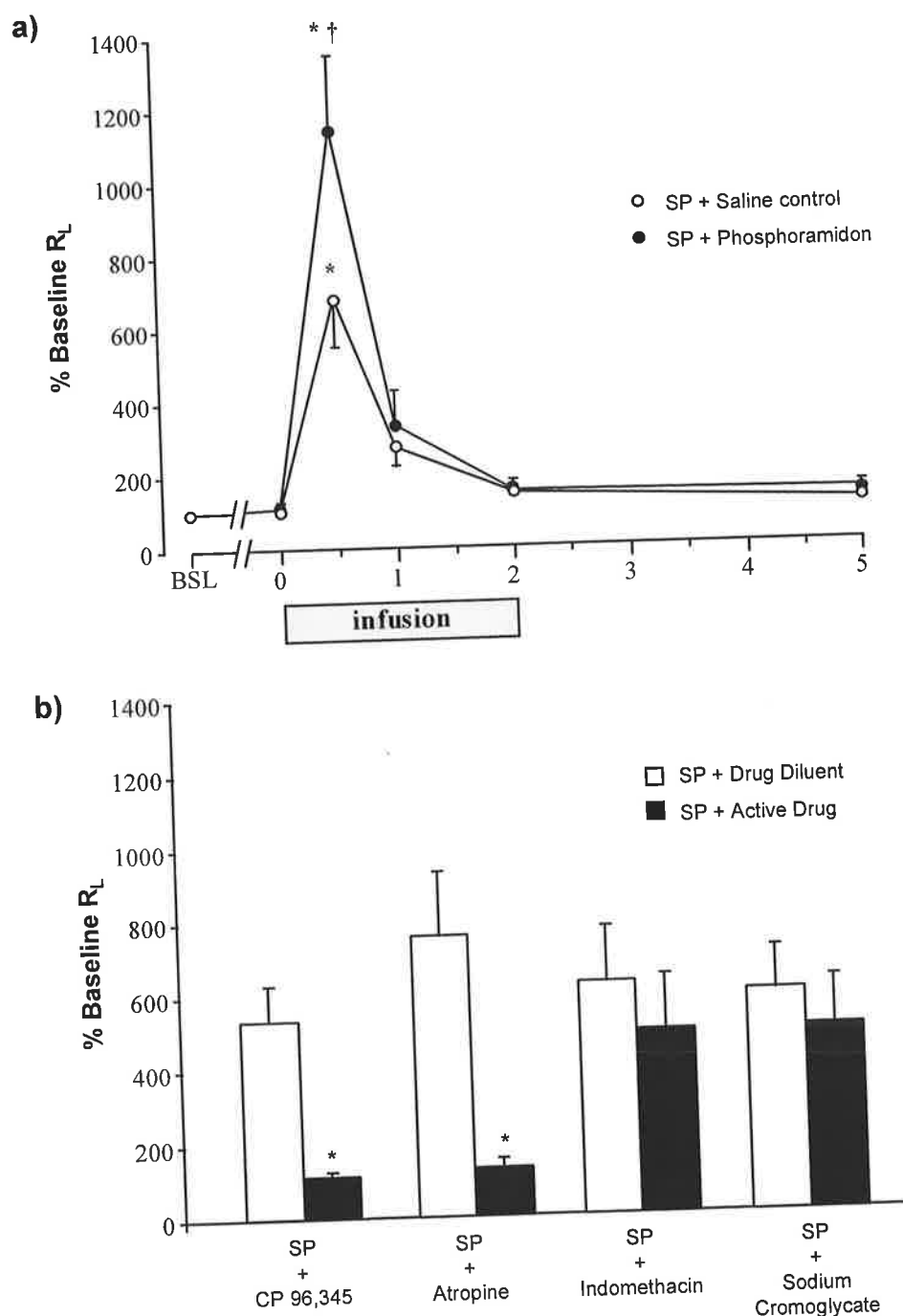
Pharmacological Interventions

The effect of several pharmacological interventions on the R_L response to SP was assessed. In each case agents were chosen in accord with specific hypotheses based upon knowledge of the tachykinin axis in the airway. Phosphoramidon is an inhibitor of the neutral endopeptidase, the enzyme chiefly responsible for SP degradation, and was thus hypothesised to increase responses to exogenous tachykinins. CP 96345 is a specific NK-1 receptor antagonist¹⁸⁵ and was therefore used to investigate whether SP responses were mediated by NK-1 receptors. There is some evidence that SP in part acts by facilitation of parasympathetic responses, thus atropine was used to investigate this. In vitro studies have also shown that SP can stimulate the release of prostaglandins which cause smooth muscle relaxation. If such a mechanism were operative in the sheep model then inhibition of cyclo-oxygenase with indomethacin might reduce prostaglandin production, leading to enhancement of SP-induced increases in R_L . In addition, there has been some evidence that sodium cromoglycate may have an effect on neurologically mediated airway responses, thus this too was investigated.

There was no significant difference between initial baseline R_L measurements and the measurements taken 20 mins after either active drug or diluent administration without administration of SP. R_L values after diluent control or drug are shown in Table 4-1. In all of the control challenges (ie diluents followed by SP) SP produced a significant rise in R_L (Figure 4-4). Pre-SP treatment with phosphoramidon significantly increased the peak response to SP: mean $\pm$ SEM R_L was $754.8 \pm 139\%$ of baseline with SP alone vs $1151.5 \pm 196\%$ with phosphoramidon + SP (Figure 4-4a). Phosphoramidon had no effect on the duration of the response however. The response to SP was completely abolished by CP-96,345 and atropine (Figure 4-4b). Indomethacin and sodium cromoglycate did not significantly change the response to SP (Figure 4-4b).

Table 4-1. Raw baseline R_L data obtained from intervention studies

Intervention Study	Pre-SP Baseline R_L (cmH ₂ O.L ⁻¹ .sec ⁻¹)	
	After Diluent	After Drug
Phosphoramidon	1.62 ± 0.12	1.86 ± 0.25
CP96,345	2.47 ± 0.26	2.14 ± 0.18
Atropine	1.87 ± 0.08	1.67 ± 0.19
Sodium Cromoglycate	2.08 ± 0.28	1.89 ± 0.16
Indomethacin	2.24 ± 0.17	2.74 ± 0.31



Intervention Study

Figure 4-4. Effect of various pharmacological interventions on the R_L response to SP. **a)** Time course of the response to SP infusion following control (saline) pre-treatment (○) or pre-treatment with phosphoramidon (●). Mean $\pm$ SEM for 6 sheep is shown. * $p < 0.05$ vs baseline, † $p < 0.05$ vs peak R_L response in control study. **b)** Effect of various agents on the peak R_L response to SP. Mean $\pm$ SEM for 6 sheep (except sodium cromoglycate, $n=5$) is shown. * $p < 0.05$ vs SP + drug diluent.

Effect of antihistamine

Because SP release of histamine from mast cells has been proposed as a mechanism for some of the airway effects of SP, the effect of anti-histamine treatment on SP responses was assessed. Pretreatment with pyrilamine blocked the R_L response to histamine, but had no effect on the response to SP (Figure 4-5).

Effect of SP compared to NKA

At a dose of 5×10^{-6} mol/kg SP significantly increased R_L , whereas at the equivalent dose, the R_L response to NKA was not significantly different to baseline (Figure 4-6a).

Effect of atropine + NKA on R_L

At the highest dose of NKA used (10^{-5} mol/kg), R_L was $279 \pm 25\%$ of baseline, an effect which was not statistically significant. Pretreatment with atropine had no significant effect (Figure 4-6b). However, statistical interpretation of these results may have been limited due to the small number of animals available for study.

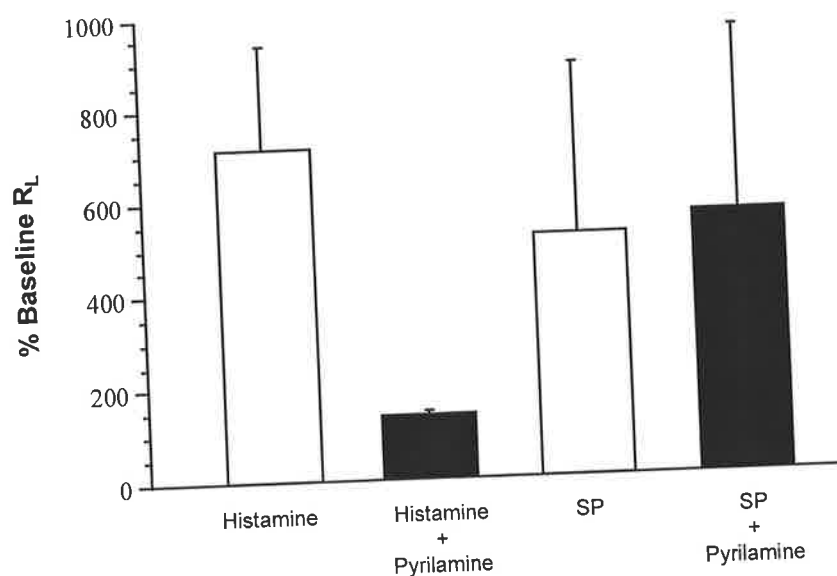


Figure 4-5. Effect of histamine pre-treatment. Peak R_L response to histamine was measured with no prior active treatment or after pyrilamine (mean $\pm$ SEM of 3 sheep). The effect of pyrilamine was then assessed on the R_L response to SP in these same three sheep.

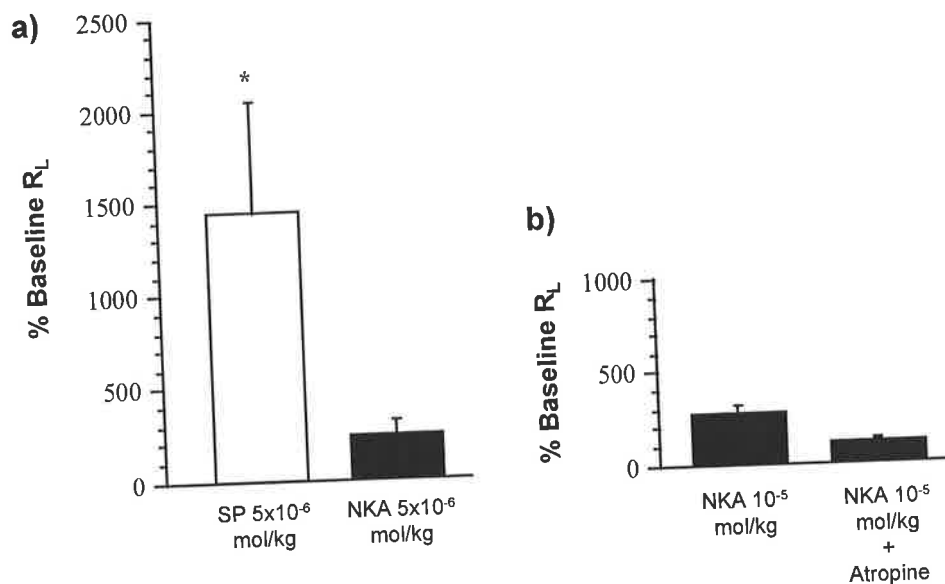


Figure 4-6. Comparison of response to SP and NKA. **a)** A bolus dose of SP was given to 4 sheep and the peak R_L response was measured. On separate days an equimolar bolus dose of NKA was given to the same 4 sheep and the R_L response again measured. Data is mean $\pm$ SEM for the group. * $p < 0.05$ vs baseline R_L . **b)** In two of these sheep a higher dose of NKA was administered, and on a separate day the effect of atropine on the response to this dose was assessed.

Discussion

In this study we have shown that intravenous tachykinins increase lung resistance in sheep and have characterised the response in a number of ways. Unlike some previous reports^{185,192} we did not find that SP had any significant effect on blood pressure, therefore none of the responses detected were confounded by reflex responses to hypotension. Although the effect of inhaled SP was investigated in preliminary experiments, like Abraham et al¹⁵² we found no significant effect. However, this was not the focus of the current study and the negative results obtained were possibly due to the low dose of SP. The use of higher doses of SP was constrained by the cost of the reagent.

A novel aspect to the current study compared to previous reports is the examination of tachyphylaxis *in vivo*. The response to bolus intravenous SP was maintained for less than one minute, with the peak effect typically lasting only 5 breaths. Possible mechanisms for the transient nature of this response include the induction of tachyphylaxis and rapid SP degradation. In an effort to distinguish between these possibilities, the effect of SP delivered by infusion was examined. The use of computerised measurement of R_L , which enables continuous on-line measurement, allowed the reliable assessment of the time course of transient airway responses. When SP was delivered by infusion, there was a slight prolongation of the airway response, but no significant prolongation of the time at peak R_L . The return of R_L to baseline while the infusion was still running clearly indicated a rapid desensitisation to SP, rather than rapid degradation of SP alone. This finding is in accord with *in vitro* studies of calcium mobilisation responses to SP⁵⁵ and with vascular permeability responses in rat blood vessels⁵⁶, but has not previously been reported for airways responses. This effect was further examined by performing repeated infusions on the same day, which showed that tachyphylaxis to the effect of infused SP persisted

for at least 2 hours after an initial infusion. At 24 hours however, there was no evidence of persistent tachyphylaxis. These findings clearly have important implications for the design of studies examining the effects of drugs or other interventions on the response to SP, as results will be confounded by tachyphylaxis if insufficient time is allowed between baseline and post-intervention SP doses. For example one report¹⁹² examined the effect of atropine on SP responses only 20 minutes after the index SP dose and only one hour after an SP dose response study. No comment was made regarding tachyphylaxis in this study, although the transient nature of the response to SP was similar to our findings. The effect of atropine on SP induced airway responses they reported was therefore likely to have been confounded by tachyphylaxis. Possible mechanisms for the development of tachyphylaxis include receptor phosphorylation and second messenger uncoupling^{54,57}. NK1 receptor internalisation, a mechanism which has been demonstrated by direct visualisation in endothelial cells⁵⁶ appears to play less of a role. Interestingly, our *in vitro* studies of isolated ovine tracheal smooth muscle did not find evidence of tachyphylaxis to SP, although tachyphylaxis was seen to the NK1 receptor specific agonist Sar⁹Met(O₂)¹¹-SP⁷⁹. Thus the *in vitro* effects of SP in that study may have been mediated largely by cross-reactivity at NK2 receptors, as NK2 activation was not subject to tachyphylaxis in the *in vitro* system. The apparent discrepancy between *in vitro* and *in vivo* responses may be due to a much larger NK1 mediated cholinergic component *in vivo*. The findings of the current study clearly indicate that the inducement of tachyphylaxis is a very powerful mechanism for limiting the airway responses to exogenous SP. It is likely that a similar effect would operate in response to the release of endogenous SP.

Inhaled phosphoramidon enhanced the increase in R_L due to SP, a finding in agreement with several studies, including those in humans⁷⁰. In light of the rapid tachyphylaxis to intravenous SP, the finding that inhibition of NEP was still able to enhance the airway response

indicates that NEP degradation of SP occurs extremely rapidly, clearly having a significant effect before being masked by the development of tachyphylaxis. It has been proposed that a reduction in NEP (due to epithelial damage) may occur in the airways of asthmatics and hence potentiate SP effects in this disease. Several studies in animal models have shown an increase in airway responsiveness following exposure to noxious agents coincides with a reduction in NEP¹³⁷, and that anti-inflammatory corticosteroids increase NEP production¹⁴⁰. However, studies reported to date have not demonstrated evidence for decreased NEP activity in asthmatics⁷⁰, although severe asthmatics have not been assessed. The findings of the current study in the sheep indicate that NEP is a powerful and rapid inhibitor of SP, and suggest that the sheep model may be useful to further examine the importance of NEP in the modulation of airways responses.

We characterised the tachykinin receptor subtype responsible for the effect of SP by comparing the effect of SP and NKA on R_L , and by using the NK1 specific antagonist, CP-96,345. The much greater effect seen with SP, and the inhibition seen with CP-96,345, indicates an NK1 receptor mediated response. The limitations of CP-96,345, with regard to calcium channel blocking activity have been discussed in chapter three. Once again, for the reasons discussed, we do not believe the principle finding was undermined by this effect. The R_L response to SP was also abolished by atropine, suggesting that the effect seen was largely due to the stimulation of cholinergically mediated bronchoconstriction. *In vitro* studies have shown that SP administration enhances cholinergic responses⁷⁷, and that this may be a post-ganglionic effect, therefore the effects we noted *in vivo* are possibly mediated by SP binding to NK1 receptors on cholinergic nerves. In addition, vagal transection and cooling studies in sheep indicate that the response to intravenous SP is mediated at least in part by myelinated fibres carried in the vagus nerve¹⁹³. Evidence for cholinergic mediation of the airway response to SP has also been noted in humans where it was found that ipratropium bromide reduced the airway

response to inhaled SP in subjects with asthma⁷¹. No significant airway response to NKA was detected, thus no comment could be made about atropine sensitivity in the context of this agent.

The current study is consistent with previous reports of ovine responses in that SP was a more potent bronchoconstrictor than NKA when administered by the vascular route. In contrast our *in vitro* studies using sheep trachea preparations showed that NKA was the more potent contractile agent, and additional receptor specific agonist studies illustrated the predominance of NK2 receptors in the mediation of the *in vitro* response⁷⁹. This discrepancy between the *in vitro* and *in vivo* findings may be due to a low concentration of NKA reaching the smooth muscle NK2 receptors when administered by the vascular route, allowing the indirect, cholinergically mediated response to SP (via NK1 receptors) to dominate in this setting. Interestingly, our *in vitro* studies not only showed that the response to Sar⁹Met(O₂)¹¹-SP was subject to tachyphylaxis, but was also sensitive to atropine, thus in accord with the *in vivo* findings of the current study.

In addition to the immediate induction of cholinergic responses, the possibility that SP may have a delayed effect on R_L was also considered. Such an effect might have important implications in airways disease. For example a delayed effect could arise as a result of SP-induced stimulation of histamine release from pulmonary mast cells, and the production of bronchoconstricting prostaglandins. Further, SP has important effects on vascular permeability¹¹ and stimulation of inflammatory cells including neutrophils²⁰⁰, eosinophils⁸⁹ and lymphocytes⁹². We therefore looked for any changes in baseline R_L at each time point during the tachyphylaxis study. Although the sheep had a total of four SP infusions over a 3 ½ hr period, no change in baseline R_L was seen. Therefore, a significant effect on airways resistance due to changes in vascular calibre or the induction of oedema is unlikely. In addition, although the reproducibility studies were not designed primarily to assess cellular influx, it is interesting to note that there was no change in baseline R_L and no increase in bronchial reactivity to SP over the three days of

this study to suggest that a significant influx of inflammatory cells was taking place.

SP increases histamine release from both human⁹⁴ and guinea pig⁹⁶ pulmonary mast cells and the release of histamine from guinea pig lung can be blocked by specific NK1 and NK2 receptor antagonists. The question of histamine release was specifically addressed in the current study by using the H1 receptor antagonist pyrilamine. It was found that pyrilamine had no effect on the airway response to SP, thus implying that SP is not acting via histamine release in this setting. During these experiments it was also noted that the bronchoconstriction in response to exogenous histamine reached a sustained plateau for at least several minutes which was quite different to the transient response to SP. Our findings indicate that histamine release was not involved in SP-induced bronchoconstriction and are in agreement with those of Crimi et al who showed that the acute bronchoconstriction response to inhaled SP in humans was not blocked by the antihistamine astemizole⁷¹.

There is some evidence that SP-induced prostaglandin production may be important in airways responses. SP has been shown to induce the production of both bronchoconstricting (eg. PGF2 α) and bronchodilating (eg. PGE2) prostaglandins under different circumstances⁸⁰. To investigate any role for SP-induced prostaglandin production, indomethacin was used to inhibit prostaglandin synthesis. No significant effect on the response to SP was seen, suggesting prostaglandin production was not important in the immediate airway response.

Studies in humans¹⁵⁰ have shown that nedocromil sodium reduces the response to inhaled NKA, which is the predominant bronchoconstricting tachykinin in human airways. The mechanism of this effect is unclear but there is some evidence that nedocromil may inhibit sensory nerve activation¹⁴⁸. There is also evidence that sodium cromoglycate may inhibit tachykinin action in the airways²⁰¹. The effect of inhaled sodium cromoglycate on SP-induced

bronchoconstriction was investigated, but no significant effect was seen.

Conclusion

In conclusion, the findings of the current study indicate that intravenous SP causes transient bronchoconstriction in sheep, that this is an NK1 receptor mediated response acting via cholinergic mechanisms, and that this response is rapidly abolished by SP degradation and the inducement of tachyphylaxis. We found no evidence for histamine or prostaglandin involvement in the acute response, nor did we find any delayed increase in R_L to implicate vascular or inflammatory cell responses. The protective effect of CP-96,345 seen in this study provides further evidence that tachykinin antagonist based agents offer therapeutic potential, although newer, more specific agents will be required for human therapy. In addition, our study highlights the need to consider the impact of tachyphylaxis on tachykinin-mediated responses, especially in studies evaluating these potential new therapies.

Chapter Five

Expression of preprotachykinin-A, neutral endopeptidase and the NK1, NK2 and NK3 tachykinin receptors in human airways.

Tachykinin containing nerves in the airways mediate protective reflexes such as cough¹⁷, and in chapter three of this thesis a role for tachykinins in the acute bronchoconstriction response to allergen was demonstrated¹⁹⁸. In addition, there is substantial supportive evidence in the literature for a role for tachykinins in the pathogenesis of airway epithelial inflammation, because of their established proinflammatory and trophic effects (reviewed in chapter one). In this regard, the increased levels of SP seen in the airways of patients with asthma^{123,124,126} or chronic bronchitis¹²⁶ are likely to have important local physiological effects including effects on the epithelium. There is some evidence for this suggestion from *in vitro* studies, which have shown that SP has a number of direct effects on airway epithelial cells including increased adherence of neutrophils¹⁰⁰, increased mucus secretion⁸⁷ and the release of neutrophil chemotactic⁹⁸, and smooth muscle relaxing factors⁸⁰. Tachykinins may reach the epithelium following their release from airway epithelial nerves by axon reflex mechanisms¹⁴³. However, although axon reflexes have been shown to occur in the skin²⁰², the existence of this mechanism has not been confirmed in human airways. An alternate possibility is that tachykinins may be released directly from the stimulated nerve terminal, but there is also evidence that tachykinins may be produced by non-neuronal cells. Reports of SP production from non-neuronal cells include production by the ciliated epithelial cells of the rabbit²⁰³, murine eosinophils¹⁰⁵, and human endothelial²⁰⁴ and neuroendocrine cells²⁰⁵. We sought evidence for the local production of SP in human airway

epithelium *in vivo*, thereby suggesting an alternate source of this peptide not reliant on axon reflex mechanisms. In order to discriminate between local SP production and that released from nerves, we initially sought evidence for the local expression of PPT-A mRNA. SP produced in nerves derives from the nerve cell bodies, principally in the jugular and nodose ganglia²⁶, which is the location of neurally derived PPT-A mRNA¹¹⁸. In contrast, if SP is produced locally, PPT-A will be detected in cells resident in the airway epithelium.

The effects of SP and the other tachykinins on airway epithelium are dependent on the presence of the appropriate receptors. The specific tachykinin receptors present in airway epithelium are incompletely characterised although physiological²⁰⁶ and molecular⁵⁹ studies are most consistent with a dominance of the NK1 receptor for the effects so far investigated. However, other receptor subtypes may be present on airway epithelium and may mediate epithelium-tachykinin interactions. Therefore, the distribution of tachykinin receptors at the epithelial level and their modulation by airway inflammation may be an important contributing factor to the pathophysiological role of the epithelium in diseases such as asthma and chronic bronchitis.

The epithelium also has an important role in the degradation of tachykinins by the enzyme neutral endopeptidase (NEP, EC 3.4.24.11.) which has been detected in airway epithelial cells¹³⁷. Changes in epithelial production of this enzyme will influence the local effect of tachykinins by causing changes in tachykinin degradation.

In addition to the investigation of human airway epithelium, it was my initial intention to investigate tachykinins, NEP and tachykinin receptors at the molecular level in sheep airways, and to compare the levels of expression in allergic responder sheep, non-responder sheep and naïve animals. Attempts to identify gene expression for tachykinins and tachykinin receptors in

sheep airways proved unsuccessful however. These analyses were performed using Northern blots and the human receptor cDNAs described in chapter two, but were likely compromised by the relatively low sensitivity of this approach. PCR and RNase protection assay were considered, but this would have required first determining the sequences for the sheep receptors, which have not been published. Further considerations included the fact that allergic responder sheep were rather scarce (only 4 of the initial 24 immunised) and sacrificing these animals would have been required. At the time a bronchoscope of sufficient length for the proper examination and biopsy of sheep airways was not available. In addition, although it has been reported that ovine airway responses can mimic human asthmatic responses by having a late phase component accompanied by inflammatory cell influx²⁰⁷, we did not find any evidence of late phase responses (at 6-10 hours post challenge) in our responder sheep, and the few epithelial biopsies we could obtain from proximal airways (left upper lobe bronchus) did not show any overt inflammatory changes. Thus, for several reasons, the investigation of the role of tachykinins at the molecular level in sheep airways was not further pursued, and the investigation of human airways became the ongoing focus.

The net effect of tachykinins in the airway will depend on the balance between several factors including tachykinin production, tachykinin receptor expression and tachykinin degradation, each of which may be modulated by airway epithelium. Despite the importance of the epithelium in airway inflammation, and the evidence for tachykinins being inflammatory mediators, there are few studies investigating the role of airway epithelium *in vivo* in the modulation of tachykinin effects or attempting to examine the balance between receptor, PPT-A and NEP expression. In addition, extrapolation of findings in some animal studies to human disease is difficult because there are important differences in tachykinin responses between species. I wished to develop a technique that could have broad application to the investigation of

components of the tachykinin axis in a range of disease states and thus did not rely on the need for autopsy or lung resection specimens. Thus, the minimally invasive approach of fibreoptic bronchoscopy coupled with airway epithelial brush biopsy was adapted to investigate gene expression for tachykinins, tachykinin receptors and NEP in freshly isolated samples of human airway epithelium. Using a molecular approach enabled both a high degree of sensitivity and complete discrimination between the receptor types (specific antibodies for the three tachykinin receptor subtypes were not available), as well as discriminating the potential for local tachykinin production from that produced by nerves.

Materials and Methods

Detailed materials and methods information can be found in the methods chapter. Where necessary, abbreviated methods are included along with results here for clarity.

Experimental protocol

We wished to examine the presence of the three important components of the tachykinin axis - tachykinins, their receptors and their principle degradative enzyme - in human airway epithelium *in vivo* using a minimally invasive procedure, thus avoiding the need to rely on resected tissues or autopsy obtained materials. To this end, we utilised the technique of harvesting samples of epithelium from subjects using a cytology brush applied via a flexible bronchoscope. We then examined these samples for evidence of expression of the molecules of interest at the level of their specific mRNAs, and where possible at the level of the proteins themselves. For this assessment, we sought volunteers among patients who were undergoing fiberoptic bronchoscopy for clinically indicated reasons (discussed in chapter two). In this phase of the study, patients were typically smokers undergoing investigation of lung opacities or minor haemoptysis, and were not taking corticosteroids. Quantitative investigation of more clearly defined disease subgroups (including normal volunteers) is presented in chapter seven.

The study protocol was approved by the Research Ethics Committee of the Royal Adelaide Hospital and informed, written consent was obtained from all participants.

Fiberoptic bronchoscopy was performed as detailed in the chapter two, with a standard cytology brush being used to obtain epithelial brush biopsy specimens. The basic approach was in accord with standard clinical practice.

Results

Characterisation of cells

Small aliquots of the epithelial cell suspension were examined directly in a cell culture dish by phase contrast microscopy to assess viability by the observation of ciliary beating and under standard light microscopy for trypan blue exclusion (Figure 5-1a, b). Rhythmic ciliary beating was seen on greater than 50% of the ciliated cells. Observation of cells after trypan blue staining revealed a close correlation between the presence of ciliary beating and trypan blue exclusion. A further aliquot of the epithelial cell suspension (50µl) was sedimented onto a microscope slide by cytosin then alcohol fixed, stained with a standard Papanicolou stain and examined under light microscopy to assess the morphological features of the cells. This technique showed that 95 - 97% of the cells were of epithelial type, and were often in sheets or clumps as described previously¹⁵⁹. In addition, a small number of specimens were assessed by transmission electron microscopy, which showed well preserved nuclear and cytoplasmic architecture in approximately 70% of cells that were in clumps or strips whereas single cells were usually non-viable (Figure 5-1c, d).

The epithelial nature of the cells obtained by brushing was further confirmed by immunohistochemical staining for cytokeratin (Figure 5-2), as detailed in the methods chapter.

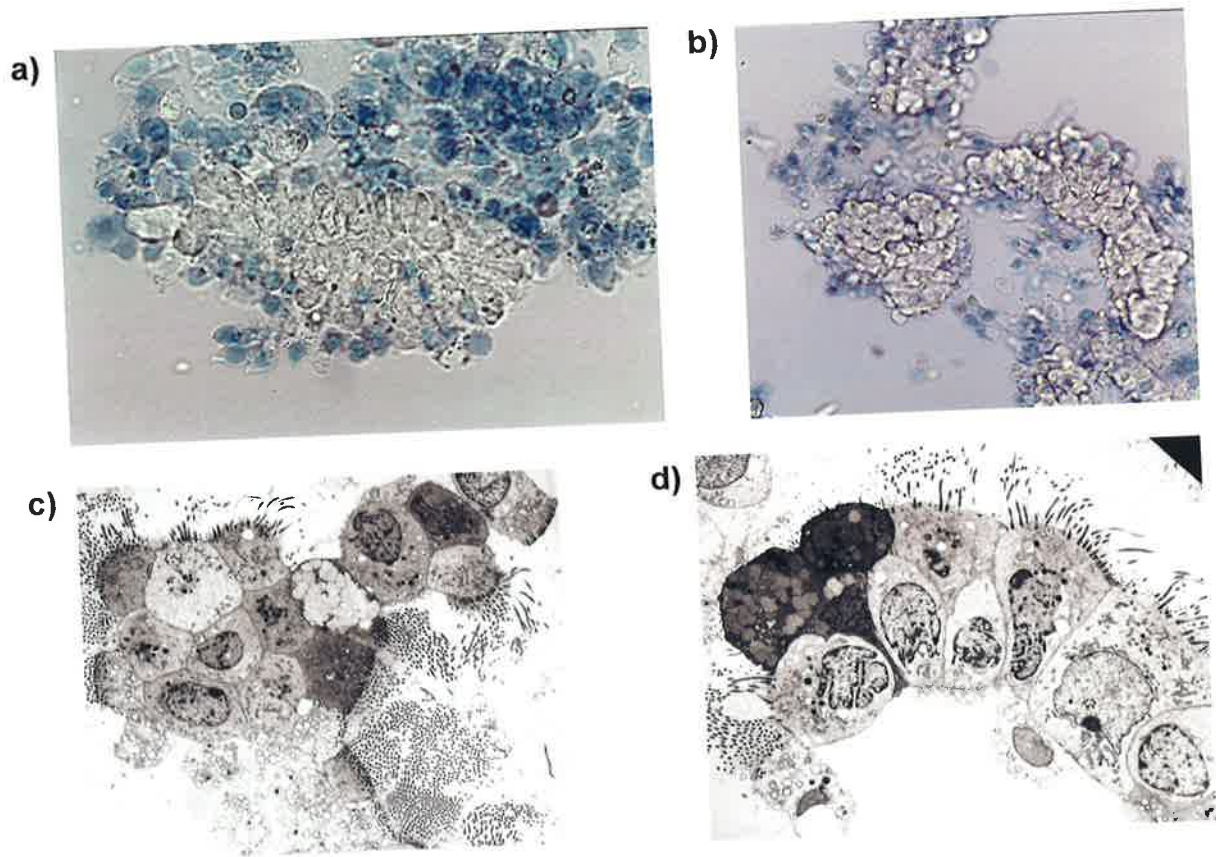


Figure 5-1. Brush biopsy of human airway epithelium. a) and b) Trypan blue exclusion, phase contrast microscopy, 100x. Viable birefringent cells can be seen along with non-viable blue stained cells. Vigorous ciliary beating could be seen on the viable cells. c) and d) Transmission electron microscopy of brushing sample. Well preserved ciliated and secretory cells can be seen, along with non-viable damaged cells (lower region panel c).

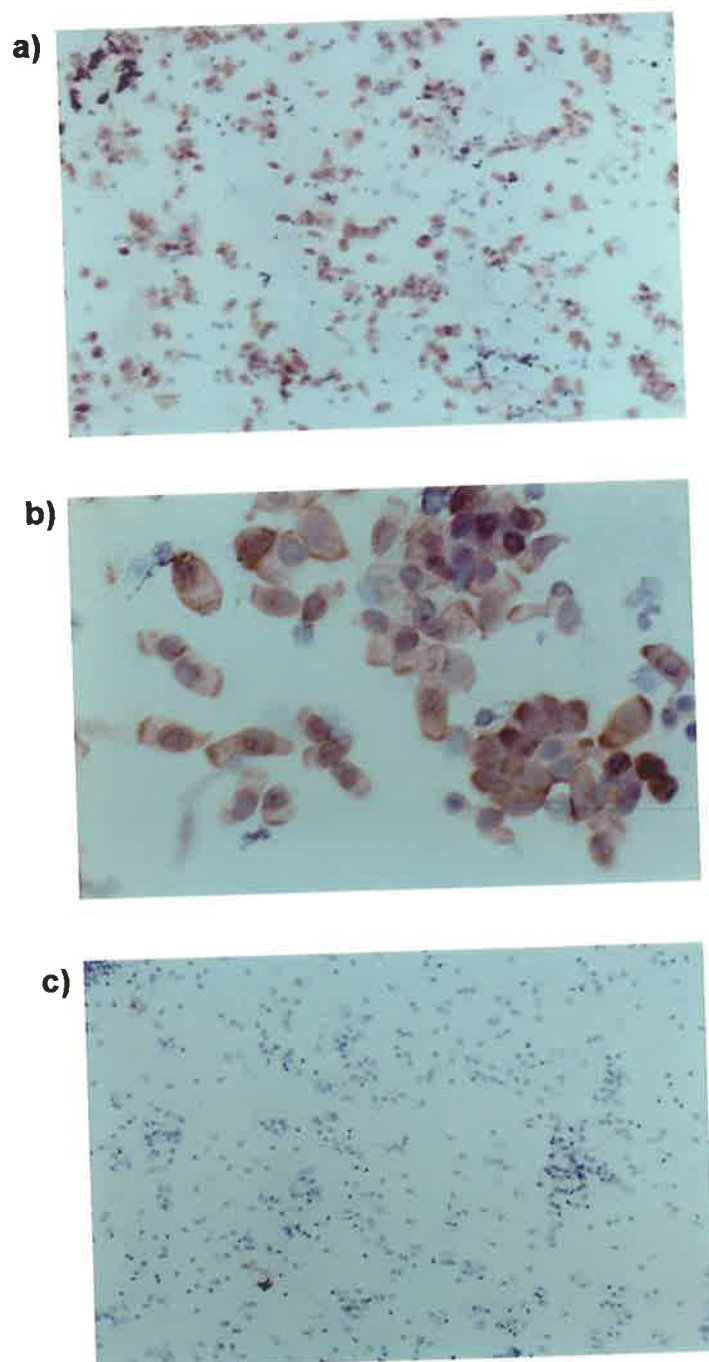


Figure 5-2. Brush biopsy of human airway epithelium. Cytokeratin staining using monoclonal Cam 5.2 anticytokeratin antibody a) 40x , b) 100x. Negative control isotype matched mouse IgG, c) 40x.

Analysis of tachykinin, tachykinin receptor and NEP gene expression in epithelial brush biopsies by reverse transcription - polymerase chain reaction (RT-PCR).

Details of the RT-PCR technique used can be found in chapter two. Briefly, total cellular RNA was extracted from the epithelial samples using a commercially available RNA extraction solution (RNAzol B). Reverse transcription and PCR were performed using a RT-PCR kit (Perkin Elmer) and oligonucleotide primers designed to be specific for the genes of interest. PCR products were analysed by gel electrophoresis in 1% agarose, then transferred to nylon membrane by Southern blotting. Blots were hybridised to a specific ^{32}P -labeled oligonucleotide probe followed by detection with autoradiography or phosphorimager scanning²⁰⁸. Where indicated, negative controls consisting of "sham" RNA extractions (ie using all reagents but no tissue sample) were used and amplified alongside the patient samples.

Primer and probe sequences are given in chapter two, Table 1. Because of the high degree of homology between the three receptor subtypes all primer pairs were not only checked for specificity by computer analysis but also by testing amplification specificity against a panel of tachykinin receptor cDNAs (gift from Dr A. Graham, Biotechnology Dept., ICI Pharmaceuticals, Cheshire, UK) (Figure 5-3). In the experiments shown, PCR analysis was performed using cDNA reverse-transcribed from five epithelial specimens (lanes 1-5), alongside plasmid DNA containing the human NK1, NK2 or NK3 tachykinin receptor sequences. Each possible mismatched combination of probe and primer pair was tested - ie NK1 amplified products were probed using the NK2 and NK3 probes, NK2 products with NK1 and NK3 probes and NK3 products with NK1 and NK2 probes. In each gel a relevant positive control was also included (ie NK1 amplified products run on gels that were to be probed with the NK1 probe, etc.). No cross-reactivity between any specific primer pair and the other receptor cDNAs was seen, nor were there any non-

specific signals seen for the epithelial samples. All matched primer/probe combinations gave the expected signal when used against the positive control matched cDNA plasmid sequence.

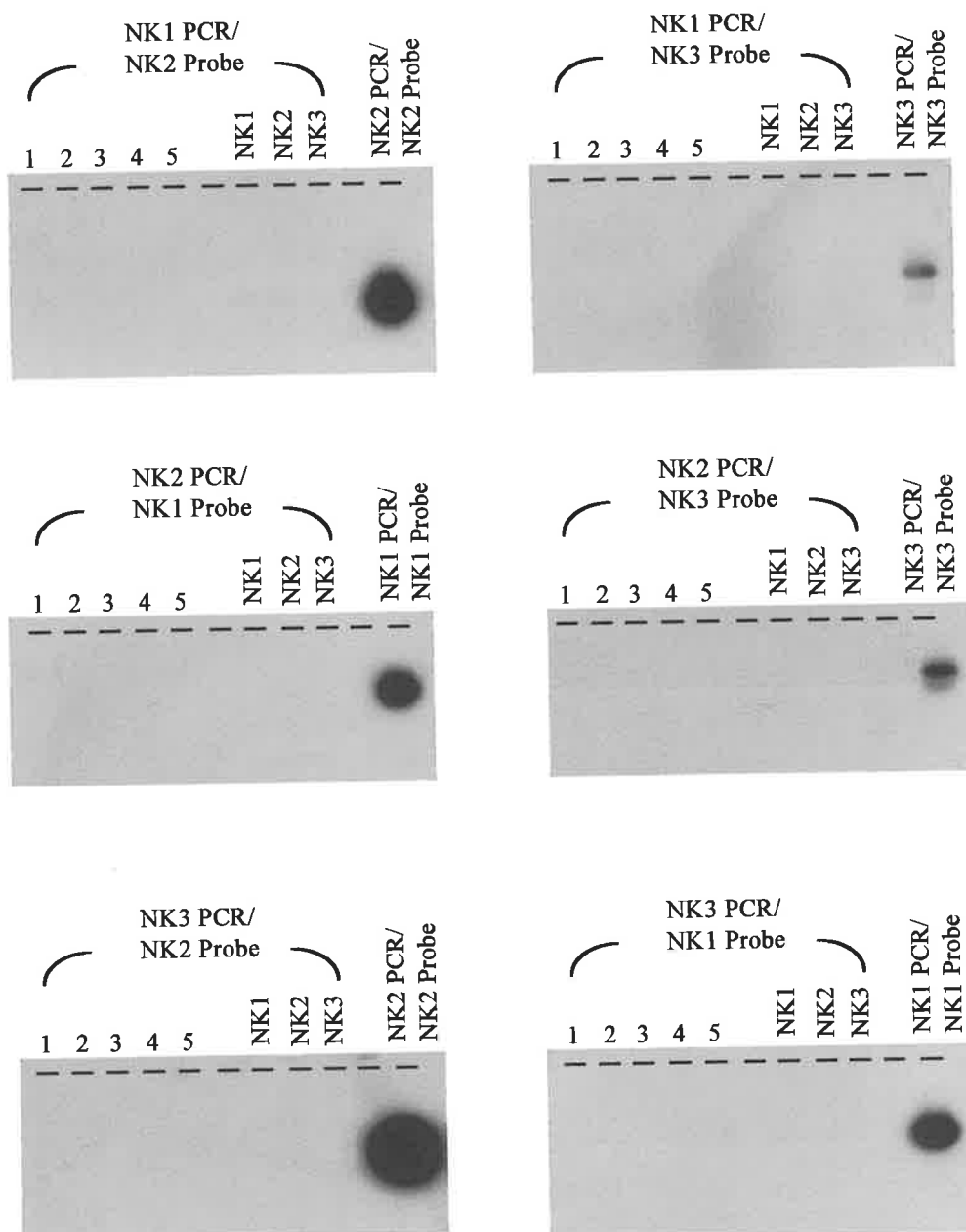


Figure 5-3. Confirmation of specificity of tachykinin receptor primers and probes. The tachykinin receptor specific PCR primers were each used to amplify cDNA obtained from 5 airway epithelial specimens (lanes 1-5) as well as plasmids containing the receptor sequences. Amplified products were then analysed by Southern blot, using mismatched probes as indicated, to check for cross-reactivity. The last lane on each autoradiograph shows the appropriate positive control.

For the initial analysis of gene expression for tachykinin receptors, NEP and PPT-A, bronchial epithelial cells were obtained from eight patients. In all of the subjects, epithelial mRNA was detected for NK1 and NK2 receptors (Figure 5-4, a and b). In four of the specimens mRNA for the NK3 receptor was detected (Figure 5-4c). No positive signal was detected in any of the negative controls. Amplification using the NK2 primer set resulted in intron amplification of contaminating genomic DNA (Figure 5-4b) as well as amplification of reverse transcribed mRNA. This was apparent from the size of the amplified bands since the difference in size between the larger product and the expected 513 base pair product corresponded to the size of the intervening intron between the amplification primer sequences (intron 1, approximately 800 base pairs). The identity of the larger band as a product of genomic DNA amplification was confirmed by performing PCR without reverse transcription, which resulted in amplification of the larger product only (Figure 5-5). Thus mRNA for all three tachykinin receptor subtypes was detected at the airway epithelial level.

In all subjects, mRNA was detected for NEP, consistent with previous reports of airway epithelial production of this enzyme (Figure 5-6a).

Importantly, mRNA for PPT-A was detected in all subjects (Figure 5-6b). This latter finding is of particular interest because it potentially indicates the local production of tachykinins at the human airway epithelial level and therefore suggests an alternate epithelial source for tachykinins in addition to c-fibre nerves (and thus also an alternative to the axon reflex hypothesis).

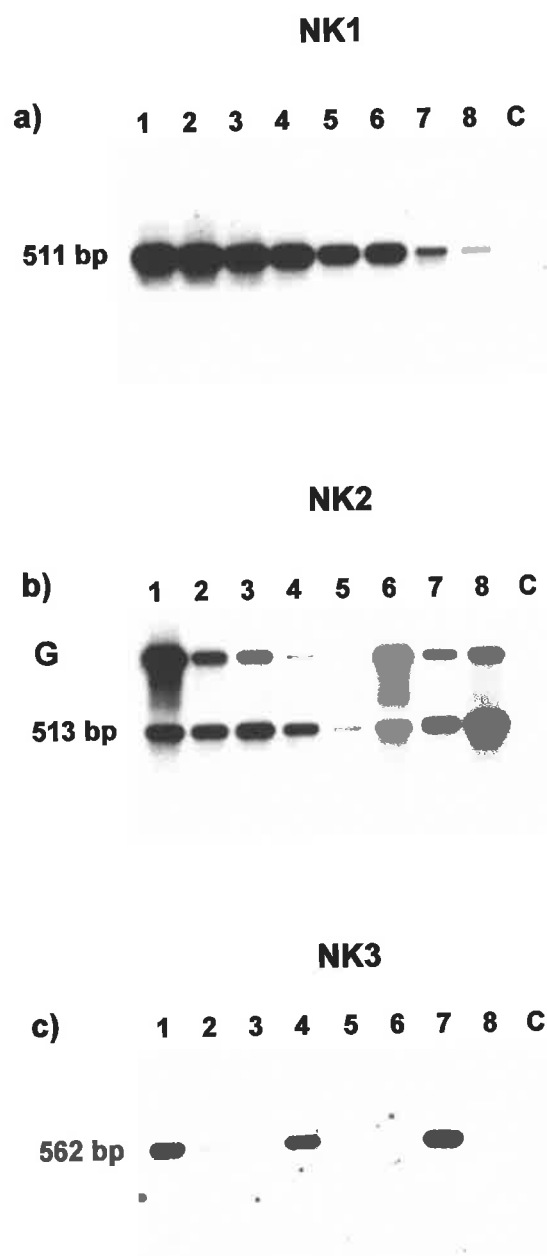


Figure 5-4. Human airway epithelial brushing samples. RT-PCR analysis for tachykinin receptor gene expression. 1-8 = patient samples, C = negative control. G = genomic DNA amplification.

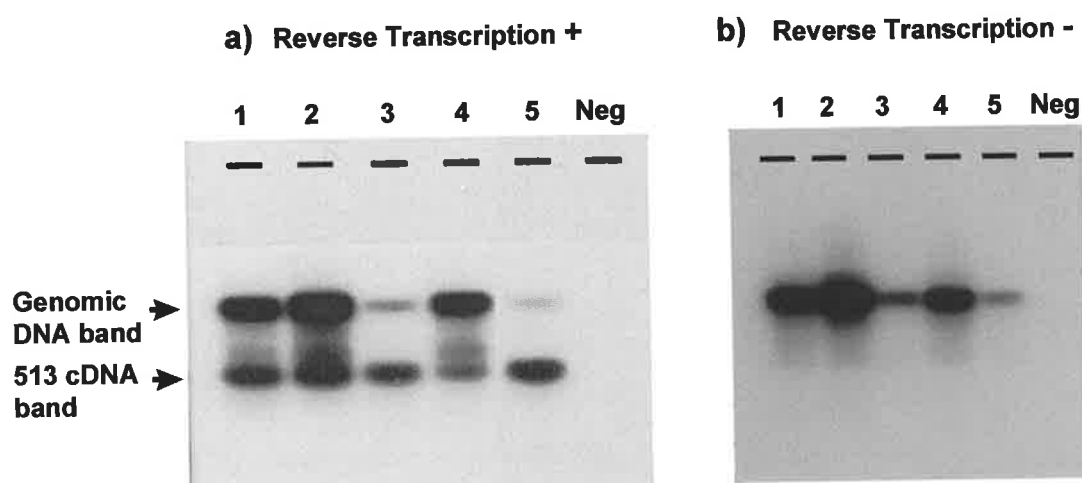


Figure 5-5. NK2 analysis by Southern blot of PCR products amplified from epithelial brush biopsy RNA samples (lanes 1-5). a) RT-PCR analysis. b) Amplification without reverse transcription confirms the upper band is due to amplification of contaminating genomic DNA, which gives a larger band due to amplification of intron sequence.

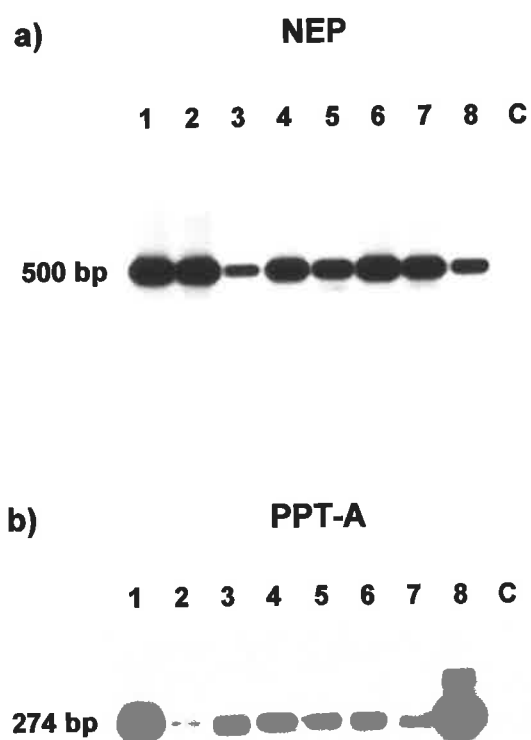


Figure 5-6. Human airway brushing samples. Analysis by RT-PCR for NEP and PPT-A. 1 - 8 = patient samples, C = negative control.

Analysis of peripheral blood

Although care was taken to avoid blood contamination of the epithelial specimens, to check for the effect of minor blood contamination on the gene expression results, whole blood and leukocytes from three normal volunteers were collected and analysed for gene expression along with the epithelial specimens. Processing of blood samples is detailed in the methods chapter. No PCR products were obtained from the whole blood specimens and the peripheral white blood cells showed a positive signal only for NEP as expected and not for any of the other mRNA species (Figure 5-7).

Primary cell culture

Following the demonstration that gene expression for the three major components of the tachykinin axis occurs at the airway epithelial level *in vivo*, we attempted to further confirm that epithelial cells themselves were capable of such expression. Although >95% of cells obtained in the samples were epithelial (based on morphology and cytokeratin staining) we did consider the possibility that the PCR findings might in part be influenced by a degree of minor contamination by other cells. We had already analysed blood and not been able to detect PPT-A or tachykinin receptor expression with this technique, but to further address this issue we established primary cultures of airway epithelium from cells obtained by brush biopsy. These cultures were established from fresh brushing samples using a modification of the technique of Lechner and La Veck¹⁶² (see chapter 2). After 2 - 3 weeks cells had become 80% confluent, and had the typical 'cobble-stone' appearance typical of cultured airway epithelial cells (Figure 5-8a). The epithelial nature of the cells was confirmed by immunohistochemistry for cytokeratin expression (using CAM 5.2 or mouse IgG control, followed by nuclear counterstaining). Cytokeratin

immunoreactivity was detected in all cells (Figure 5-8b). Cellular RNA was analysed for PPT-A, tachykinin receptor and NEP expression as before, alongside actin expression which acted as a control (Figure 5-9). We were able to detect NK1, NK2 (2 of three samples) PPT-A and NEP expression but not NK3 expression. Thus, airway epithelial cells do express the relevant genes for a broad role in the modulation of tachykinin effects.

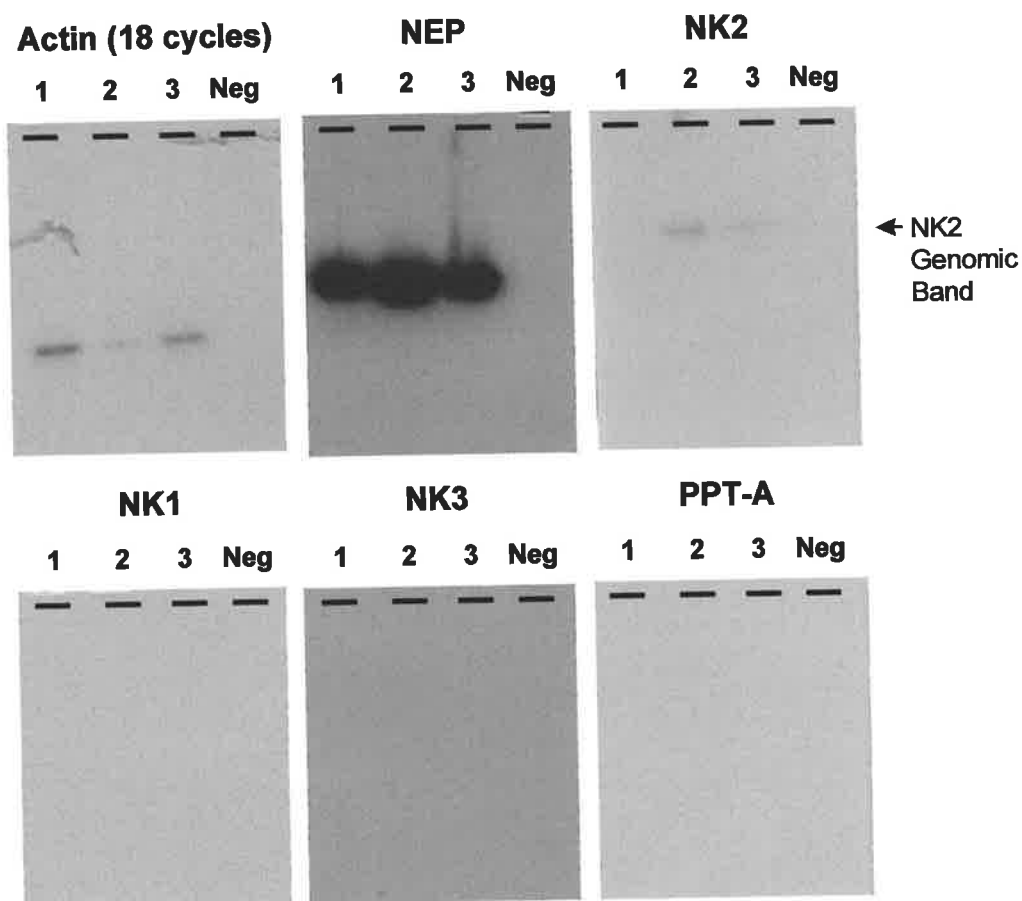


Figure 5-7. RT-PCR analysis of RNA extracted from the peripheral leukocytes of three subjects. Actin amplified 18 cycles, other genes for 35 cycles. Autoradiographs show positive signal only for actin, NEP and the genomic band seen with NK2 analysis.

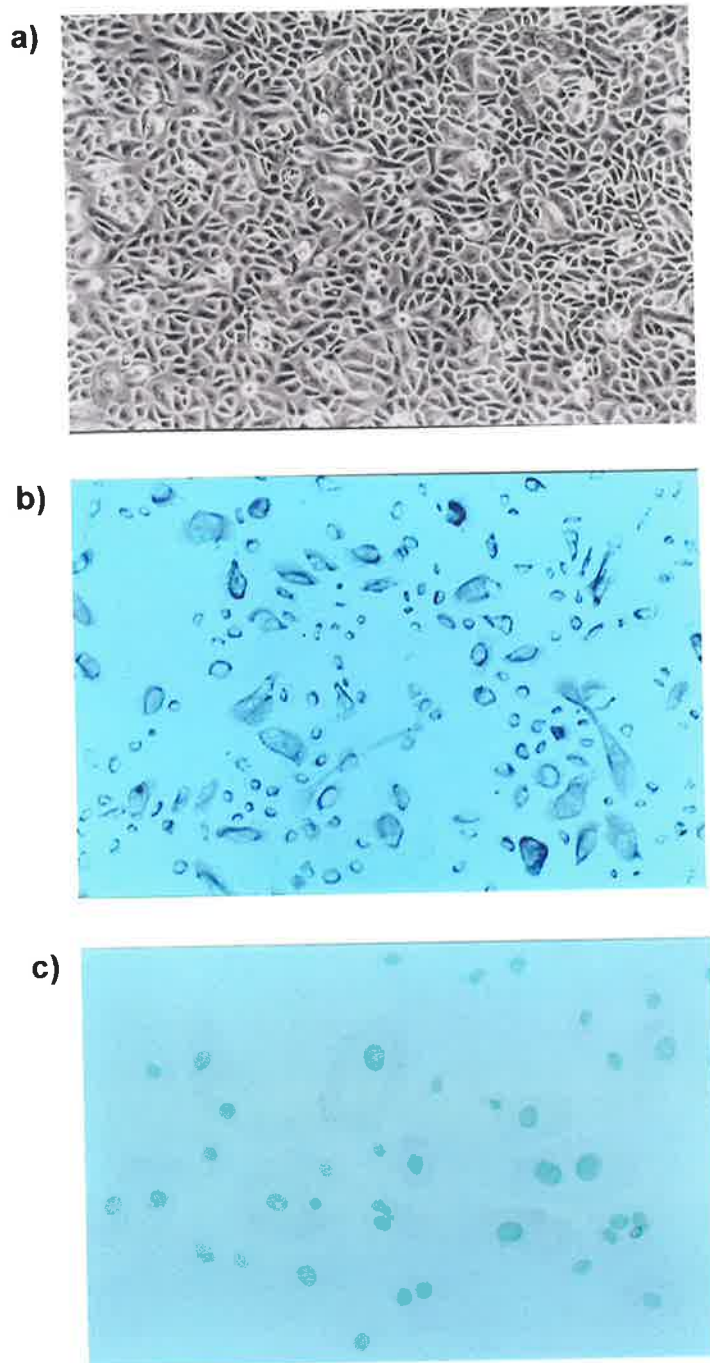


Figure 5-8. Primary culture of human airway epithelium. a) Phase contrast microscopy. b) Cells were trypsinised and cytopspin preparations were stained for cytokeratin using monoclonal anti-cytokeratin antibody, followed by nuclear counterstaining, 100x. All cells present were positive for cytokeratin. c) Negative control mouse IgG, 100x.

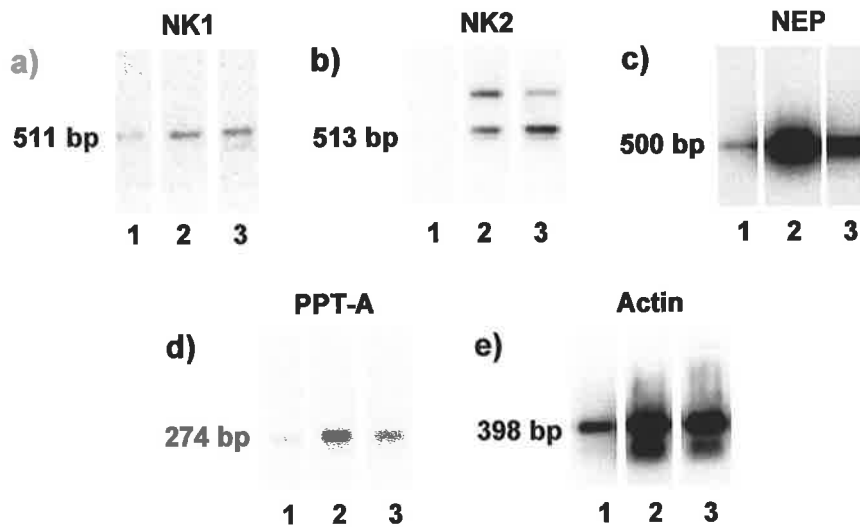


Figure 5-9. RT-PCR analysis of RNA obtained from primary cultures of human airway epithelium. Positive signals for NK1, NK2, NEP, PPT-A and Actin are shown.

NK1, NEP and SP protein detection

The use of highly sensitive RT-PCR allowed us to detect gene expression for all major components of the tachykinin axis in the small samples obtained by epithelial brush biopsy. We wished as far as possible to correlate these findings with detection of the relevant protein. For this evaluation we used commercially available antibodies against SP and NEP and an antiserum raised against the c-terminal of the rat NK1 receptor (amino acids 376-407) generated by Dr J. Krause, which has been characterised elsewhere ¹⁸². Cross-reactivity of this antibody for the NK1 receptor was predicted based on the high degree of homology between the rat and human NK1 sequences and had been confirmed by Dr Krause in the human nervous system (personal communication).

NK1 receptor

Cytospin preparations of fresh airway epithelial brushing samples were prepared then immediately fixed in 4% (w/v) paraformaldehyde in PBS for 15 min at 4°C. Primary anti-NK1 receptor antisera was applied at 1/200 dilution overnight at 4 °C. Positive signal was detected using a FITC-conjugated anti-rabbit secondary antibody followed by visualisation with immunofluorescent microscopy. In the fresh epithelial samples, fluorescent signal was detected at the apical border of ciliated cells, in the region of tight junctions. (Figure 5-10 c-f). These findings are morphologically similar to those described for rat airways ⁶⁰. Concurrent staining with the two negative controls (normal rabbit serum (Figure 5-10 g,h) and without addition of primary antibody) showed no such staining.

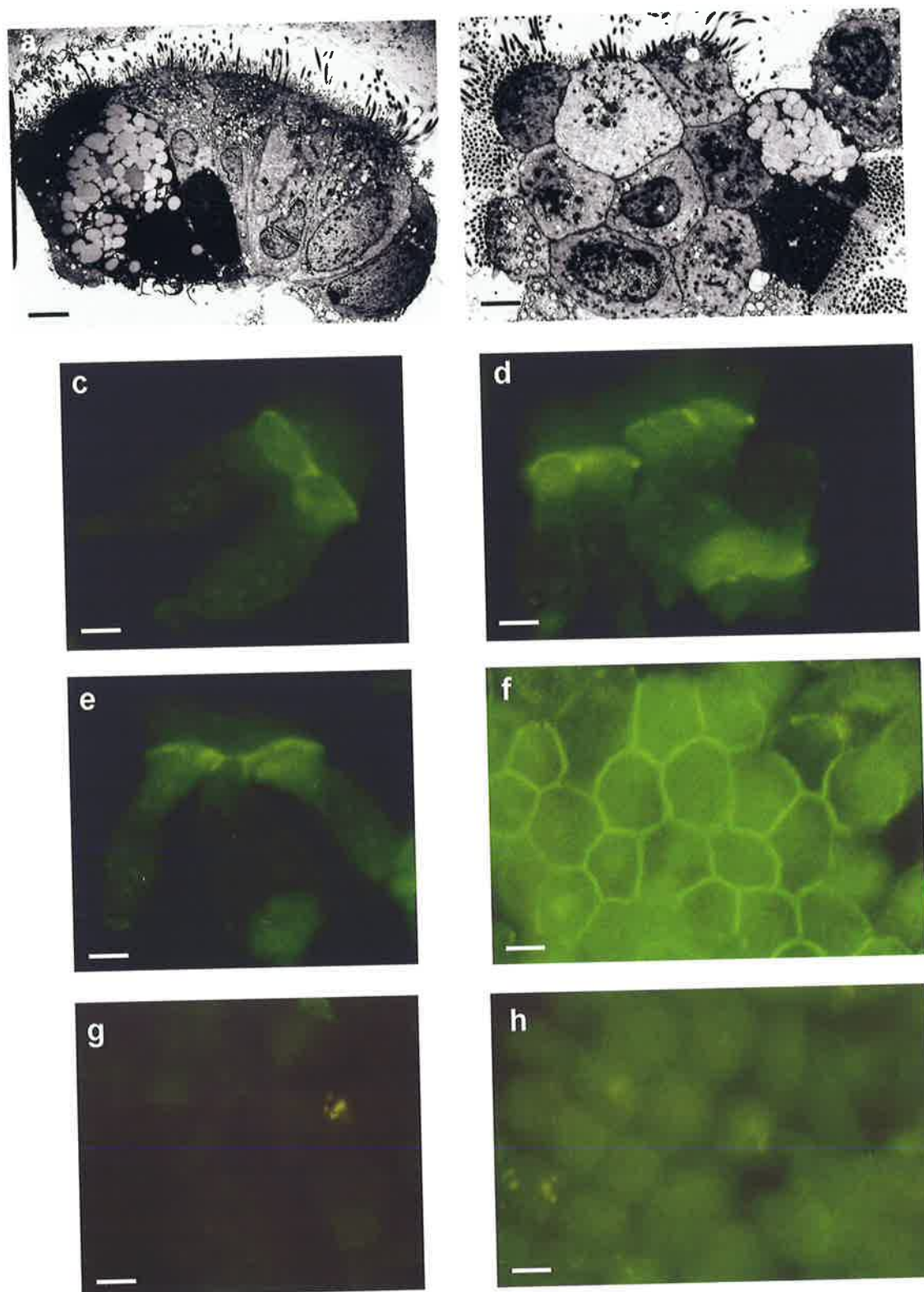


Figure 5-10. Human airway epithelial samples analysed by transmission electron microscopy a) longitudinal section, b) horizontal section. Immunofluorescence for NK1 receptor performed on cytospin preparations seen in longitudinal aspect c), d), e) and en face aspect f). Control serum g) and h). Bar = 4 μ m.

Neutral Endopeptidase

Neutral endopeptidase was detected with a mouse J5 monoclonal antibody (Coulter Immunology, Hialeah, FL USA.) directed against the human Common Acute Lymphoblastic Leukemia Antigen (CALLA) CD 10, which is identical to NEP. Cells were cytospun as above then fixed 4% paraformaldehyde. Primary antibody was applied overnight at room temperature. Signal was detected using a secondary biotinylated rabbit anti mouse antibody, followed by streptavidin - HRP and DAB. Specificity of the staining methods was determined by omitting primary antisera or replacement with comparable isotype sera. Positive brown staining was detected in all samples (Figure 5-11).

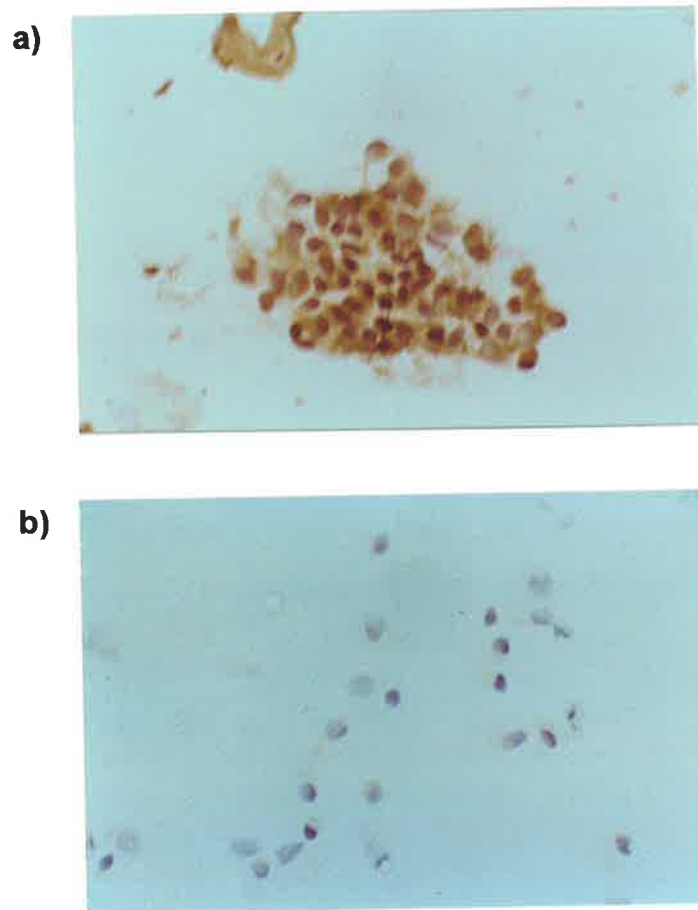


Figure 5-11. Brush biopsy of human airway epithelium. a) Staining for NEP using monoclonal anti-NEP antibody, 40x. b) Negative control mouse IgG, 40x.

Substance P

Rabbit anti SP antibody, directed against the C-terminus of SP was purchased from AUSPEP (Melbourne, Australia). We attempted immunohistochemical detection of SP using this antibody but were unable to detect specific staining in the fresh epithelial cell samples. These results may have been compromised due to technical difficulties with high background, although staining in a positive control (sheep jejunum, data not shown), could be seen. In view of this we used a highly sensitive ELISA to examine extracts from the brushing samples. This ELISA also used the Auspep polyclonal anti-SP antibody.

Cells were collected from twelve subjects in an identical manner to that previously described except that immediately after collection the cell suspensions were transferred to tubes containing 10^{-5} M phosphoramidon (to inhibit degradation of SP by NEP). Samples were thoroughly washed prior to analysis for SP. Because of the small number of epithelial cells obtained from each subject, samples were pooled so that there were at least 10^6 cells available for analysis per ELISA per sample. This resulted in six cell pools as follows : A (2 patients), B (2 patients), C (3 patients), D (2 patients), E (2 patients), F (1 patient). Cells were pelleted by centrifugation, washed in PBS, then boiled in 10 volumes of 1M acetic acid / 10mM b mercaptoethanol for 10 min. Then SP was extracted by reversed phase chromatography using Sep-Pak C18 cartridges (Millipore Australia Pty. Ltd., Kent Town, SA, Aust.). This assay and the SP extraction technique have been described in full in the methods chapter and elsewhere¹⁸³. The lower limit of detection using this ELISA is 5 femtomoles per well. A mean ($\pm$ SEM) SP concentration of 0.16 ± 0.04 pmoles per 10^6 cells was detected (n=6 pooled samples).

Bronchoalveolar lavage cells

To further characterise tachykinin production in the airways we also evaluated cells obtained by bronchoalveolar lavage. SP has been detected in rat alveolar macrophages ¹⁰⁶, thus we were particularly interested to determine whether human alveolar macrophages might be a significant source of SP.

Alveolar macrophages are the predominant cell obtained by bronchoalveolar lavage (BAL) except in certain diffuse interstitial lung diseases or sarcoidosis. Thus, for this analysis, BAL was performed, initially on 10 subjects. In each case, lavage was performed in an area of the lung with no gross radiological abnormality. Subjects were typically smokers with either an undiagnosed cough or a radiological opacity for investigation and patients with diffuse interstitial lung disease or sarcoidosis were not included. Lavage was performed by wedging the tip of the bronchoscope into a subsegmental bronchus, then sequentially instilling and aspirating three aliquots of normal saline, 50 mLs per aliquot. To reduce the possibility of significant contamination by airway epithelial cells, only the third aspirate was analysed. This aspirate was transferred into a 50mL tube containing phosphoramidon (final conc. 10^{-5} M). A 5mL aliquot of the sample was forwarded to the cytology laboratory of the IMVS, where cells were stained using Papanicolou stain and cell profile was assessed under light microscopy by a cytologist experienced in assessing such samples. The remainder of the sample was centrifuged at 400g for five minutes, then the cell pellet washed with PBS. Extraction and analysis of SP content was performed as for the epithelial brush biopsy samples.

Mean ($\pm$ SEM) cell profile of the lavage specimens (n=10) was as follows - macrophages 86.7 ± 2.4 %, lymphocytes 9.6 ± 2.7 %, neutrophils 3.3 ± 1 % and eosinophils 0.4 ± 0.2 %.

Mean ($\pm$ SEM) SP content in cells obtained by lavage was $0.0152 \pm .0042$ pmoles per 10^6 cells, which was 10 fold less than the concentration seen in the epithelial brush biopsy samples ($p = 0.013$, epithelium vs BAL cells) (Figure 5-12c)

To gain a more complete assessment of the potential for SP production by human alveolar macrophages, we also extracted RNA from the cell pellets and used RT-PCR to detect PPT-A gene expression. RNA was readily obtainable from the lavage cells with total yields of up to 10 fold (up to 80 mg total) greater than we obtained from epithelium, and of at least equivalent purity as determined by UV absorbance. In the initial analysis of 10 samples we were only able to detect faint signal for PPT-A in 2 patients (Figure 5-12a), whereas signal for GAPDH (as a control) was strongly detected in all samples (not shown). We subsequently investigated a further 5 patients and found very weak signal in only one of these (Figure 5-12b), although signal for NEP (Figure 5-12c) was detected in all samples. For each analysis, a positive controls of epithelial RNA were analysed for PPT-A expression in parallel. In summary, faint signal for PPT-A was detected in only 3 of 15 BAL samples.

Thus, on the basis of both protein and gene expression analysis it appears that airway epithelium is a richer source of SP in the airways than are cells obtained by BAL and that little, if any, SP is produced by human alveolar macrophages, at least in areas of the lung which are radiologically normal.

Although it appeared that macrophages did not produce much SP, they may play an important role in the modulation of tachykinin effects by the production of NEP. Production of NEP in macrophages has previously been described, and we were readily able to detect gene expression for NEP in bronchial lavage cell samples (Figure 5-12c). Gene expression for tachykinin receptors was detected in some BAL samples, but this was much less consistent than

for the epithelium (Figure 5-13). Tachykinin receptor expression has been described in the alveolar macrophages of guinea pigs, where SP was shown to stimulate gelatinase production via both NK1 and NK2 activation¹⁰¹. Kaltreider et al, using a murine model of parenchymal lung inflammation, showed that tachykinin receptor expression on alveolar macrophages was upregulated in response to in the inflammatory response⁶⁴. It is possible that human alveolar macrophages may also be stimulated by tachykinins.

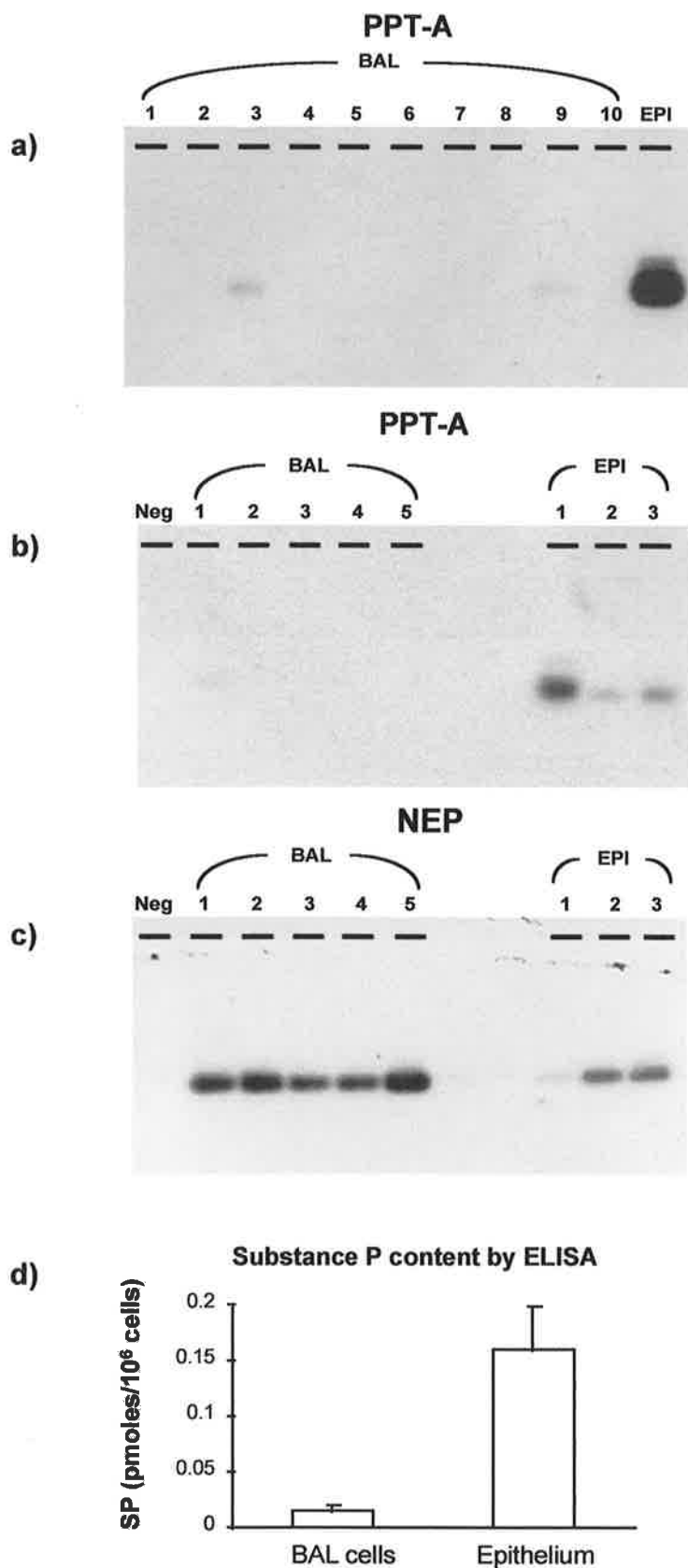


Figure 5-12. Analysis of cells obtained by BAL. a) and b) RT-PCR analysis for PPT-A RNA. c) Analysis for NEP mRNA. EPI = airway epithelial cell RNA positive control. d) SP content measured by ELISA in extracts from BAL cells vs airway epithelial samples (mean $\pm$ SEM).

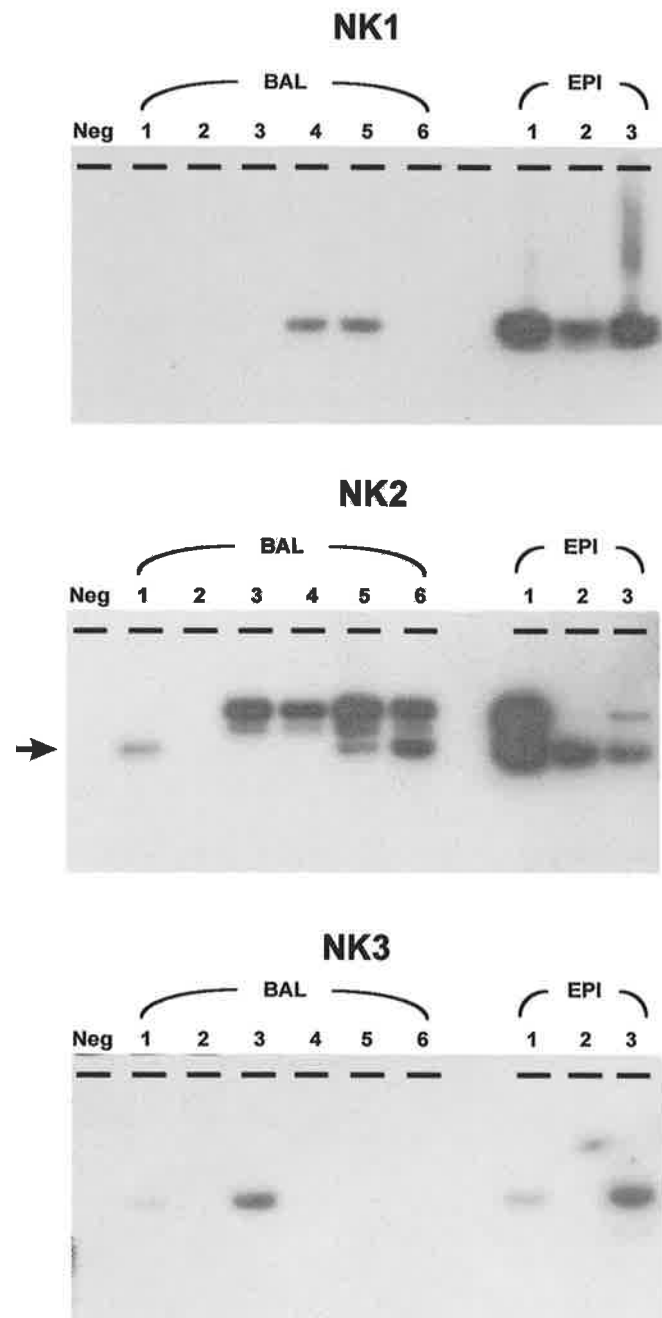


Figure 5-13. Analysis of cells obtained by BAL. BAL cells were analysed by RT-PCR for the NK1, NK2 and NK3 tachykinin receptors. EPI = epithelial brush biopsy positive controls.

Arrow = NK2 cDNA band.

Airway smooth muscle culture

In addition to the analysis of epithelium and alveolar macrophages, we had a limited opportunity to analyse primary cultures of airway smooth muscle. This material was kindly provided by Dr J. Black and Dr P. Johnson, Department of Pharmacology, University of Sydney. The culture technique and characterisation of cells has previously been reported ²⁰⁹. In brief, cells were obtained from resected human lungs by microdissection, then propagated and characterised and supplied to us as growing cultures.

Physiological studies have shown that contraction of human smooth muscle in response to tachykinins is predominantly an NK2 mediated response, and radioligand studies have shown moderate binding of labelled NK2 agonists to guinea pig airway smooth muscle ²¹⁰ but this has not been reported for human cells. Thus initially, NK2 receptor gene expression was investigated in these human primary cultures. Two cultures were analysed by RT-PCR alongside two airway epithelial brush biopsy samples. Expression of NEP by smooth muscle has previously been described, and was readily detected in the cultures (Figure 5-14a). NK1 and NK2 receptor gene expression was also detected (Figure 5-14b,c). Two additional cultures were analysed for NK1 and NEP as well as NK3 receptor and PPT-A gene expression, this time alongside an immortalised airway epithelial cell line, 1HAEo- (Figure 5-15). This analysis confirmed NEP and NK1 expression and interestingly showed gene expression for PPT-A. This latter finding was unexpected, but was clearly not due to contamination from previously amplified samples because analysis with all reagents but omitting the reverse transcription step did not give any signal. Analysis of the initial two cultures also showed strong signal for PPT-A. As a further control, an irrelevant human cell line, the hepatoma line HepG2, was also analysed for PPT-A gene

expression and was negative. The observations in smooth muscle provides further evidence that the production of SP in human airways is not confined to nerves

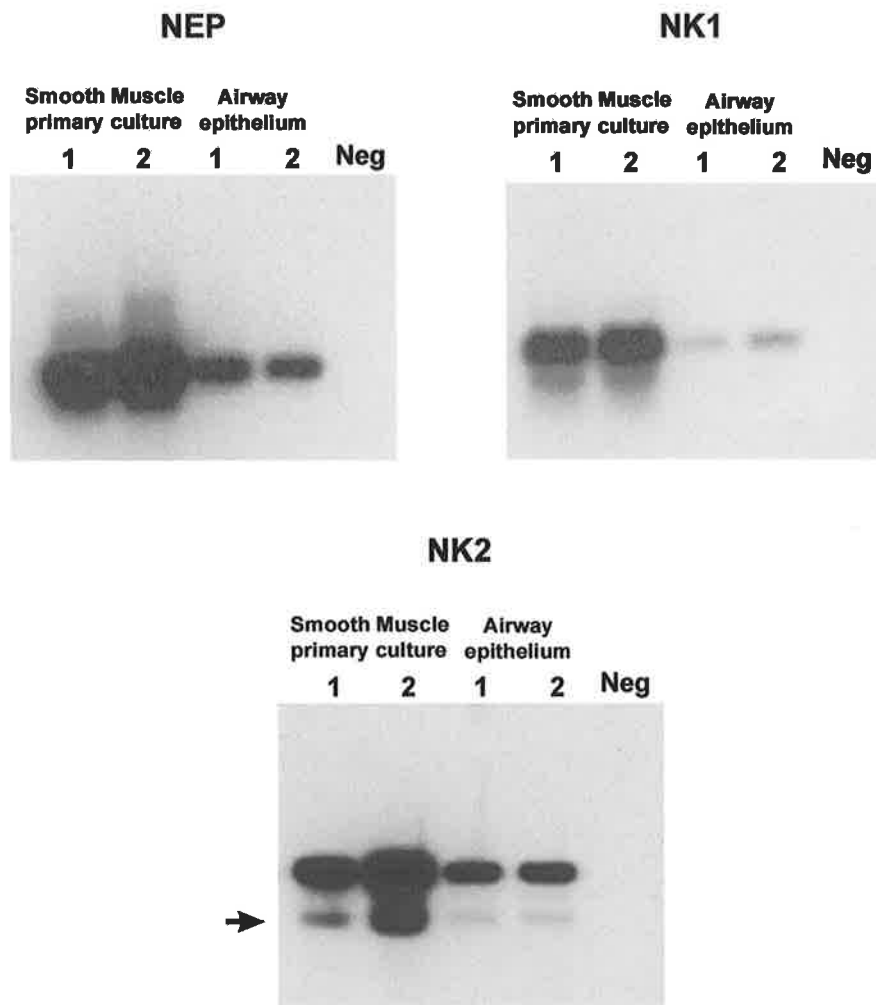


Figure 5-14. Analysis of RNA from two primary smooth muscle cultures alongside two airway epithelial brush samples. Expression is shown for NEP, NK1 receptor and NK2 receptor (arrow = cDNA band). Neg = negative control

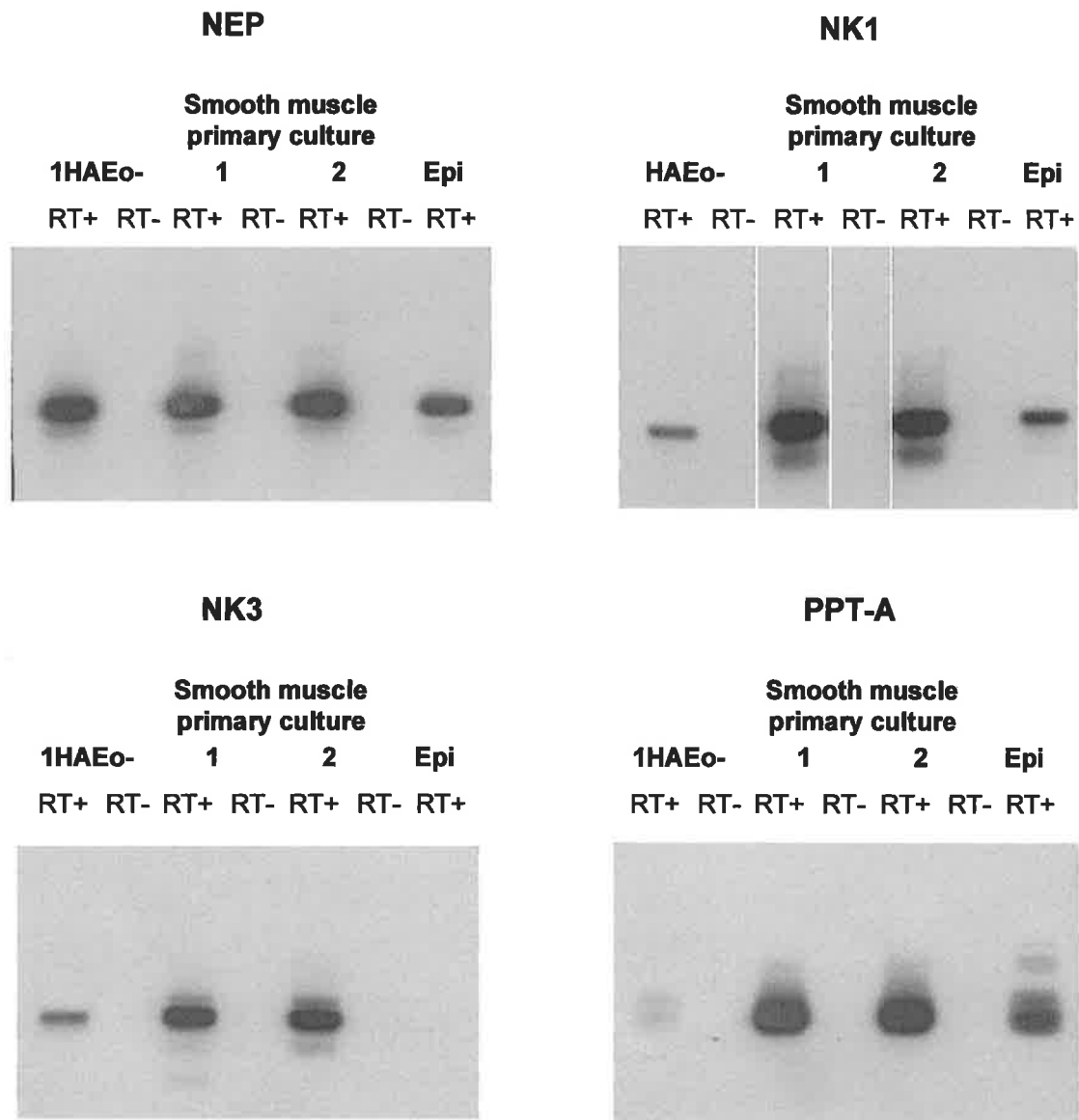


Figure 5-15. Further analysis of RNA from human airway primary smooth muscle cultures. Analysis for NEP, NK1 receptor, NK3 receptor and PPT-A is shown alongside analysis of an immortalised airway epithelial cell line (1HAEo-) and an epithelial brush biopsy sample. RT+ = with reverse transcription, RT- = negative control (RNA sample analysed without reverse transcription).

Discussion

Understanding changes that occur in the airway epithelium is critical to investigations of inflammatory airways diseases. In this study we have utilised the minimally invasive technique of airway epithelial brushing to investigate the tachykinin axis in human airway epithelium *in vivo*. We have used qualitative analyses to demonstrate gene expression for the three principle tachykinin receptors, NEP and PPT-A, and used immunohistochemistry and ELISA techniques to corroborate the presence of the NK1 receptor and PPT-A respectively.

The technique of airway epithelial brushing has been reported in several previous studies, but not previously for the investigation of tachykinins. Consistent with previous reports^{158,159}, we found that sufficient quantities of viable epithelial cells were readily obtainable and the procedure was well tolerated. This approach has clear advantages over reliance on organ donor material or resected specimens and samples are both more specific to the epithelium and obtainable over a wider area than can be achieved with endobronchial biopsy.

It has been postulated that tachykinins are released by axon reflex mechanisms, whereby impulses from endings in the epithelium are transmitted both centrally and down arborised axon branches in an antidromic fashion, leading to release of neuropeptides in the region of adjacent structures¹⁴³. This mechanism has been shown to release tachykinins in the skin, but no anatomical correlate to support this hypothesis has been detected in the airway. More recently however, changes in neuroendocrine elements in the epithelium in response to airway inflammation have been noted²¹¹, and such changes may have a local role. Killingsworth et al¹¹⁸ used a rat model of chronic bronchitis to investigate the role of C-fiber nerves in this disease. Interestingly they showed an increase in airway SP levels in response to sulfur dioxide but did not see a corresponding increase in PPT-A mRNA in the dorsal root ganglia. While post-

translational mechanisms or changes in NEP might be a factor, local airway production of SP should also be considered. Our findings demonstrating PPT-A mRNA in airway epithelium clearly suggest SP is produced locally in addition to being transported to the epithelium by axons from distant nerve cell bodies. Importantly, the detection of PPT-A mRNA in the primary cultures and an immortalised airway epithelial cell line is compelling evidence that the airway epithelial cells themselves can contribute to local SP levels.

The investigation of PPT-A gene expression was extended to include cells obtained by bronchoalveolar lavage and, to a limited extent, to airway smooth muscle. PPT-A gene expression has been described in alveolar macrophages in the rat ¹⁰⁶, thus, we were particularly interested to investigate whether human alveolar macrophages also expressed PPT-A. Cell samples obtained by bronchoalveolar lavage, were confirmed to consist mostly of macrophages. Despite investigating fifteen subjects in this way, we were able to detect very minimal signal for PPT-A in only three cases. This contrasted to our experience with airway epithelium, where PPT-A was detected in all cases. The presence of SP was further investigated using a sensitive ELISA. In an effort to avoid confounding our findings by the detection of SP that may have been released from nerves, epithelial and lavage samples were washed thoroughly. It was noteworthy that the level of SP per 10^6 cells in the BAL cells was 10 fold less than that seen in the epithelial samples. Thus it appears that as far as local production is concerned, the epithelium is a richer source of SP than are alveolar macrophages. It remains to be investigated whether SP production by macrophages can be upregulated in disease states.

We had limited opportunity to evaluate primary cultures of airway smooth muscle, but interestingly we were able to readily detect PPT-A gene expression in these cells. These findings would suggest there may be an even wider potential for local non-neuronal SP production in the lung.

For tachykinins to have any local effect, their specific receptors must also be present. There have been several previous reports concerning tachykinin receptors in the airways. Early work, using radiolabelled SP, detected binding in airway epithelium, submucosal glands, vascular endothelium and airway smooth muscle⁵⁸. The use of radioligand studies was initially compromised by the degree of cross-reactivity of the ligands for the three tachykinin receptor subtypes, but improvements in specific synthetic ligands in recent years has allowed better discrimination^{29,210}. Nevertheless, these studies still require the use of resected specimens. Molecular analyses using RNase protection assays, also using resected specimens, detected NK1 and NK2 mRNA in lung, but not NK3 mRNA, and in situ mRNA hybridization studies detected only NK1 mRNA⁵⁹. Our study differs by selectively sampling airway epithelium then using a more sensitive molecular assay, RT-PCR, which may explain our ability to detect all three receptor subtypes. In addition, we have used anti-NK1 receptor antisera and been able to demonstrate NK1-like immunoreactivity at the apical margin of airway epithelial cells, which is the first report of this approach in human airway tissues. We extended our analysis of tachykinin receptor gene expression to the smooth muscle cultures, and here we also found mRNA for all three receptor subtypes. In our analysis of bronchoalveolar lavage cells, tachykinin receptor expression was detected in some subjects, although with less consistency than for epithelial samples. It is possible that subtle differences in underlying disease state may have accounted for these differences, as upregulation of tachykinin receptors in cells obtained by BAL has been reported in a murine model of parenchymal inflammation⁶⁴.

The third component of the tachykinin axis we investigated was the enzyme principally responsible for degradation in the airways, NEP. We found NEP mRNA in all tissue samples we assessed, including the epithelium, primary epithelial cultures, bronchoalveolar lavage cells, smooth muscle cultures and peripheral leukocytes. We also detected NEP-like immunoreactivity

in airway epithelium samples. Several earlier reports of NEP immunohistochemistry analysis of airway epithelium appeared to show localisation of staining to the basal cells¹³⁷. More recently however, NEP has been detected in both the apical and basal regions of columnar cells¹⁴⁰. Our findings are more in keeping with this latter report. Aside from the question of localisation of staining within the epithelium however, our findings are all in accord with previous reports and the wide distribution of this enzyme is consistent with the hypothesis that NEP is a key factor in the control of responses to SP. These findings are thus also in accord with our studies using the ovine model, where inhibition of NEP with phosphoramidon led to significantly increased airway responses to both exogenous SP and allergen challenge. Based primarily on what is known about the pro-inflammatory effects of tachykinins, it has been proposed that loss of NEP due to epithelial damage in the setting of airway inflammation may lead to potentiation of SP effects and hence amplification of the airway pathology²¹². However, no evidence for reduced NEP activity has been found in the airways of asthmatics⁷⁰. With the increasing realisation of the protective role of C-fiber nerves^{112,116} and the trophic effects of SP^{13,117}, it might be just as reasonable to propose that in certain circumstances influx of neutrophils (and thus NEP) at sites of inflammation might actually potentiate epithelial damage by reducing local SP levels.

Conclusion

In conclusion, this study has served to illustrate the feasibility of using a minimally invasive procedure to investigate the tachykinin axis in human airway epithelium *in vivo*, thus enabling the study of the role of tachykinins in a much broader range of subjects than can be achieved if relying on resected lung specimens. The findings reported here suggest that the role of epithelium in modulating inflammation potentially includes effects on all three components of the tachykinin axis - tachykinins, their receptors and their major degradative enzyme.

Chapter Six

***Ex vivo* modulation of human airway epithelial brush biopsies.**

I. SP increases IL8 mRNA in human airway epithelium

In the previous chapter we demonstrated that the major molecules involved in tachykinin biology are all expressed at the level of the airway epithelium. We next sought evidence for tachykinin receptor function in freshly obtained airway epithelium and to investigate one possible way in which tachykin - epithelium interactions may be involved in the modulation of local inflammatory responses.

It has been shown that airway epithelium performs a number of important biological functions in the airway including physical defence functions (protective barrier, ciliary clearance), mucus and electrolyte secretion, and the production of a number of immunomodulatory cytokines²¹³. Among the cytokines produced by epithelial cells is interleukin 8 (IL8), a powerful chemoattractant and activating factor for neutrophils^{214,215}. IL 8 production by epithelial cells in culture is upregulated by pro-inflammatory cytokines such as TNF α and IL1 β ²¹⁶. Increased IL8 levels have been detected in several inflammatory processes *in vivo* including chronic bronchitis^{217,218}.

In vitro studies using cultured epithelial cells have shown that SP can stimulate these cells to release factors which are chemotactic for neutrophils and enhance neutrophil attachment to epithelium^{98,100}. IL8 is a candidate molecule which could play a role in this process. In this regard, a definite link between SP stimulation and IL8 release has been demonstrated in

astrocytoma cells^{219,220} and neutrophils²²¹. In this latter case, SP both directly stimulated IL8 secretion from neutrophils as well as enhancing TNF α -induced IL8 secretion . Furthermore, it was shown that increased IL8 secretion from these cells was preceded by an increase in IL8 mRNA, indicating SP was acting via transcriptional regulation. Studies in astrocytoma cells have also indicated a component of post-transcriptional regulation as evidenced by an increase in IL8 mRNA half-life²¹⁹. The response to SP in astrocytoma cells is an NK1 mediated effect and strongly inhibited by the NK1 specific antagonist SR 140333²²⁰.

We hypothesised that SP stimulation may lead to an increase in IL8 production in airway epithelium. As a prelude to testing this hypothesis we first established whether IL8 mRNA could be detected in airway epithelial samples and whether such samples could be used in an *ex vivo* context for short term experiments investigating the response of IL8 mRNA to inflammatory stimuli. For this initial analysis we used two previously established stimulators of IL8 production in airway epithelial cells, TNF α and IL1 β . Following this assessment we then addressed the question of whether SP upregulated IL8 expression in brush biopsy samples, then attempted to further investigate the effects of SP on IL8 using immortalised airway epithelial cell lines.

Experimental Protocol

Airway brush biopsy samples

Airway epithelial brush biopsy samples were obtained from patients undergoing bronchoscopy for clinically indicated reasons as previously described. Subjects were typically smokers undergoing investigation of radiological opacities or minor haemoptysis. Patients taking oral or inhaled corticosteroids were specifically not included because steroids are known to decrease IL8 expression²¹⁶. Samples were transported to the laboratory within 20 mins of collection, and examined for morphology and viability as previously described. Cell suspensions were divided into two equal aliquots and sedimented by centrifugation at 400g for 5 min. Aliquots were then resuspended in 1ml of serum free RPMI cell culture media containing either the stimulant under investigation or its diluent as a control, and plated into six well plates. In this way, cells from each subject acted as their own control. Stimulants used were TNF α (Amgen), 20 ng/ml final concentration, IL1 β (Amgen), 10 ng/ml final concentration (both diluted from stocks dissolved in sterile water and stored at -80C) and SP. SP was dissolved in 10% ethanol/0.1% BSA to a stock concentration of 10^{-3} M and stored aliquotted at -70C. SP experiments were performed using a final concentration of SP of 10^{-5} M, plus phosphoramidon 10^{-6} M (to inhibit SP degradation by NEP), or the corresponding control which consisted of phosphoramidon 10^{-6} M and the SP diluent. Cell suspensions were placed into the wells of a 24 well tissue culture plate and incubated at 37°C in 95% air / 5% CO₂ atmosphere in a tissue culture incubator for four hours. After this time, RNA was extracted from the cells using the RNazol B technique as previously described. RNA was reverse transcribed to cDNA, then serially diluted cDNA was analysed by semi-quantitative RT-PCR, Southern blotting, probing and phosphorimager analysis as described. Analysis was performed for both IL8 mRNA (22 cycles of amplification) and actin

mRNA (18 cycles of amplification). The IL8 / actin ratio for the samples incubated with SP was then compared to the control incubated samples. For each sample the IL8/actin ratio was taken as the mean IL8/actin ratio of three serially diluted cDNA samples.

Airway epithelial cell lines

Following the analysis of brush biopsy specimens, IL8 production in response to SP stimulation was assessed in two immortalised airway epithelial cell lines in which we had detected gene expression for NK1 receptors - 16HBE14o-¹⁶¹ and 1HAEO-¹⁶⁰ (chapter five). For these analyses we investigated the effect of a range of concentrations of SP from 10^{-4} to 10^{-11} M. We also investigated whether stimulation by SP had any priming effect on the subsequent stimulation of epithelial cells by $\text{TNF}\alpha$, an effect previously noted in neutrophils²²¹. We developed a new ELISA to measure IL8 levels specifically for this purpose (chapter two), and used RNA slot blot analysis to investigate levels of IL8 mRNA. For these analyses cells were plated at 100,000 per well in 35mm tissue culture dishes in RPMI/10%FCS with antibiotics. 48 hours later cells were initially incubated with phosphoramidon 10^{-6} M for 30 min, then in media containing phosphoramidon plus SP at various concentrations from 10^{-4} to 10^{-11} M. To assess whether SP had any priming effect on $\text{TNF}\alpha$ - induced IL8 production, $\text{TNF}\alpha$ was added into the wells 30 min after SP to a final concentration of 1, 5, 10 or 20ng/ml. Supernatants were collected 24 hours later and analysed for IL8 content by ELISA.

Data were compared using Student's t-test and significance accepted at $p < 0.05$.

Results

Specific signal for IL8 mRNA was readily detected in all brush biopsy samples. Amplification for 22 cycles gave quantitative signals as determined by the analysis of serially diluted input cDNA (Figure 6-1a).

Increased amounts of IL8 mRNA were detected in samples incubated with either TNF α (n = 6) or IL1 β (n = 4), when compared to paired control samples incubated with diluent alone. Mean ($\pm$ SEM) IL8/actin ratio in TNF α incubated cells was $214 \pm 22\%$ of control (p = 0.036), and IL8/actin ratio in IL1 β incubated cells was $221 \pm 42\%$ of control (p = 0.042) (Figure 6-1b).

Cells obtained from 15 subjects were incubated with SP 10^{-5} M or control. In this analysis, there was also an increase in IL8 mRNA, mean ($\pm$ SEM) IL8/actin ratio in SP treated samples was $137 \pm 29\%$ of controls (p = 0.002) (Figure 6-1b).

Using the newly developed ELISA, IL8 was detected in the culture supernatants from both 16HBE14o- and 1HAEO- cells. A consistent dose related increase in IL8 was detected in both cell lines following stimulation with TNF α (Figure 6-2), consistent with previous reports using other airway epithelial lines ²¹⁶. An increase in IL8 mRNA was detected at various time points (1.5, 3 and 8 hours) by RNA slot blot using a labelled IL8 cDNA probe (1.5 hour time point shown, Figure 6-3). No increase in IL8 mRNA in response to SP was detected.

No increase in IL8 was detected by ELISA when cell lines were stimulated with SP at doses from 10^{-4} to 10^{-11} M. No significant changes in IL8 mRNA were seen at any time point using either slot blot analysis or the more sensitive SQ-RT-PCR approach. In addition, no dose of SP had any significant effect on the subsequent TNF α -induced IL8 production at either 1, 5, 10 or 20 ng/ml doses of TNF α (Figure 6-4 and 6-5).

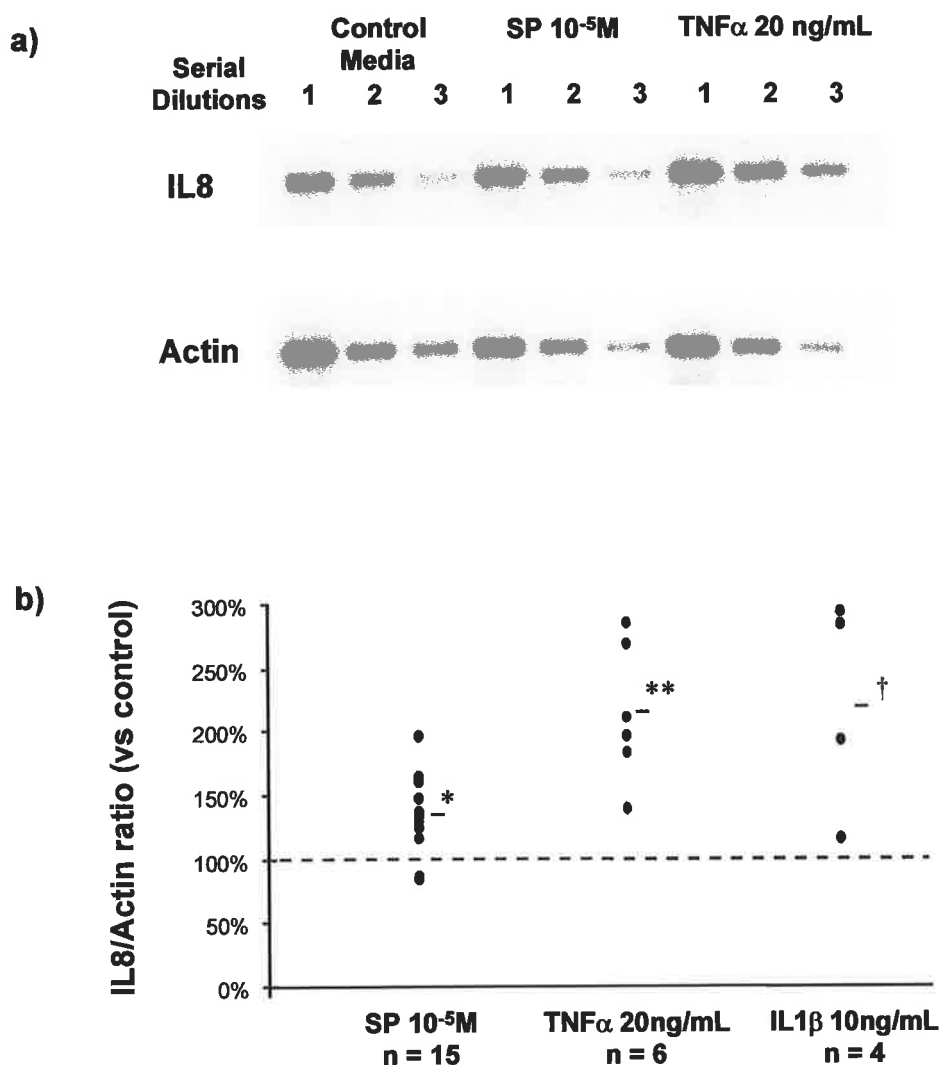


Figure 6-1. a). Representative autoradiographs from SQ-RTPCR analysis of IL8 response to SP and TNF α in airway epithelial brush biopsy specimens. Specimens were incubated with either control medium, SP or TNF α for 4 hours then RNA extracted, reverse transcribed and PCR amplification of serially diluted cDNA was performed. **b).** Effect of SP, TNF α and IL1 β on the IL8/actin ratio, normalised for the control cells of the same subject. Each point represents an individual subject, bar = mean. * $p = 0.002$, ** $p = 0.0036$, † $p = 0.04$.

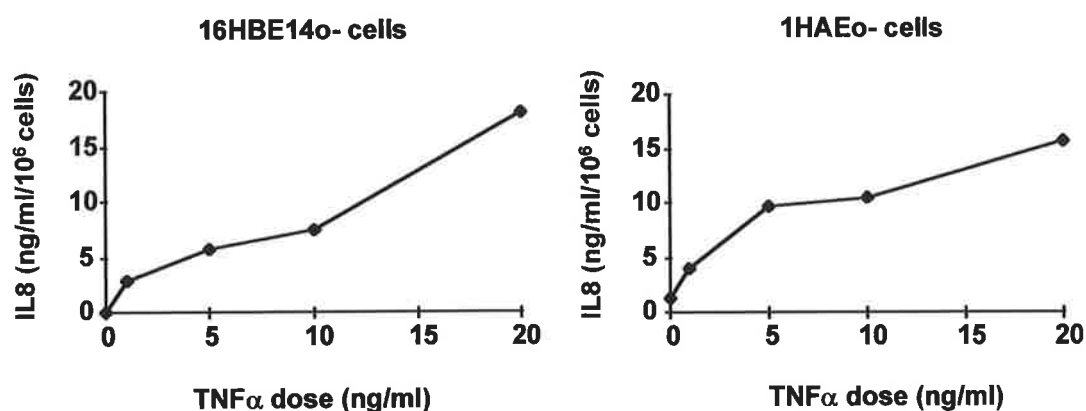


Figure 6-2. IL8 production by two airway epithelial cell lines in response to TNF α . Cells were incubated with medium containing the indicated amount of TNF α for 24 hours, then supernatants were collected and analysed for IL8 content by ELISA.

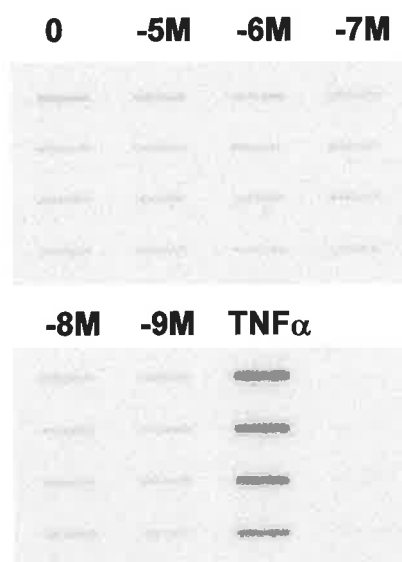


Figure 6-3. IL8 mRNA response to TNF α . 16HBE14o- cells incubated with either SP (log Molar dose indicated) or TNF α (20ng/ml). RNA was extracted at 1.5 hours and analysed by slot blot, probed with an IL8 cDNA labeled probe.

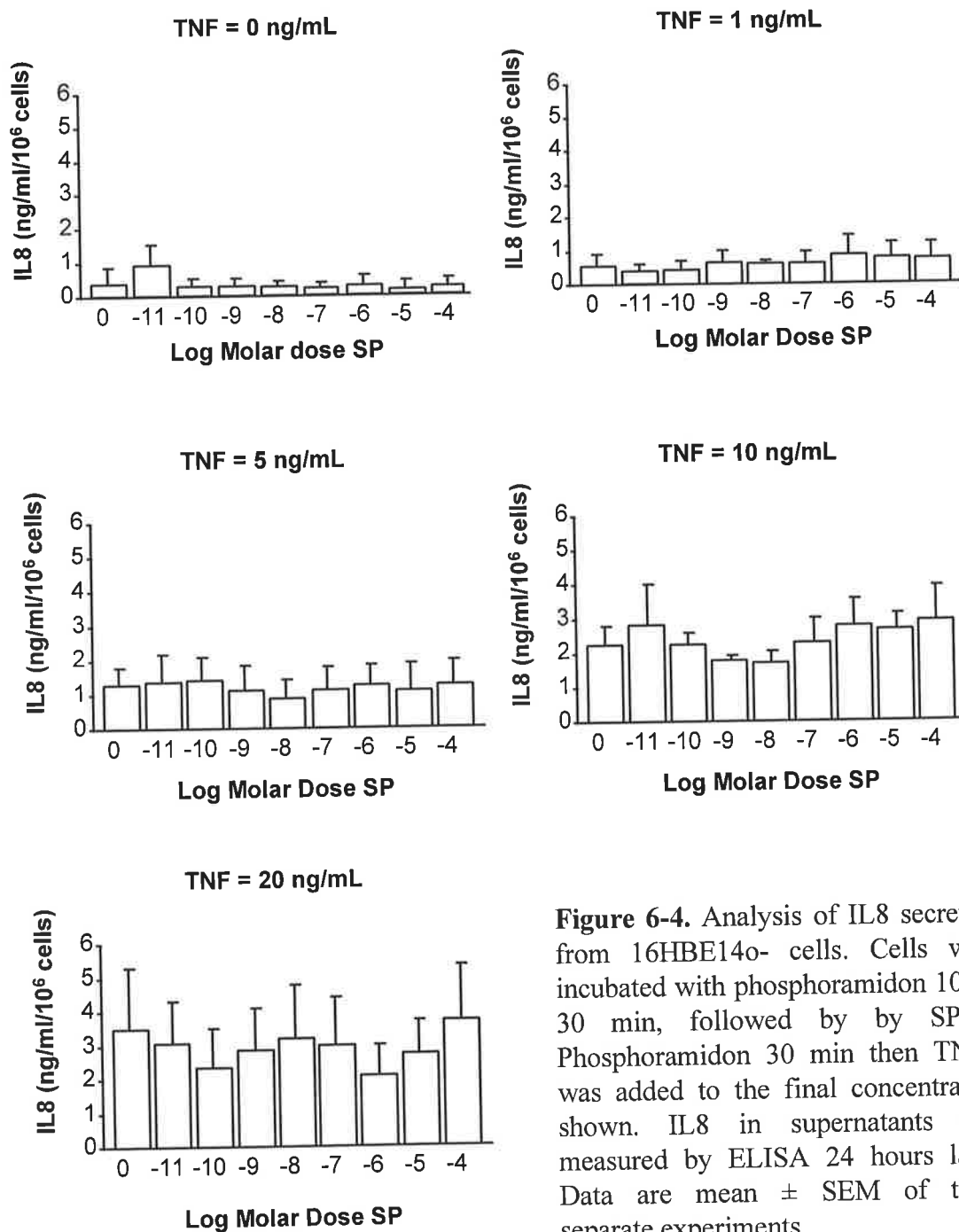


Figure 6-4. Analysis of IL8 secretion from 16HBE14o- cells. Cells were incubated with phosphoramidon 10⁻⁶M 30 min, followed by by SP + Phosphoramidon 30 min then TNF α was added to the final concentration shown. IL8 in supernatants was measured by ELISA 24 hours later. Data are mean $\pm$ SEM of three separate experiments.

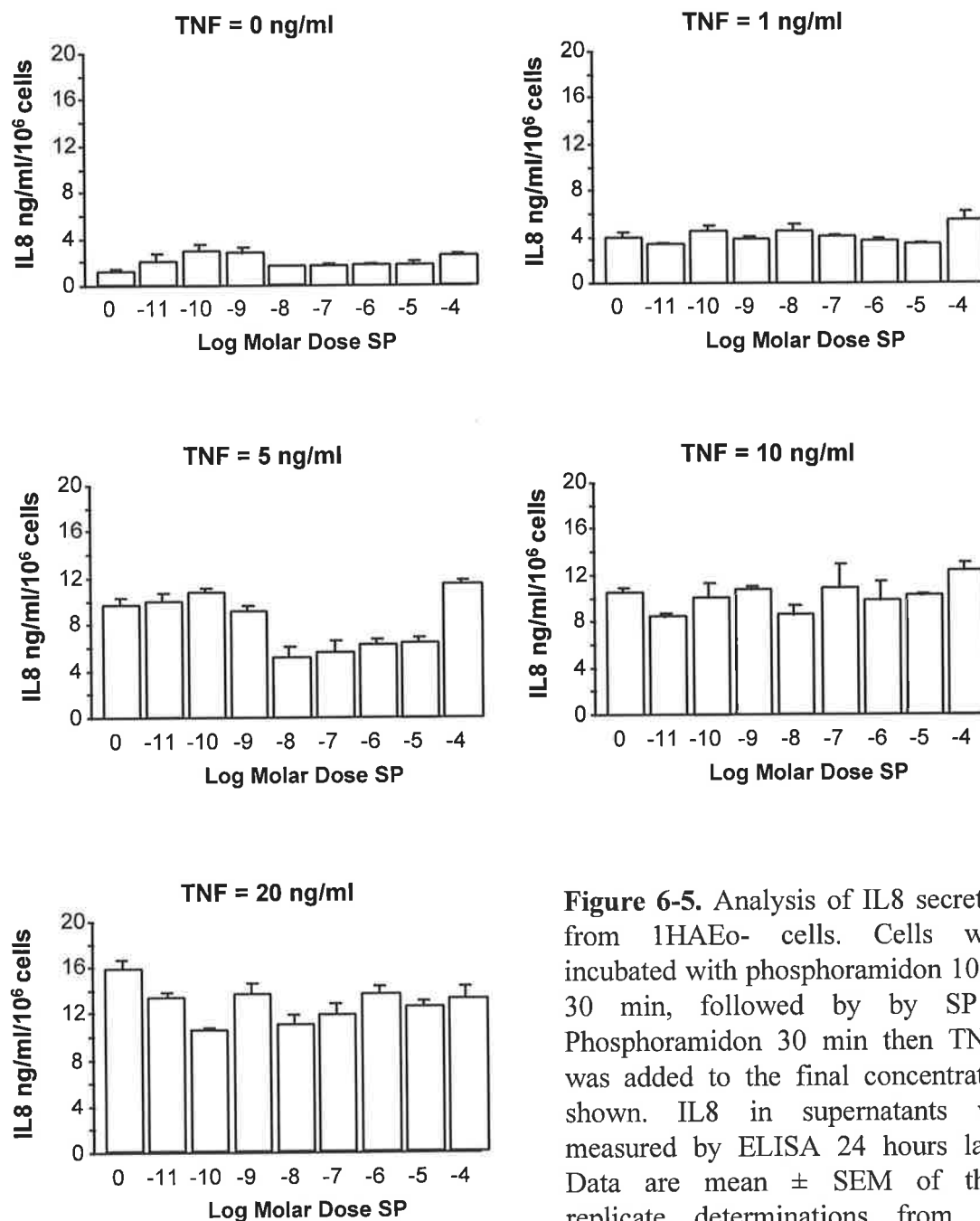


Figure 6-5. Analysis of IL8 secretion from 1HAEo- cells. Cells were incubated with phosphoramidon 10^{-6} M 30 min, followed by by SP + Phosphoramidon 30 min then TNF α was added to the final concentration shown. IL8 in supernatants was measured by ELISA 24 hours later. Data are mean $\pm$ SEM of three replicate determinations from one experiment.

I. Effect of glucocorticosteroid on NK1 receptor mRNA levels

There is evidence that at least some of the pro-inflammatory and trophic effects of SP on the airway epithelium are mediated via NK1 receptors. There is also evidence in some systems that glucocorticosteroids downregulate NK1 receptor expression^{48,65}. Using explant cultures of lung from organ donors Adcock et al showed a reduction in NK1 mRNA in response to incubation with dexamethasone⁶². Our technique of airway epithelial brushing potentially enables a more specific evaluation of NK1 modulation at the level of the airway epithelium. We thus sought to determine whether a reduction in NK1 mRNA could be induced by incubation of brush biopsy samples with dexamethasone. Such a response would provide new insights into the therapeutic effect of inhaled steroids in asthma.

Experimental protocol

Brush biopsy samples were obtained from seven patients in the manner that has been described. These subjects were similar to those previously described, principally patients with a history of tobacco smoking who were undergoing investigation of radiological opacities. No subjects were taking inhaled or oral steroids.

A stock solution of 10^{-2} M dexamethasone (Sigma) was prepared in 100% ethanol then diluted in culture medium as required. An equivalent volume of ethanol in medium was used as a diluent control. Patient samples were incubated in RPMI medium containing diluent or 10^{-6} M dexamethasone. After 24 hours of incubation, RNA was extracted and analysed by SQ-RT-PCR. Serial dilutions of input cDNA determined a quantitative relationship between input sample and phosphorimager signal using 30 cycles for NK1 and 18 cycles for actin. Amplified products were

processed by agarose gel electrophoresis, Southern blotting and phosphorimager analysis as previously described. For each subject sample, the NK1 mRNA signal relative to actin mRNA signal was determined. The NK1 mRNA levels in the dexamethasone treated samples were expressed as a percentage of the control treated samples.

Results

Specific mRNA for NK1 receptor was detected in all samples. Comparison of dexamethasone vs control incubated samples revealed a modest decrease in NK1/Actin ratio in the dexamethasone treated cells to $76 \pm 6\%$ of control $p < 0.05$ (Figure 6-6).

We also investigated the effect of a higher dose of dexamethasone, 10^{-4}M , which had been shown to decrease NK1 mRNA in lymphoblastoma cells⁴⁸. Samples from five subjects were analysed. Dexamethasone at this concentration actually resulted in an increase in NK1/Actin ratio to $187 \pm 9\%$ of control, $p < 0.05$.

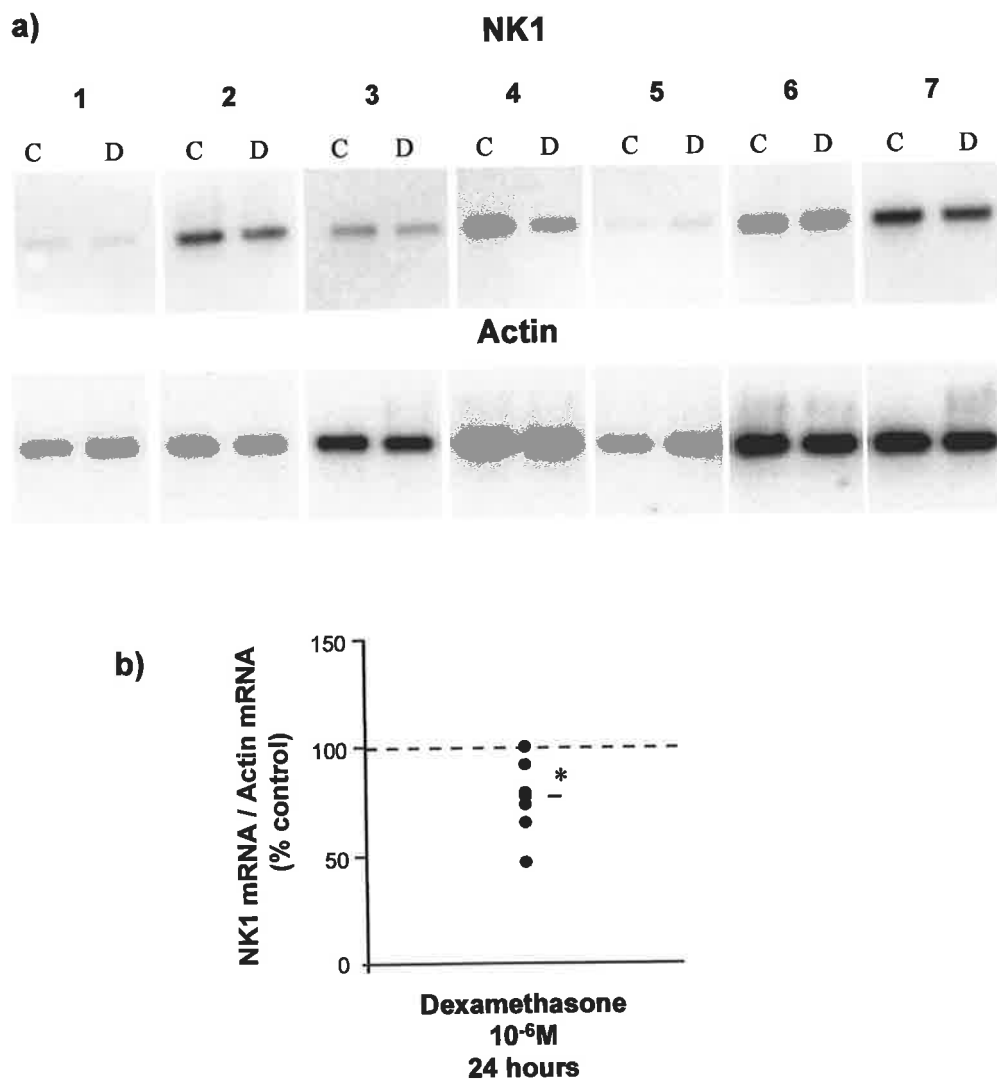


Figure 6-6. a) Representative autoradiographs from SQ-RTPCR analysis of NK1 mRNA response in airway epithelial brush biopsy specimens. Cells were incubated in either control medium (C) or medium containing 10^{-6} M dexamethasone (D), for 24 hours. **b).** Phosphorimager analysis of SQ-RTPCR: NK1 mRNA levels relative to Actin mRNA was determined and is expressed as the percentage of the value determined for the control treated cells. Each point is the result from one subject and bar = mean. * $p < 0.05$ dexamethasone vs control.

Discussion

In this study we sought to determine whether epithelial brush biopsy specimens could be used as a system for the evaluation of tachykinin responses in airway epithelium. The principle findings were that SP induced an increase in IL8 mRNA in freshly obtained samples (but not in immortalised cell lines) and that dexamethasone had a variable effect on NK1 mRNA levels depending on the dose used..

To facilitate the investigation of airway epithelial cell responses in a variety of biological systems, many immortalised airway epithelial cell lines have been used ^{161,222}. Such an approach offers the advantage of a robust system in which a variety of experimental variables can conveniently be assessed. However, the ability to extrapolate from such cultures to the airway epithelium *in vivo* is difficult to predict and will naturally vary depending upon the particular cell line being used. For instance steroids have been reported to induce either an increase in NEP expression²²³ or to have no effect ¹³⁹, depending on the line being used. Primary airway epithelial cell cultures²²⁴ may more accurately reflect *in vivo* biology but even in early passage these cultures are substantially de-differentiated in comparison to the complex array of specialised cell types present in mature epithelium. Previous reports had suggested that modulation of brush biopsy explants was feasible²²⁵ and might potentially be a closer representation of *in vivo* responses. In the first instance, we determined whether we could reproduce effects in brush biopsy samples that had previously described in primary cultured cells. To this end, we investigated the effect of TNF α and IL1 β on IL8 expression and found that we could detect an increase in IL8 mRNA in response to both of these stimuli. We next investigated whether SP induced an upregulation of IL8 production from the epithelium. Such an effect might in part explain some of the pro-inflammatory effects of SP. We found a small but significant increase in

IL8 mRNA in response to SP at a concentration of 10^{-5} M. Although this dose is high, it is plausible that such levels may be reached locally in the airway epithelium, either as a result of local intraepithelial production as suggested by the findings in chapter five, or as a result of SP release from the C-fibre nerve endings, which are located in close proximity to the epithelium. Furthermore, this dose is comparable to those used in the studies of SP stimulation of neutrophils^{91,221} and smooth muscle cells¹⁰⁹. These findings thus suggest that IL8 may play a role in SP-induced release of neutrophil chemotactic activity from cultured airway epithelial cells, and in the SP induced increases in adherence of neutrophils to epithelium.

Although epithelial brush biopsies may have an advantage over cultured cells in terms of the representation of airway epithelium *in vivo*, there are clearly disadvantages in attempting to use this system in extensive evaluations of biological responses. Aside from the logistics of collecting specimens, limitations are principally due to the small number of cells available for evaluation, which greatly restricts the number of variables that can be assessed in any one experiment. In practice, it was found that attempting to divide samples into more than two or three aliquots greatly compromised subsequent analysis. Thus, in an effort to further characterise the mechanisms of IL8 induction by SP, two transformed airway epithelial cell lines were evaluated. Messenger RNA for NK1 receptors was detected in both of these lines, and it was confirmed that the lines had the capacity to produce IL8 in response to a known stimulus ($\text{TNF}\alpha$). However, the brush biopsy findings with regard to SP stimulation of IL8 mRNA could not be confirmed in these lines. In addition to the evaluation of direct stimulation of IL8 production by SP, we investigated whether SP had the capacity to prime cells for an increased response to a subsequent incubation with $\text{TNF}\alpha$, an effect reported in neutrophils²²¹. No such effect was found in these cell lines over a broad range of SP concentrations. A number of variables were manipulated to determine if they had any impact on the cell lines' response to SP. These variables

included incubations at various time points from 30 minutes to 48 hours, differing the length of time between passage and SP stimulation (1 - 4 days), use of different concentrations of fetal bovine serum in the culture medium, the use of serum free medium, starving cells for 24 hours prior to stimulation and growing cells on a coating of collagen / fibronectin / BSA rather than plastic. In every case IL8 production could be stimulated by $\text{TNF}\alpha$, which served as a positive control, but none of these manoeuvres had any convincing impact on IL8 production in response to SP. Thus, for this reason these lines could not be used to further characterise the findings seen in the fresh airway epithelial samples.

The other aspect of tachykinin biology we investigated in the airway epithelial brush biopsies was the response of NK1 receptor mRNA to dexamethasone. There is evidence in the literature that dexamethasone downregulates NK1 mRNA, although the effect in human airway epithelium had not been assessed. Such an effect would be of relevance to the mechanism of action of inhaled steroids. We found a slight decrease in NK1 mRNA in response to steroids of about 25%, which is consistent with although less marked than the findings of Adcock et al, who found an 85.4% decrease in lung tissue explants⁶². In rat pancreatic acinar cells, Ihara reported > 95% reduction in NK1 mRNA expression by a 4 hour incubation with dexamethasone⁶⁵ and in lymphoblastoma cells Gerard reported approximately 50% reduction at 24 hours in response to a high dose of dexamethasone of 10^{-4}M ⁴⁸. Ihara's studies showed that the effect of dexamethasone was probably at the point of transcription initiation and not an effect on mRNA half-life, which was quite short at approximately 30 minutes. They went on to show that the rapid turnover of NK1 mRNA was dependant on protein synthesis, and that blocking protein synthesis resulted in a substantial increase in NK1 mRNA, an effect which was present at 1 hour and persisted for 8 hours. We found that incubating brush biopsy specimens with high dose dexamethasone (10^{-4}M) actually led to an increase in NK1 mRNA. This was unexpected although an increase in NK1

mRNA in response to steroids has been noted in astrocytoma cells. The mechanism of this response in the epithelial specimens is uncertain, although downregulation of a protein involved in NK1 mRNA degradation is a possibility. In any case, levels of steroid achievable at the airway epithelium *in vivo* are thought to be closer to 10^{-6} M than 10^{-4} M⁶². Nevertheless, to more clearly define the effect of dexamethasone on epithelial NK1 mRNA levels will require further studies using a greater number of doses on cell samples obtained from individual subjects, and until such studies are done the effect is properly considered indeterminate at this time. It is interesting to note that a typical glucocorticoid response element has not been identified in either the rat or human NK1 genes⁵².

Conclusion

In conclusion these studies using epithelial brush biopsy explants have provided information on a possible mechanism of action of SP in inducing airway inflammatory responses and on a mechanism by which the effect of steroids may reduce responses to SP. Further, a discrepancy between the effect of SP on the mature epithelial samples and on immortalised cell lines was noted.

Chapter 7

Tachykinin receptor, NEP and preprotachykinin A gene expression in the airway epithelium of subjects with airways disease.

In chapter five data was presented indicating that tachykinins, their receptors and their principle degradative enzyme are produced in airway epithelium, and the potential roles of tachykinins in the airways, both as mediators of inflammatory responses and as mediators of protective and healing responses have been extensively discussed. Some evidence for an SP-induced pro-inflammatory effect in terms of IL8 production was presented in chapter six. The next aim was to use molecular analysis of brush biopsy samples to gain some understanding of the way in which production of PPT-A, NEP and tachykinin receptors are modulated in disease states in airway epithelium. As has been discussed, the evidence available favours a nett increase in tachykinin activity in inflammatory states. However, studies to date have generally focused on the individual components of the tachykinin axis in isolation. For example, although SP levels are increased in the sputum and bronchoalveolar lavage fluids of patients with asthma or chronic bronchitis^{124,126}, downregulation of receptors or an increase in NEP at the epithelial level would clearly impact upon the nett effect. In this regard, both an increase and a decrease in tachykinin receptors has been reported in different studies of airway inflammation^{59,62,63}. In addition, although it has been shown that airway epithelial dysfunction in certain inflammatory states is accompanied by a reduction in NEP activity^{136,137}, there is as yet no evidence for such a reduction in human airway epithelium *in vivo*⁷⁰. Furthermore, although increased airway SP was detected in a rat model of chronic bronchitis, no corresponding increase in PPT-A mRNA was seen in the dorsal root ganglia¹¹⁸. However, in neonatal rats, hyperoxia caused an increase in lung

SP content and lung PPT-A mRNA levels, although the source of this RNA was not determined¹²⁸. A more complete understanding of the net effect of disease on the tachykinin system could be achieved by the simultaneous investigation of NEP, PPT-A and tachykinin receptor expression in human airway epithelium *in vivo* in the one analysis.

The use of brush biopsy allows for a minimally invasive approach to this issue, thus allowing for the evaluation of a broader range of subjects than is possible if relying on autopsy or resection material. In addition, the use of a semi-quantitative PCR approach offers a high degree of sensitivity and specificity and by analysing for PPT-A mRNA, local epithelial tachykinin production can be distinguished from that produced in distant nerve cell bodies.

Experimental protocol

As for the previous qualitative evaluations reported in chapter five, subjects were recruited from patients undergoing bronchoscopy for clinically indicated reasons. Patients were assigned to particular disease groups based upon clinical history and investigations leading up to the bronchoscopy procedure. To allow comparisons to be made to a group of subjects with normal pulmonary function, volunteers were specifically recruited to form this subject group. The specific details of the bronchoscopy procedure and subsequent analysis of airway epithelium gene expression by semi-quantitative RT-PCR analysis has been described in detail in the methods chapter. In brief, airway epithelial brush biopsy samples were obtained using a standard cytology brush from areas of the lung not involved in an obvious pathological process on radiological examination. The principle reason for bronchoscopy in a number of the recruited subjects was a radiological opacity on chest x-ray examination, in most cases suspicious of malignancy. In such cases airway samples were obtained from the opposite lung and were obtained before any manipulation in the region of the suspected tumor, to avoid the potential for contamination of samples with malignant cells. All samples were transported to the laboratory within 20 minutes of collection, where they were quickly assessed for morphology and viability by phase contrast microscopy as previously described. Contamination with blood was rare, but in such cases samples were discarded. All samples used for this analysis were determined to contain at least 50% viable cells based on ciliary beating and trypan blue analysis of a small aliquot. Extraction of RNA was then performed using the RNazol B technique as previously described. Reverse transcription using MoMLV was performed as described and the resultant cDNA was stored at -70°C until required for PCR analysis. When comparisons were made between groups for any of the genes of interest, a single master mix of reagents was prepared and all samples were amplified in the same PCR run. Amplification was performed for the number of cycles previously

determined to yield quantitative data. For each amplification of patient samples in this analysis, a standard curve of serially diluted cDNA (1:2 dilutions) was amplified in the same run to ensure amplification was quantitative. Following amplification, PCR products were analysed by Southern blotting, radiolabeled oligonucleotide probing and phosphorimager detection as previously described. For each gene of interest data is presented as the signal intensity normalised for actin expression.

Subject Recruitment

Subject details are given Table 7-1. Subjects were assigned to groups based on clinical history, examination and radiological findings. Pulmonary function tests (as a minimum forced expiratory volume, forced vital capacity, response to bronchodilator) were available for the majority of subjects.

The 'normal' group consisted primarily of volunteers recruited specifically for the study. These subjects were all non-smokers with no history of pulmonary disease and had normal lung function. A single patient subject was also included. This was a man who was investigated for minor haemoptysis which was likely of nasal origin. He was a non-smoker with no history of pulmonary disease and bronchoscopy revealed no endobronchial abnormality.

Subjects with paroxysmal cough were patients who had undergone a number of investigations for non-productive cough but in whom no formal diagnosis had been made. These subjects had had normal chest x-ray examination, normal pulmonary function tests (with no evidence of asthma). They had had a persistent cough for at least six weeks with no overt evidence of viral infection for at least that length of time although viral serology was not performed. They had often had a trial of inhaled β_2 agonists and inhaled steroids but had found

the cough did not respond, and were not taking steroids at the time of bronchoscopy. In each case the bronchoscopy examination failed to find any overt pathology.

Patients assigned to the asthma group were subjects undergoing bronchoscopy for a variety of reasons as indicated in Table 7-1. Four out of the six subjects were current smokers. Diagnosis of asthma was based on clinical history / case note review and pulmonary function tests. This group was clearly heterogeneous in nature and there was likely co-existence of asthma and smoking related chronic bronchitis in some. All but one were current users of inhaled steroids, although compliance with this medication is uncertain. There was no clear history of recent viral infection.

The chronic bronchitis group consisted of subjects who were all smokers with a history of sputum production for at least three months in two consecutive years. None of these subjects were taking steroids and none had a clear history of viral infection in the preceding six weeks. The principle symptom which brought them to medical attention was a worsening cough and/or haemoptysis and the most common diagnosis was a primary pulmonary malignancy. Pulmonary function tests typically showed a degree of airflow obstruction without significant response to bronchodilator. As discussed below five patients were initially assessed in this group then a further four subjects were included on repeat examination.

Three subjects who had radiological signs consistent with sarcoidosis (newly diagnosed and subsequently confirmed on pathological examination) were also assessed.

Analysis of gene expression

In the first analysis airway epithelial samples were analysed for gene expression for PPT-A, NK1, NEP and intercellular adhesion molecule 1 (ICAM-1). ICAM-1 analysis was included because it has previously been described that ICAM-1 expression is upregulated in inflammatory states and in particular in brush biopsy samples taken from subjects with asthma and chronic bronchitis compared to normals^{226,227}. Thus, examination of ICAM-1 expression was performed in an effort to validate our approach. A second analysis was also performed in which we focused more specifically on the group with chronic bronchitis. This was principally due to the greater number of patients available with this disease. In this second analysis we recruited an additional four subjects and expanded our investigations to include analysis of NK2 and interleukin 8 (IL8). IL8 examination was included as a further confirmation of our approach, as it has previously been reported that IL8 is upregulated in the airways in chronic bronchitis²¹⁸.

Statistics

In the first analysis data was analysed by Student's t-test with Bonferoni's adjustment. In the second analysis Student's t-test was used for normally distributed data and Wilcoxon test for non-normally distributed data. In each analysis significance was accepted at $p < 0.05$.

Table 7-1. Characteristics of subjects included in the study

	Normal	Sex	Age (years)	Reason for bronchoscopy / underlying disease	Lung function	Smoking history (pack-years)	Steroids
1		M	34	Volunteer	Normal	0	no
2		M	28	Volunteer	Normal	0	no
3		F	39	Volunteer	Normal	0	no
4		M	24	Volunteer	Normal	0	no
5		M	24	Volunteer	Normal	0	no
6		M	77	Haemoptysis (?nasal)	Normal	0	no
Mean Age			37.7				
Sarcoidosis							
1		F	30	Sarcoidosis	Low MMEF	0	no
2		F	22	Sarcoidosis	Normal	7	no
3		M	41	Sarcoidosis		0	no
Mean Age			31.0				
Asthma							
1		F	47	Interstitial opacities FI	Moderate obstruction. >15% FEV1 response to BD	20	yes
2		M	28	Haemoptysis (no specific lesion)	Normal FEV, > 20% PEFr response to BD	10	no
3		F	52	Haemoptysis (no specific lesion)	Slight obstruction. >15% FEV1 response to BD	40	yes
4		F	45	Mucus plug	Normal, marginal FEV1 response to BD	0	yes
5		M	63	Poorly differentiated carcinoma	Moderate obstruction. >15% FEV1 response to BD	30 (ex 20 yrs)	yes
6		F	69	Squamous cell carcinoma	Severe obstruction, >15% FEV1 response to BD	40	yes
Mean Age			50.7				
Paroxysmal Cough							
1		M	68	Cough	Normal	0	no
2		F	55	Cough	Normal	0	no
3		F	60	Cough	Normal	0	no
4		F	61	Cough	Normal	0	no
Mean Age			61.0				
Chronic Bronchitis							
1		M	51	Hoarse voice (sluggish vocal cord.)	Normal	20	no
2		F	63	Adenocarcinoma.	Slight obstruction, no response to BD	40	no
3		F	78	Large cell carcinoma	Slight obstruction, no response to BD	30	no
4		M	56	Large cell carcinoma	Slight obstruction, no response to BD	20	no
5		F	76	Squamous cell carcinoma.	Unable to perform test (too ill)	120	no
6*		F	30	Normal	Normal	10	no
7*		M	60	Cough / SOB FI	Moderate obstruction.	30	no
8*		M	72	Large cell carcinoma	Severe obstruction, no response to BD	50	no
9*		M	28	Haemoptysis (no specific lesion)	Normal FEV, > 20% PEFr response to BD	10	no
Mean Age			57.1				

* = included in second analysis

BD = bronchodilator (salbutamol)

Results

Signal detection of serially diluted cDNA for each of the genes of interest is shown in Figure 7-1. For each target gene quantitative amplification is shown except for NK2, where persistent amplification of genomic DNA likely affected results.

Mean PPT-A mRNA was higher in the disease groups compared to normals but in the initial analysis the values for chronic bronchitis and asthma did not reach statistical significance (Figure 7-2a). Mean ($\pm$ SEM) signal intensity (PPT-A/Actin ratio) was: normals - 0.05 ± 0.01 , cough - 0.55 ± 0.07 , asthma - 1.11 ± 0.35 , chronic bronchitis - 0.73 ± 0.26 and sarcoidosis 0.22 ± 0.2 .

ICAM-1 mRNA was significantly increased in the cough and chronic bronchitis group compared to normals (Figure 7-2b). Mean ($\pm$ SEM) signal intensity (ICAM-1/Actin ratio) was: normals - 0.11 ± 0.03 , cough - 0.36 ± 0.05 , asthma - 2.05 ± 1.10 , chronic bronchitis 2.28 ± 0.50 and sarcoidosis - 0.26 ± 0.07 .

No significant differences were seen in NK1 or NEP expression (Figure 7-2 c,d).

The finding of an increase in PPT-A expression in airway inflammation was further investigated by repeating the PCR analysis in the normal and chronic bronchitis group. An additional four chronic bronchitis subjects, bringing the total to 9, were included in this analysis. One of the additional subjects included in this analysis had borderline evidence of asthma but was a smoker and had not been taking inhaled steroids. In this comparison, analysis was extended to IL8 and NK2 expression. In this analysis the PPT-A/Actin ratio was > 10 -fold higher in the chronic bronchitis group compared to normals (mean ($\pm$ SEM) PPT-A/Actin ratio 0.62 ± 0.19 for normals vs 6.6 ± 2.0 for chronic bronchitis, $p = 0.019$, Figure 7-3c) Increased ICAM-1

and IL8 mRNA in comparison to normals was seen ($p = 0.001$ and $p = 0.0067$ respectively) and is consistent with previously published reports in which upregulation of gene expression for these molecules has been noted^{218,226,227}.

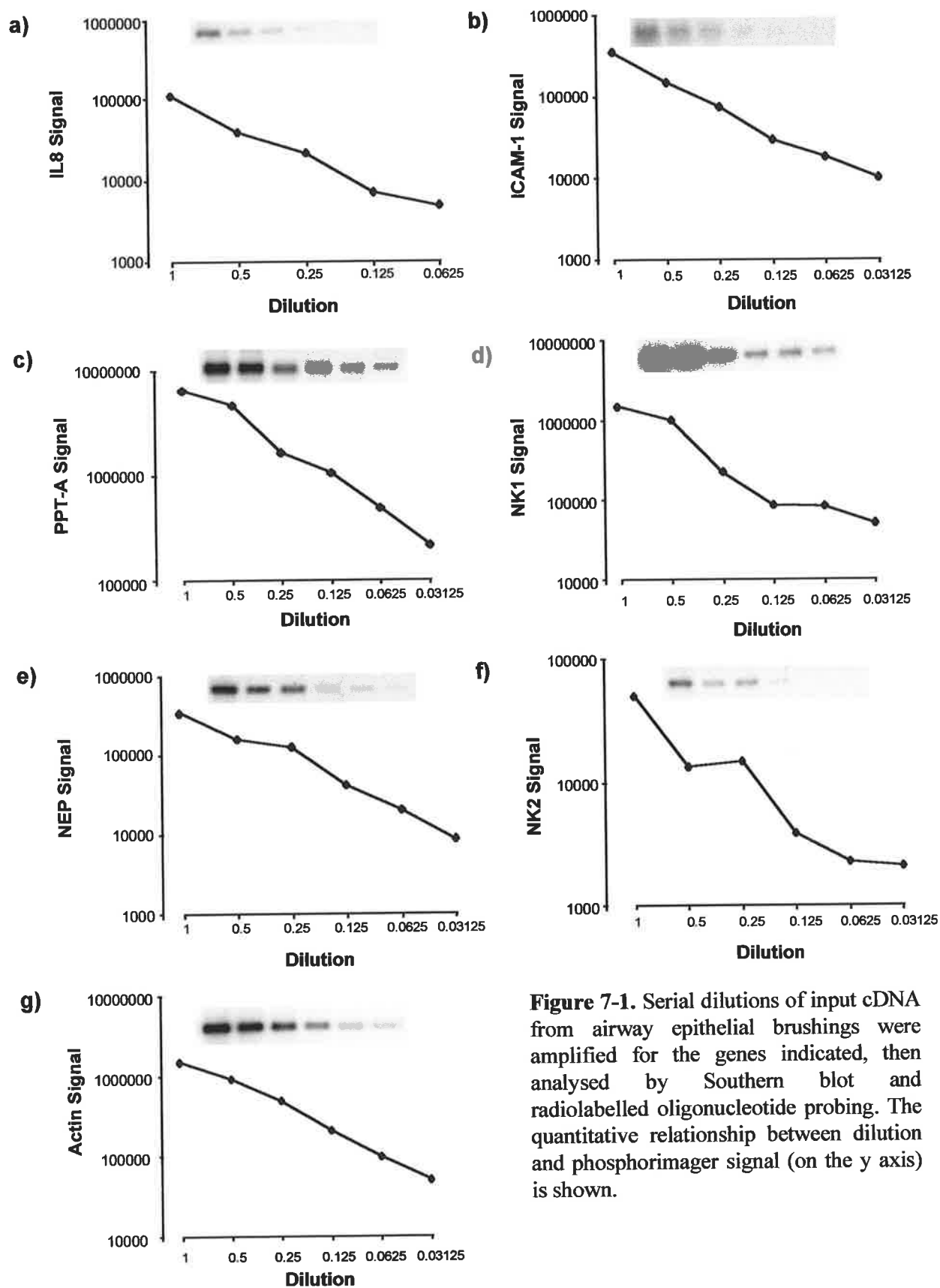


Figure 7-1. Serial dilutions of input cDNA from airway epithelial brushings were amplified for the genes indicated, then analysed by Southern blot and radiolabelled oligonucleotide probing. The quantitative relationship between dilution and phosphorimager signal (on the y axis) is shown.

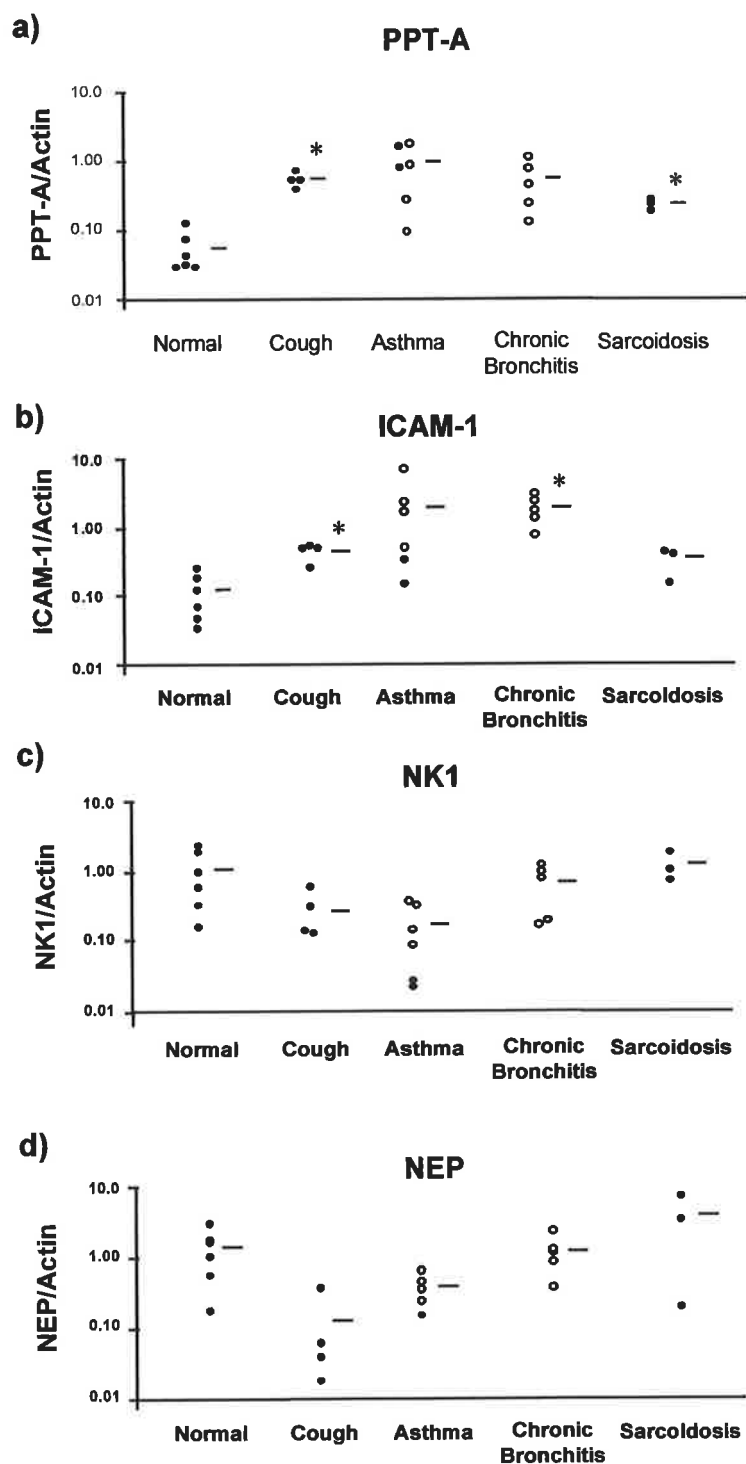


Figure 7-2. Semi-quantitative RT-PCR analysis of airway epithelium specimens from subject groups for a) PPT-A b) ICAM-1, c) NK1 receptor, d) NEP. Data shown is the signal detected by phosphorimager scanning of Southern blots, normalised for actin expression. Each point represents an individual subject. Bar = mean. Open circles = smokers.

* $p < 0.05$

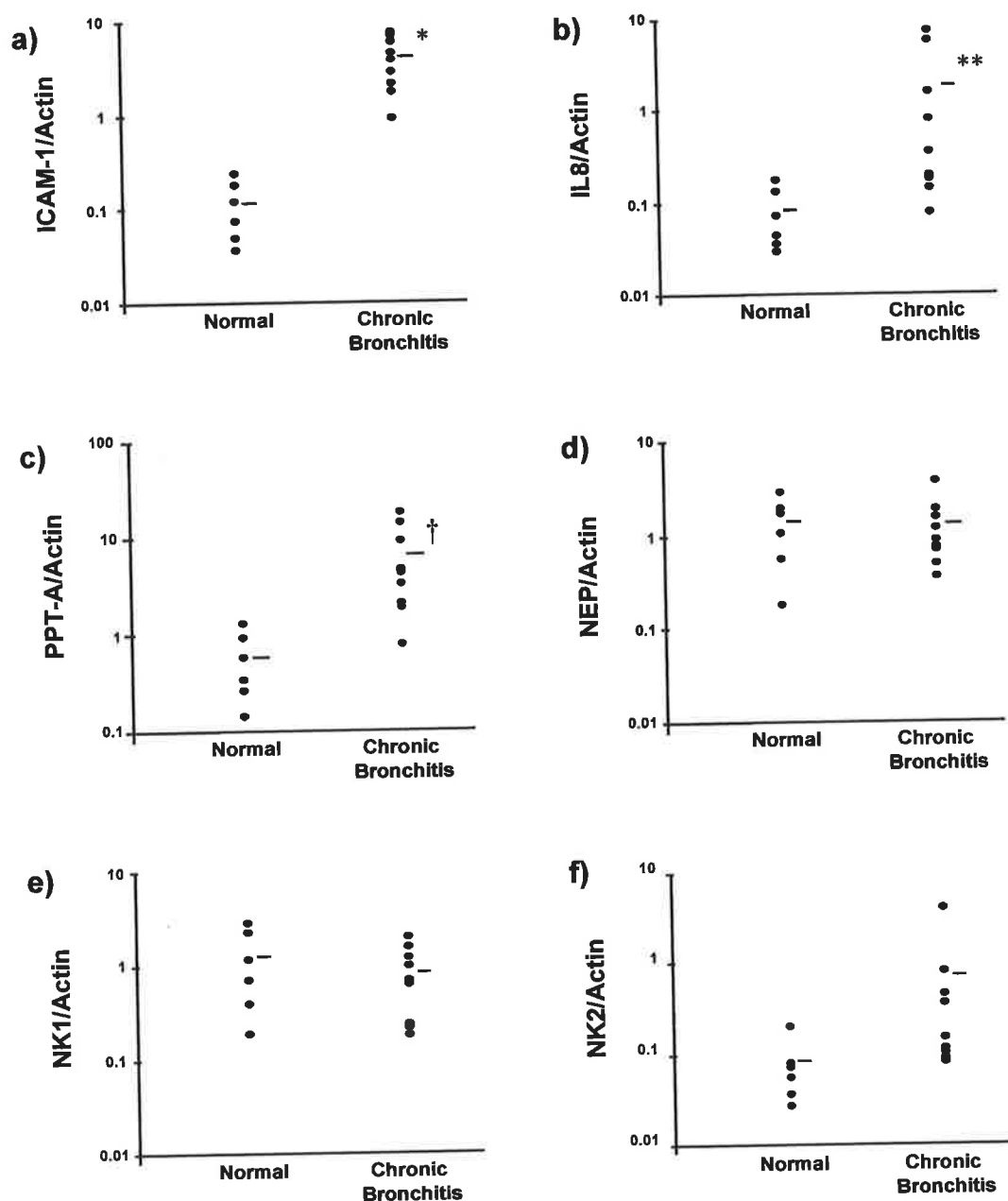


Figure 7-3. Airway epithelial gene expression in subject groups, normalised for actin expression. Airway epithelium was obtained from normal subjects (n = 6) and subjects with chronic bronchitis (n = 9). Samples were analysed by RT - PCR and signal intensity determined by phosphorimager analysis of Southern blots probed with ^{32}P labelled internal oligonucleotide probes. Signals normalised for actin are shown, mean for group indicated by bar.

* p = 0.001, ** p = 0.008, † p = 0.019.

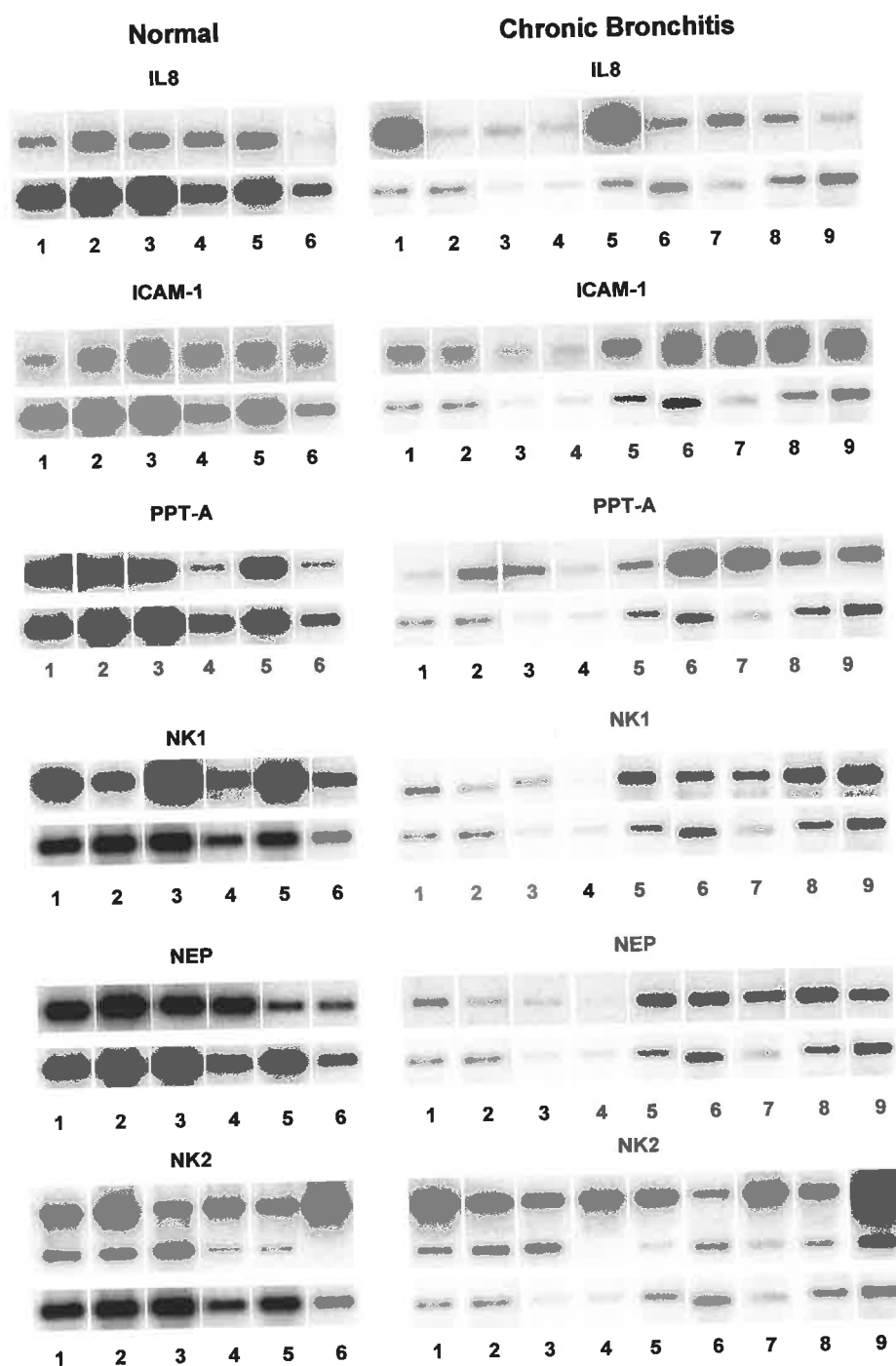


Figure 7-4. Representative blots from each of the subjects in the normal and chronic bronchitis groups analysed in figure 3. In each block the top row is the signal for the gene of interest, as indicated, and the bottom row is the signal for actin.

Discussion

Previous studies have examined individual components of the tachykinin axis, but simultaneous examination of the principle tachykinin precursor, tachykinin receptors and the principle tachykinin degradative enzyme in human airway epithelium *in vivo* has not been reported.

In this study we used our approach of epithelial brush biopsy coupled with semi-quantitative RT-PCR analysis to investigate the changes induced in PPT-A, NEP and tachykinin receptor mRNA levels in subjects with airways disease compared to normals. The principle finding was an increase in epithelial PPT-A mRNA in the subjects with chronic bronchitis.

The principle goal in this analysis was to compare normal to inflamed airways. For this study we relied on recruiting our subjects with disease from among patients who were undergoing diagnostic fiberoptic bronchoscopy for clinically indicated reasons. In so doing, we were able to identify a group of subjects with chronic bronchitis based on standard clinical criteria and who were not taking corticosteroids. We were unfortunately not able to recruit well characterised asthmatics in this way however. Ideally we would have preferred to enroll such subjects specifically for the study, but this proved not to be logistically possible. As a result, we were unable to analyse subjects with asthma in the absence of potential confounding factors such as smoking and corticosteroid medication. The group with chronic paroxysmal cough had had extensive clinical investigation, but in the absence of a specific final diagnosis may represent a heterogeneous group. The sarcoidosis patients all had a histologically proven diagnosis although activity of disease did differ among them. Despite these limitations we clearly had a group of normal subjects with which to compare those with disease.

In an effort to validate our approach, we not only analysed samples for PPT-A, NEP and tachykinin receptors but also for molecules that had previously been reported as being upregulated in chronic bronchitis and /or asthma and other inflammatory states. Vignola et al ²²⁷ reported an increase in ICAM-1 expression in brush biopsy specimens obtained from asthmatics and patients with chronic bronchitis. We also found this to be the case in our chronic bronchitis group. In addition, it has been shown that cigarette smoke induces the production of IL8 in bronchial epithelial cells, and that IL8 is increased in the airways of patients with chronic bronchitis ²¹⁸. Consistent with these reports we were also able to detect an increase in IL8 expression in our chronic bronchitis group.

It has been postulated that NEP may have an important role in airway inflammation. In view of the fact that epithelial cells produce NEP, it has been speculated that airway epithelial cell dysfunction or loss consequent to inflammation may lead to a net decrease in NEP activity and hence a potentiation of tachykinin effects. Thus far, *in vivo* studies of airway responsiveness in humans with asthma have failed to support this hypothesis⁷⁰ and there are no reports specifically addressing the issue of NEP activity in patients with chronic bronchitis, although cigarette smoke has been shown to decrease NEP in animal models^{134,135}. We did not find any change in NEP mRNA in airway epithelium from our subjects with chronic bronchitis, although we did find changes suggesting that airway inflammation was indeed present (increases in IL8 and ICAM-1 mRNA). Our results in the asthma group may have been confounded either by co-existent smoking or the use of inhaled steroids. In regard to this latter point it has been shown that the use of inhaled steroids does increase NEP expression in asthmatics¹⁴⁰, although whether NEP in asthmatics not taking steroids is really less than normal subjects remains unknown. Thus, although previous reports certainly indicate that NEP modulation of tachykinin effects does occur in the airways, and that sputum SP levels are increased in chronic bronchitis, a downregulation of

NEP gene expression did not appear to play an important pathogenic role in our subject group. In fact, no significant reduction in NEP expression was seen for any of the groups, although it was interesting to note that three out of the four paroxysmal cough patients had expression levels lower than all of the other subjects. It is possible that undiagnosed viral infection (a recognised cause of paroxysmal cough) may have been a factor, as viral infection in animal models has lead to reduced NEP expression^{133,228}. Further studies in a larger group of subjects will be required to evaluate this possibility further.

The levels of expression of tachykinin receptors in airway inflammation have also been investigated. Early work, using radiolabelled SP detected binding in airway epithelium, submucosal glands, vascular endothelium and airway smooth muscle ⁵⁸. Bai et al ⁵⁹ examined resected specimens and donor organs from normal subjects and subjects with asthma or chronic bronchitis. They found a modest increase in NK1 receptor mRNA in smokers without airflow obstruction, but this appeared to revert toward a normal level in those with obstruction. No change in NK1 expression was seen in asthmatics, although NK2 expression was increased four fold in asthmatic lung samples containing membranous airways. In contrast, in another study of a smaller group of less well characterised asthmatic organ donors ⁶², NK1 mRNA was increased in lungs of asthmatics compared to normals. Our study differs by having its focus on the airway epithelium. We did not detect any significant increase in NK1 expression in our subjects with chronic bronchitis, although we did not have sufficient subjects to allow subgroup analyses based on obstruction (in fact at least five out of our nine subjects did have evidence of obstruction). We also did not see any increase in NK1 in our asthmatic subjects although we do not know what impact smoking or steroid use may have had on these findings. Interestingly, although NK2 quantitation was problematic, we did find a suggestion of increased expression in chronic bronchitis, consistent with Bai's findings. Although we detected NK3 mRNA we did not find

NK3 in all subjects and levels appeared to be quite low. Reliable quantitative comparisons were not possible.

Among the components of the tachykinin axis we investigated, the most striking difference between normals and subjects with chronic bronchitis was a relative increase in PPT-A gene expression in the latter group. Elevation in the SP content of BAL fluids and sputum from patients with chronic bronchitis and asthma has been noted by others^{124,126}. By analysing induced sputum, Tomaki et al found increases in SP levels of approximately 25 and 17 fold compared to normal in subjects with chronic bronchitis and asthma respectively¹²⁶. Changes in PPT-A gene expression in airway epithelium have not been previously reported however, and it has generally been accepted that SP is released from nerve endings. An increase in SP containing nerves was reported in the airways of asthmatics compared to autopsy material from normal subjects¹¹⁹. However, in another study comparing actual SP content in tracheas, subjects with mild asthma had lower SP levels than normals¹²². It is possible however, that this finding may be a reflection of a dynamic process whereby SP is released before significantly accumulating. This study also found no differences in SP content of pulmonary parenchyma between the groups. Interestingly, some authors, using immunohistochemistry techniques, have been unable to detect any SP containing nerves at all in bronchial biopsies of either asthmatics or normals¹²⁰. We also were unable to detect SP by immunohistochemistry in the epithelial samples we obtained, but could do so using a more sensitive tissue extraction and ELISA approach (chapter five). The only reported study to investigate PPT-A mRNA levels in chronic bronchitis used a rat model and investigated PPT-A mRNA in dorsal root ganglia¹¹⁸. Despite showing an increase in SP content of bronchoalveolar lavage fluid, there was no corresponding increase in PPT-A mRNA, which lead the authors to propose post-translational mechanisms to explain the increase in SP. A study of the effect of hyperoxia in neonatal rats showed an increase in PPT-A mRNA levels in the lung, but

this was not further localised¹²⁸. The current study indicates that local PPT-A mRNA production at the airway epithelial level may explain these findings.

Conclusion

The present study suggests that of the multiple elements of the tachykinin axis operative at the epithelial level in inflammation, an increase in tachykinin production rather than changes in NK receptor levels or changes in NEP may be the most important factor in increasing tachykinin effects in the local milieu of subjects with chronic bronchitis. In most discussions, it has been proposed that such an increase may be detrimental, leading to a worsening of local inflammatory responses. This hypothesis has led to proposals for novel therapies. In this regard, there has been considerable effort spent on the development of specific tachykinin receptor antagonists as potential anti-inflammatory therapies for asthma. In preliminary reports there has been some evidence to support the contention that such an approach may have therapeutic efficacy^{85,144,145}, however, a completed phase three study of such an agent has not yet been reported. When considering such an approach it is important to note that tachykinins also have trophic effects and appear to play a role in healing and protection of airway epithelium^{13,117}. Thus, an increase in local tachykinin production may in fact represent a protective response to injury. As with many biological phenomena the degree to which the potentially positive and negative effects of tachykinins are held in balance will be likely be determined by the net result of several complex interactions, which will need to be considered when designing tachykinin antagonist therapies.

Chapter Eight

Conclusions

In this thesis I have examined a number of aspects of tachykinin biology relevant to airways disease.

The specific questions I addressed were:

- 1) Are tachykinins involved in acute allergic responses to inhaled allergen in a large animal model relevant to human airways disease?
- 2) What are the mechanisms involved in the acute airway response to SP in this model?
- 3) Which components of the tachykinin “system” are expressed in human airway epithelium *in vivo*?
- 4) Is there evidence for a functional effect of SP on freshly harvested human airway epithelium?
- 5) How are the components of the tachykinin “system” modulated at the epithelial level in human airway inflammation?

The initial study using an ovine model of asthma demonstrated evidence for a role for tachykinins in the acute allergic airways response. Evidence for the involvement of tachykinins was obtained in three ways. First, the response was enhanced by inhibition of tachykinin degradation by using phosphoramidon, an inhibitor of NEP. Second, the response was inhibited by the drug CP-96,345, a specific inhibitor of the NK1 tachykinin receptor. Thirdly, the response

was inhibited by prior depletion of tachykinins with the agent capsaicin. These findings indicate that the sheep model of asthma is a useful tool for the investigation of the roles of tachykinins in airways disease, and provide evidence that tachykinin receptor agonists may have a therapeutic role in acute bronchoconstriction responses. Because of its cardiodepressive activity, CP-96,345 will not be suitable for human therapy, but newer agents which lack this limitation have been developed and hold greater promise. One of the limitations to the model was the relatively low percentage of immunised sheep which developed reproducible airways responses. In view of this, modifications to the immunisation protocol are the subject of ongoing studies. Nevertheless, the key question of the relevance of tachykinins in acute allergic airways responses was addressed and the data shows that these neuropeptides play an important role.

In the second study I sought to more specifically characterise the ovine airways response to SP. The principal findings were the demonstration of an acute, transient bronchoconstriction response, which was potentiated by NEP inhibition and inhibited by CP-96,345, findings in accord with the first study. Further, it was discovered that this response was subject to very rapid receptor desensitisation, so rapid in fact that airways resistance returned to baseline values within two minutes, even in the face of an ongoing infusion of SP. To our knowledge this is the first demonstration of desensitisation to the airway response to SP *in vivo*. The effect of this tachyphylaxis persisted for at least two hours after the initial infusion, which has important implications for the design of studies of candidate tachykinin antagonist therapies in this model. Taking this into account, subsequent studies sought to further characterise the mechanism of the response. These further studies indicted that the response to SP was largely mediated via a cholinergic mechanism, which could be entirely blocked with atropine. We also investigated whether histamine release was involved in the response, but found no evidence for this because the response could not be blocked by anti-histamine. No significant alteration of the response was

seen by prior treatment with indomethacin, thus there was no evidence for a role for prostaglandin release in the acute response to SP. A direct comparison of the effect of equimolar concentrations of SP and NK2, showed that SP was the more potent bronchoconstrictor, which, along with the effect seen with CP-96,345, indicates that NK1 receptors are primarily involved in the response. These studies, in conjunction with the *in vitro* analysis of sheep airway contractility undertaken by Dr Ann Reynolds illustrate certain similarities between human and ovine responses to tachykinins (evidence for SP acting via cholinergic mechanisms, and NK2 being the most potent bronchoconstrictor *in vitro*) and suggest the sheep as an ideal model system for the evaluation of new therapies which modulate tachykinin effects. Although SP was a more potent bronchoconstrictor than NKA when given intravenously, it is not possible to compare this directly to human responses, as the human response to NKA or SP administered intravenously has not been reported.

Following the physiological studies in the sheep model, the next series of investigations focused on the analysis of tachykinin and tachykinin receptor expression at the molecular level in airway epithelium. Initially it was proposed to examine both human and ovine airways, the latter to see if airways responses in "asthmatic" sheep could be correlated to expression of tachykinin receptors at the cellular and molecular level. However, for the reasons discussed in chapter five, further investigation of sheep airways in this way was limited by the relatively low number of sheep which had reproducible allergic responses, and because of technical limitations with the molecular biology analyses. Investigation of human airway epithelium was felt to be ultimately more relevant to the role of tachykinins in human disease. In fact there was actually little published information regarding the expression of these molecules at the molecular level in human airway epithelium. I ultimately wished to compare normal subjects to those with disease, therefore, I wished to develop a strategy which was not dependant upon the use of resected lung

specimens. Samples were therefore obtained by airway epithelial brush biopsy via fiberoptic bronchoscopy. Using a sensitive RT-PCR assay, mRNA for all three tachykinin receptor subtypes - NK1, NK2 and NK3 - was detected in the human airway epithelium, the first clear demonstration of these mRNAs at this site. NK1 receptor expression was also detected at the apical margin of ciliated columnar epithelial cells by immunohistochemistry. Importantly we also detected mRNA for the principal tachykinin precursor, PPT-A, in airway epithelial brush biopsy samples and in cultures of primary airway epithelial cells, suggesting that the airway epithelium itself may produce tachykinins. This finding suggests an alternate hypothesis to the release of tachykinins by axon reflexes, ie local airway epithelial production. Apart from the analysis of airway epithelium we also examined cells obtained by bronchoalveolar lavage. In these analyses, mRNA for PPT-A was rarely detected, and when we analysed these cells by ELISA, the amount of SP we detected was less than that we had detected in airway epithelial samples. Thus, although it remains possible that alveolar macrophages may upregulate PPT-A expression in inflammatory processes, we found that in areas of lung which are radiologically normal, little if any SP is produced by these cells. The results from this study indicated that the expression of all of the major molecules involved in tachykinin biology were expressed in airway epithelium.

Having demonstrated the presence of the key molecules involved in tachykinin biology in airway epithelium, I sought evidence for a functional effect of SP on this tissue. Based on published evidence that SP stimulates the release of chemotactic activity from epithelial cells I hypothesised that SP might upregulate IL8 production in airway epithelium. I found a small but consistent increase in IL8 mRNA when these samples were incubated with SP, thus consistent with the hypothesis that NK1 receptor expression detected by RT-PCR and immunohistochemistry indicated the presence of functional receptors. Further studies using airway epithelial cell lines were planned to fully characterise this response but unfortunately the lines

chosen could not be induced to increase IL8 production in response to SP. Whether the responses in explants is a particular feature of the complex mature epithelium, or whether alternate immortalised lines might show a similar response is uncertain. In further *ex vivo* studies I investigated whether steroids could induce a downregulation in epithelial NK1 receptor mRNA expression, as had been reported in other NK1 expressing tissues. Although a small decrease was found at steroid concentrations likely to be achievable *in vivo* (consistent with the notion that inhaled steroids might exert some of their anti-inflammatory effects via a reduction in tachykinin induced effects), high dose dexamethasone actually lead to an increase in NK1 mRNA, thus further dose ranging studies are required to clarify this issue.

In the final study I investigated whether disease states modulated the levels of expression of mRNA for the molecules involved in tachykinin biology. This study differed in its aims from previously published material in its attempt to simultaneously examine the expression of the various molecules and to attempt to draw conclusions based on the relative balance of expression between them. Although this study was limited due to difficulties in recruiting well characterised subject groups, which virtually precluded any conclusion to be made about the modulation of these molecules in asthma, we were able to recruit a group of subjects who clearly fit the criteria for smoking related chronic bronchitis. This is a relevant group because increased levels of SP had been reported in the sputum of patients with this disorder. The net effect of tachykinins in the airways will depend upon the production of tachykinins themselves, their degradation, principally by NEP, and the level of expression of tachykinin receptors. The principal finding was a relatively higher level of expression of PPT-A in the airway epithelium of subjects with chronic bronchitis than in our normal control group. This finding is important in two regards. Firstly it suggests that the PPT-A expression in airway epithelium described in chapter five is likely to be of functional importance. An upregulation in PPT-A mRNA is quite consistent with much of the

published literature which shows an increase in SP in airway inflammation and also the more recent data indicating that SP may have important healing effects in the airways. Our data adds a new aspect to this by providing evidence that local SP production rather than enhanced release from hypersensitive nerve endings alone may account for these findings. It is interesting to note that hyperoxic lung injury in rats lead to an increase in lung SP and PPT-A mRNA, whereas in a separate study using an alternate rat lung injury model, PPT-A mRNA was only assessed in the dorsal root ganglia, where no increase was seen, despite an increase in lung SP content. In addition, we did not find any significant alterations in the other relevant molecules we assessed - tachykinin receptor or NEP.

Thus a further refinement of the role of tachykinins in airways disease can be proposed (Figure 8-1). In this schema, cells resident in the airway epithelium itself may produce tachykinins in response to noxious stimuli and exert a local paracrine effect. This mechanism may co-exist with the presence of axon reflexes, but of itself does not depend on the presence of the anatomical arrangement of nerve fibres required for the axon reflex hypothesis, an arrangement which has not been demonstrated in the human airway. This schema suggests that modulation of tachykinin effects may also be achieved by the development of locally acting agents which regulate tachykinin production, in addition to agents which antagonise receptors.

Tachykinins as locally derived cytokine mediators

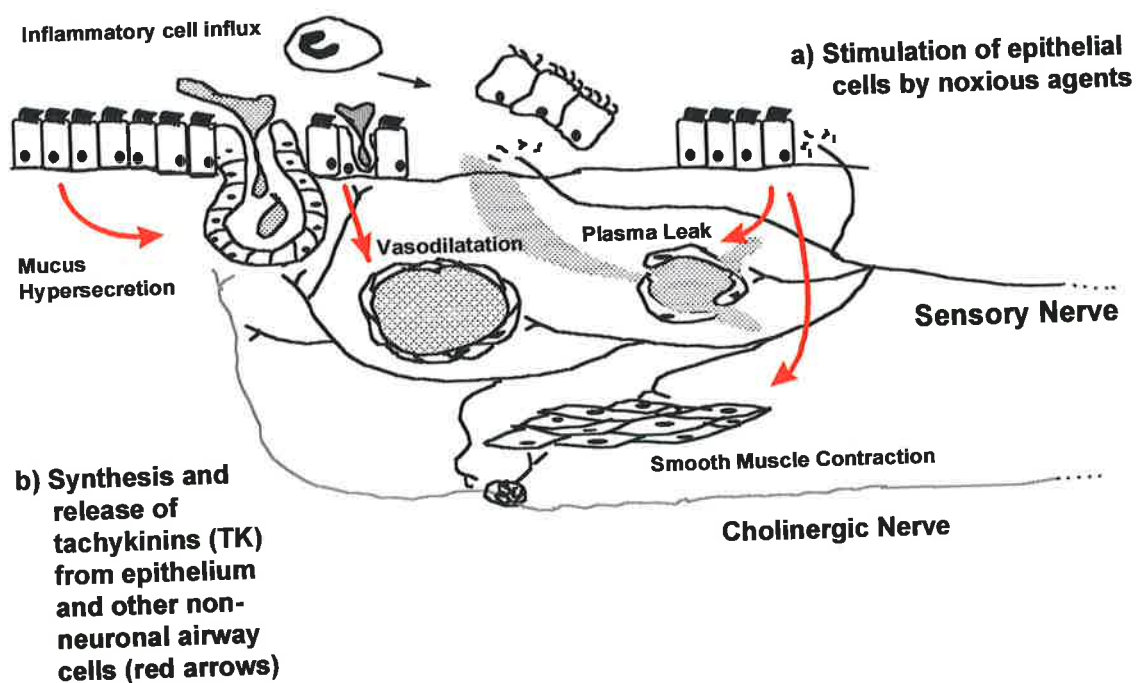


Figure 8-1. Evidence from the studies conducted in this thesis indicates a potential for local tachykinin production at the level of airway epithelium and perhaps other cellular components including smooth muscle. Further, gene expression for tachykinins was increased in the epithelium in airways inflammation. Thus, a local paracrine mechanism may in part account for the role of tachykinins in airways disease. This mechanism would not require the anatomical arrangement of sensory nerve fibres needed for an axon reflex mechanism.

Future Directions

There is much more scope for the investigation of tachykinin gene expression and production in non-neuronal cells, both *in vitro* and *in vivo* in the ovine and human settings. Further, the effects of disease on tachykinin receptors at the level of desensitisation and recycling has so far received little attention. The similarities between the reported human airways responses to tachykinins and the ovine responses discussed here illustrate the value of this model in assessing the modification of acute airways responses by new tachykinin receptor antagonist therapies. However for the investigation of allergic responses, a more reliable technique of sensitisation is desirable. New sensitisation protocols are the subject of ongoing studies. At present there is almost no information concerning tachykinin and tachykinin receptor expression at the cellular level in this model, thus further studies in this area are warranted if the model is to be maximally utilised.

The presence in the epithelium of the molecules important in tachykinin biology, and the ability to investigate them by the minimally invasive epithelial brush biopsy approach clearly will allow for more detailed analysis of the ways in which these molecules are regulated in specific disease states. Recruitment of more well characterised patients with asthma, paroxysmal cough and other diseases will greatly improve our understanding of the role of tachykinins in these diseases and the responses to current therapies. Technological advances such as the development of more antibodies specific for the tachykinin receptor subtypes, and the development of more streamlined molecular analysis approaches (such as "real time" PCR using fluorescent probes) will facilitate these studies. In this way the development of potential new therapies based on modulation of tachykinin responses will be enhanced.

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Appendix

Publications

Reynolds PN and Scicchitano R. (1996). Neuropeptides - Targets for asthma therapies? Current Therapeutics 37 (3) 13-15, 1996

Reynolds PN, Holmes MD and Scicchitano, R. (1997) The role of tachykinins in bronchial hyper-responsiveness.

Clinical and Experimental Pharmacology and Physiology. 24 (3-4): 273-280.

Reynolds PN, Rice AJ, Reynolds AM, Thornton, AT, Holmes MD and Scicchitano, R. (1997) Tachykinins contribute to the acute airways response to allergen in sheep actively sensitized to *Ascaris suum*. Respiriology. 2: 193-200.

Reynolds PN, Holmes MD and Scicchitano, R. Preprotachykinin A mRNA is expressed in airway epithelium and upregulated in inflammation

Paul Reynolds, Raffaele Scicchitano and Mark Holmes. Submitted.

Rice AJ, Reynolds PN, Reynolds AM, Holmes MD, Scicchitano R. Substance P induced increase in lung resistance is mediated by NK1 receptors, is atropine sensitive and exhibits tachyphylaxis. Submitted.

Abstracts

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