

Assessment and Mechanism of the Refractory Period in Asthma

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Abstract

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Hypothesis

The primary hypothesis of this thesis is that the refractory period in asthma is due to release of bronchoprotective mediator(s) within the airway and that these may be one or more of the pro-resolving lipid mediators such as lipoxins or resolvins.

Methods

1. Development of a LC-MS assay for the detection of pro-resolving mediators in sputum.
2. Development of an *in vitro* airway model using porcine and human bronchial rings.
3. A clinical study of mannitol-induced refractoriness with collection of urine and sputum to assess changes in prostaglandin and pro-resolving mediator levels.

Key Results

1. Approximate LC-MS limits of detection were 0.002, 0.01 and 0.04 pg/ μ l for PGE₂, LXA₄ and RvD₁ respectively. These LOD compare favourably with ELISA.
2. Serial LC-MS analysis of spiked sputum samples showed that LXA₄ appeared stable, while PGE₂ and RvD₁ showed some degradation over 5 months.
3. LXA₄, RvD₁ and MaR₁ had no significant effect on the histamine response of porcine bronchial rings.
4. Bronchoconstriction with high concentration mannitol rendered an airway refractory to further contraction with direct ASM agonists *via* an unknown mechanism.
5. In human bronchial rings, neither 10 nM LXA₄ nor 10 nM MaR₁ had any effect on bronchoconstriction induced by mast cell Fc ϵ R₁ activation.
6. In the clinical study, with bolus dosing the mean refractory index for mannitol was 24%. The degree of refractoriness to mannitol did not correlate with markers of disease severity.
7. Urinary PGD₂ and PGE₂ metabolite levels measured by ELISA showed no significant change in response to mannitol challenge. Sputum LXA₄ and RvD₁ levels measured by ELISA showed no consistent change in response to mannitol challenge.

Conclusions

This study has found no evidence of a role for pro-resolving mediators in the mechanism of refractoriness.

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Chapter 1 Introduction

1.1 Asthma

Asthma is a disease characterised by variable airflow obstruction and bronchial hyper-responsiveness associated with underlying airway inflammation. The clinical expression is very variable but symptoms will include one or more of wheeze, shortness of breath, chest tightness and cough (NHLBI, 2007). Acute episodes of bronchospasm on a background of chronic symptoms are characteristic. Acute bronchospasm may occur associated with triggers such as exercise or allergen. The severity of acute and chronic symptoms is highly variable between patients and with time in any given patient. Patients may experience exacerbations of their asthma, sometimes associated with an airway insult such as a respiratory tract infection, during which their symptoms worsen for a period of days or weeks; again, the frequency and severity of such exacerbations varies greatly from person to person.

The pathology and aetiology of asthma are still incompletely understood. Chronic airway inflammation is the key feature. It causes airway obstruction via oedema and increased mucus secretion, and in the long term can induce hyperplasia and hypertrophy of airway smooth muscle (ASM) – so-called airway remodelling - resulting in fixed airflow obstruction (Lemanske Jr & Busse, 2010). A temporary increase in the degree of inflammation, for example as a result of super-added infection, may be associated with an exacerbation of asthma symptoms; in fact markers of airway inflammation are a better predictor of exacerbations than day to day symptoms in some low symptom patient groups (Haldar et al., 2008). Inflammation also leads to airway hyper-responsiveness (AHR), in which people with asthma exhibit an exaggerated bronchoconstriction response to certain inhaled stimuli, and hence the symptoms of acute bronchospasm. AHR is discussed more fully in Section 1.2 below.

In the majority of (but not all) patients with asthma, inflammation is predominantly eosinophilic. Eosinophils release leukotrienes, promoting bronchoconstriction, and also a range of pro-inflammatory cytokines (Lemanske Jr & Busse, 2010). Other cells contribute to the maintenance of chronic inflammation. Helper T-lymphocytes release the Th2 subset of cytokines, particularly interleukin (IL)-4, IL-5, IL-10 and IL-13. These have a range of pro-inflammatory roles, such as promotion of eosinophil survival and IgE formation. Mast cells release bronchoconstricting mediators as described above, but also help to maintain an inflammatory state via cytokine release. Macrophages can also be activated by allergens to release pro-inflammatory mediators. Dendritic cells act as antigen-presenting cells for allergens, and stimulate switching of T-cells to the Th2 phenotype (Barnes, 2008).

Asthma is not only a disease of the immune system; structural airway cells play a major role in maintenance of the asthma phenotype. As well as being the organ of bronchoconstriction, ASM releases its own pro-inflammatory mediators. It can also undergo hypertrophy and hyperplasia in response to growth factors released as part of the inflammatory process (Pelaia et al., 2008). Epithelial cells can also generate inflammatory mediators, and the inflammatory process may damage epithelium, leading to repair processes which may themselves contribute to airway obstruction. Normal epithelium has a down-regulatory effect on ASM contraction and mast cell degranulation, which may be disrupted in asthma (Yang, Wardlaw, & Bradding, 2006). The genesis of the abnormal inflammatory process in asthma is poorly understood, but appears to be a mix of genetic and environmental factors, with susceptible individuals more prone to development of a pathological Th2-type cytokine profile in response to early-life exposure to challenges such as allergens and respiratory viruses (Busse & Lemanske Jr, 2001).

Treatment of asthma can be broadly divided into therapies aimed at modifying the underlying inflammatory process and those which provide symptomatic relief via bronchodilation. Mainstays of the former class are the corticosteroids, in both inhaled and oral form. More recently, biologic therapies targeting specific inflammatory cytokines have been developed, such as the anti-IL-5 mepolizumab (Barnes, 2008; Pavord et al., 2012). Most widely used of the bronchodilators are the inhaled β_2 -agonists such as salbutamol, but others include muscarinic antagonists, leukotriene receptor antagonists, and theophylline (British Thoracic Society, 2012). Many patients achieve extremely good control of their asthma symptoms using these drugs. However, control remains poor for many, and a better understanding of asthma pathophysiology and potential new targets for therapy is needed. The next section discusses airway hyper-responsiveness in more detail before considering the natural bronchoprotective phenomenon known as the refractory period. It is hoped that increased understanding of this relatively little-studied feature of asthma may reveal potential new avenues for therapeutic intervention.

1.2 Airway hyper-responsiveness and the refractory period

1.2.1 Airway hyper-responsiveness

The phenomenon of AHR was first noted by Alexander & Paddock (1921). It can broadly be divided into two components – a baseline 'persistent' component, thought to be related to airway remodelling, which does not vary greatly from day to day, and a more variable 'episodic' component which is considered to be more related to the current state of airway inflammation and may be exacerbated by factors such as recent allergen exposure. AHR is seen with two broad categories of stimuli – those which act

directly on ASM and those which act indirectly, via other mechanisms such as mast cell mediator release or neural stimulation. Very broadly, the persistent component of AHR is most strongly demonstrated by the response to direct challenges and the episodic component by the response to indirect challenges, though this is by no means a clear division and there is variability in AHR to direct challenges (Cockcroft & Davis, 2006). The most important directly acting agents are histamine and the muscarinic agonist acetylcholine; synthetic analogues of acetylcholine such as methacholine and carbachol are frequently used in clinical research. The most important indirectly-acting agents are probably allergens, which amongst other effects cause cross-linking of Immunoglobulin E (IgE) receptors on mast cells, triggering release of directly acting bronchoconstrictors such as histamine (Busse & Lemanske Jr, 2001). Exercise is another common indirect AHR trigger - the most likely mechanism is drying of the airway surface fluid, with the resulting change of osmolarity triggering release of inflammatory and bronchoconstrictor mediators from a variety of cell types, including mast cells (S. D. Anderson & Daviskas, 2000).

As stated above, the persistent component of AHR is considered to result primarily from features of airway remodelling; these include increased ASM mass and contractility, sub-basement membrane collagen deposition and reduced airway lumen diameter (Cockcroft & Davis, 2006); there is experimental evidence for *in vitro* hyper-responsiveness of asthmatic ASM (de Jongste et al., 1988). There is good evidence for the link between variable AHR and underlying airway inflammation, particularly eosinophilic inflammation. For example, an increase in airway sensitivity to histamine post-allergen challenge is associated with increased levels of eosinophils and mast cells in bronchoalveolar lavage fluid in asthmatic humans (Pin et al., 1992); AHR to adenosine monophosphate (an indirect challenge) is associated with sputum eosinophil

levels (Van Den Berge et al., 2001); inhalation of Th2 cytokines such as IL-4 and IL-5 is associated with increased AHR (Shi, Deng, et al., 1998; Shi, Xiao, et al., 1998); suppression of eosinophil recruitment reduces AHR to methacholine in an allergic mouse model (John, Thomas, Berlin, & Lukacs, 2005) and anti-IgE reduces AHR to allergen in humans (Fahy et al., 1997). Experiments in mice suggest Th1 cytokines may also be involved in AHR (Cui et al., 2005). However, the mechanism by which airway inflammation leads to AHR is still not well understood. Hypotheses include a link with airway neurological abnormalities, the increased availability of bronchoconstricting mast cell mediators resulting from increased mast cell levels in the airway, a role for the Th2 cytokine IL-13 acting directly on ASM, and epithelial damage causing a range of effects including exposure of sensory nerve endings, increased permeability to antigen, osmolarity changes and loss of an epithelium-derived bronchoprotective agent (Cockcroft & Davis, 2006; Morrison, Gao, & Vanhoutte, 1990). This thesis is particularly concerned with the last of these hypotheses, and this will be discussed in greater detail below, following an introduction to the phenomenon of the refractory period in asthma.

1.2.2 The refractory period

A poorly understood characteristic of airway hyper-responsiveness to bronchoconstricting stimuli is that in some individuals with asthma the magnitude of the bronchoconstriction diminishes on repeat challenge; this phenomenon has long been recognised, and is known as the refractory period (McNeill, Nairn, Millar, & Ingram, 1966). Figure 1.1 illustrates this; FEV₁, a simple clinical measure which declines with bronchial obstruction, falls following the first challenge and then recovers; on

administration of a second challenge the FEV₁ falls again but to a lesser extent. A refractory period was first noted in patients with exercise-induced asthma, approximately 50% of whom exhibit the phenomenon (Anderson & Schoeffel, 1982; N. G. Belcher, O'Hickey, Arm, & Lee, 1988). It usually lasts 2-4 hours (Edmunds, Tooley, & Godfrey, 1978; N. G. Belcher et al., 1988). The refractory period has been relatively little-researched, and such evidence as exists is often inconsistent; nevertheless it is an important phenomenon to study, as unravelling its mechanism may shed light on new aspects of asthma pathophysiology.

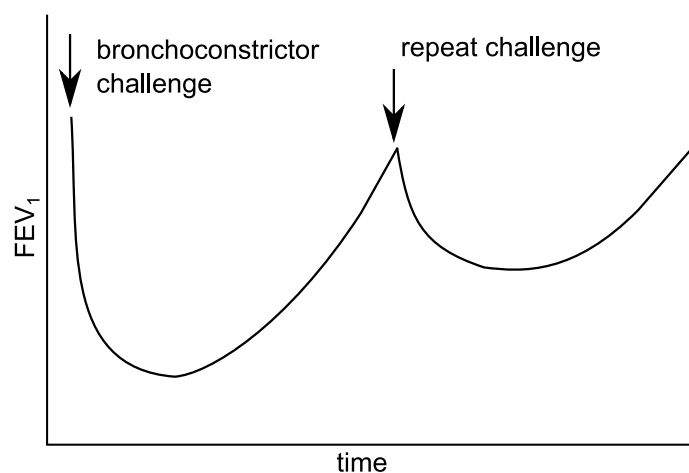


Figure 1.1. The refractory effect in asthma.

A wide range of indirect airway challenges are said to induce refractoriness; exercise is the most widely-studied, although there is disagreement as to whether induction of significant bronchoconstriction during the first challenge is necessary for refractoriness to a subsequent challenge (Hahn, Nogrady, Burton, & Morton, 1985; B. Wilson, Bar-Or, & Seed, 1990). Eucapnic hyperventilation has been used as a substitute for exercise challenge, and found by most, but not all, studies to induce refractoriness (Ben-Dov, Gur, Bar-Yishay, & Godfrey, 1983; Stearns, McFadden, Breslin, & Ingram, 1981). More recently inhaled mannitol has been used as an exercise surrogate, and a refractory period is seen (Larsson et al., 2011). Other bronchoconstrictor challenges known to

cause a refractory period include: cold dry air (Soto, Schnall, & Landau, 1985); hypertonic saline (N. Belcher, Rees, Clark, & TH, 1987); inhaled water (Kivity, Shalit, Greif, & Topilsky, 1989); adenosine monophosphate (AMP) (Daxun, Rafferty, Richards, Summerell, & Holgate, 1989); sulphur dioxide (Sheppard, Epstein, Bethel, Nadel, & Boushey, 1983); bradykinin (Fuller, Dixon, Cuss, & Barnes, 1987); sodium metabisulphite (Pavord et al., 1994) (although Wright, Zhang, Salome, & Woolcock (1990) found no refractoriness to this challenge); prostaglandin $F_{2\alpha}$ (Fish, Jameson, Albright, & Norman, 1984); leukotriene D_4 (Patrick J Manning, Watson, & O'Byrne, 1993) and inhaled ovalbumin in guinea pigs (Dorsch & Frey, 1981). A number of studies have demonstrated cross-refractoriness between different bronchoconstrictor challenges, whereby subjects who are refractory to the effect of one challenge also have a reduced response to a second stimulus (for example metabisulphite and exercise, Pavord et al. (1994); bradykinin and hypertonic saline, Rajakulasingam, Makker, Howarth, & Holgate (1995); exercise and allergen, Weiler-Ravell & Godfrey (1981).

A number of studies claim to show a refractory period induced by directly-acting bronchoconstrictors. For example P J Manning, Jones, & O'Byrne (1987) demonstrate tachyphylaxis to histamine challenge in 8 subjects with mild asthma. However, this is not borne out in other studies such as that of Ruffin, Alpers, Crockett, & Hamilton (1981). Polosa, Finnerty, & Holgate (1990) and Connolly, Stenton, Avery, Walters, & Hendrick (1989) concluded that refractoriness to histamine occurs in those with a mild degree of bronchial hyper-responsiveness but not in those with more severe disease. Tachyphylaxis to histamine has also been noted in non-asthmatic human airway smooth muscle strips in vitro (Knight, Stewart, & Thompson, 1992). Removal of the airway epithelium ablated the tachyphylaxis, as did pre-treatment with the H_2 -antagonist

ranitidine, suggesting that histamine tachyphylaxis may involve epithelial H₂ receptors and is not a direct smooth muscle effect.

More controversially several studies have claimed to show induction of refractoriness to the acetylcholine analogue methacholine. Magnussen, Reuss, & Jörres (1986) found that the magnitude of refractoriness to exercise was correlated with a reduction in sensitivity to methacholine given before and after the two exercise challenges. A study of seven patients by Inman, Watson, Killian, & O'Byrne (1990) found that inhaled methacholine induced protection against bronchoconstriction during exercise; a large retrospective analysis of 6 years of methacholine test data by Beach, Stenton, Connolly, Walters, & Hendrick (1995) found evidence of refractoriness as late as 24 hours, but not 48 hours. However, other studies such as that of P J Manning & O'Byrne (1988) found no refractoriness to acetylcholine.

As discussed in the previous section, AHR is associated with airway inflammation but its mechanism is still not completely understood; this clearly hinders attempts to gain a good understanding of refractoriness. Nevertheless, a number of possible mechanisms for the refractory period have been proposed. An early theory was that it may involve depletion of bronchoconstricting mediators (Edmunds et al., 1978). However, N G Belcher et al. (1988) measured histamine and neutrophil chemotactic activity levels following repeated exercise challenges and found no difference in levels after the first and second challenge. There was also no difference in catecholamine levels, implying that release of protective catecholamines following the first challenge was not responsible for refractoriness. Similarly, Broide et al. (1990) measured mast cell mediators in bronchoalveolar lavage fluid before and after exercise and found no evidence of depletion. More recently, Larsson et al. (2011) measured urinary leukotriene and PGD₂ metabolites after repeat mannitol challenges and found levels

increased to a similar degree after both challenges, again implying that refractoriness was not due to mast cell mediator depletion.

Another proposed mechanism for exercise-induced refractoriness is that of reduced airway water and heat loss during the second challenge – a study by Anderson & Schoeffel (1982) found no evidence for this. Polosa, Rajakulasingam, & Holgate (1991) demonstrated bradykinin-AMP cross-refractoriness, suggesting that this implied a common mechanism involving neuropeptides; however, as described above, other bronchoconstrictor agents not acting via neuropeptides also induce refractoriness, implying a common downstream mechanism. Other proposed mechanisms include down-regulation of the sensitivity or expression of receptors for bronchoconstricting substances, or increased metabolism of the bronchoconstrictors themselves (Kern, Smith, Patterson, Krell, & Bernstein, 1986). However, given the range of agents provoking refractoriness (and cross-refractoriness) a common pathway seems necessary. One unifying explanatory hypothesis is that the refractory period is due to a down regulation of bronchoconstrictor pathways occurring as a result of release of a bronchoprotective substance during the initial challenge.

Two substances have previously been proposed as the potential bronchoprotective agent. Nitric oxide (NO) was investigated by Hamad et al. (1999) but a NO synthase-inhibitor had no effect on metabisulphite-induced refractoriness. The other candidate is the cyclo-oxygenase (COX) product prostaglandin E₂ (PGE₂) (Pavord & Tattersfield, 1995). In favour of this hypothesis, inhaled PGE₂ has been shown to inhibit the airway response to several bronchoconstrictor challenges that are known to become refractory (Pavord et al., 1991). Additionally the refractory period is abolished by the cyclo-oxygenase inhibitor indomethacin in either oral (Wilson, et al, 1994) or inhaled form (Shimizu et al, 1997). A similar effect is seen in guinea-pigs sensitised to ovalbumin,

and refractoriness can be restored with inhaled PGE₂ (Dorsch & Frey, 1981). However, there is also contrary evidence, and it remains possible that other factors are involved in the pathogenesis of the refractory period; in another study a different cyclo-oxygenase inhibitor, flurbiprofen, did not show inhibition of refractoriness (O'Connor et al, 1994).

1.2.3 Epithelium-derived relaxing factors

In addition to the clinical phenomenon of the refractory period there is a wealth of evidence from laboratory studies for an epithelium-derived relaxing factor (EpDRF) which protects against bronchoconstriction in human and animal airway. This was first demonstrated by Flavahan, Aarhus, Rimele, & Vanhoutte (1985) who found that removal of bronchial epithelium from canine bronchial rings increased their contractile response to acetylcholine and histamine. The effect has since been extensively studied in a number of other species including humans (Fernandes, Preuss, Paterson, & Goldie, 1990), sheep (Hatziefthimiou et al., 2009) and pig (Morrison et al., 1990). Loss of this factor as a result of epithelial damage is a long-standing hypothesis for the mechanism of AHR in asthma (section 1.2.1 above).

It may be that this EpDRF and the factor responsible for the refractory period are one and the same, the hypothesis being that in mild asthmatics there are lower levels of bronchoprotective factor constitutively secreted in the airway but the potential remains for increased synthesis following bronchoconstriction. However, the notion of a single, diffusible EpDRF has been criticised as being implausible both for the range of bronchoprotective/relaxant effects it reputedly mediates and the requirement for it to diffuse considerable distances across the sub-epithelial layers in larger airways (Vanhoutte, 2013). It is possible that a range of bronchoprotective EpDRFs exist, which

may act directly on airway smooth muscle or via other messengers released from other cell types in the sub-epithelium. These factor(s) may be synonymous with, or overlap with, those responsible for refractoriness. To explain protection against indirect bronchoconstrictor challenges acting via different mechanisms, the most satisfying hypothesis is that these putative bronchoprotective agent(s) act downstream of all these bronchoconstriction pathways, presumably in a common pathway of airway smooth muscle contraction. It is of course possible that they have multiple sites of action, for example an effect on mast cells and on sensory nerves, resulting in refractoriness to challenges operating via different pathways.

A number of molecules have been proposed as EpDRFs; these include nitric oxide, arachidonic acid metabolites such as PGE₂, and the neurotransmitter γ -aminobutyric acid (GABA) (Vanhoutte, 2013). Clinical evidence for and against the role of nitric oxide and PGE₂ in refractoriness has been discussed in section 1.2.2 above. Here we propose that another group of candidates for the role of bronchoprotective factor(s) in the refractory period are the lipid molecules collectively known as pro-resolving mediators, and these are discussed fully in the next section.

1.3 Pro-resolving lipid mediators

1.3.1 Background

Resolution of inflammation has until recently been regarded as a passive process, involving a gradual decline in the concentration of pro-inflammatory mediators and cells (Serhan et al, 2008). It is now recognised, however, that a number of hitherto unknown classes of pro-resolution lipid mediators actively promote resolution. These mediators include molecules of the lipoxin, resolvins, protectins and recently discovered

maresin classes; they have been found in a wide variety of mammalian tissue types, including airway epithelium (Serhan, 2010). The structure, biosynthesis and effects of the various resolving mediator classes are considered in detail below.

1.3.2 Lipoxins

The lipoxins are the earliest known class of pro-resolving mediator, first characterised by Serhan in 1984 (Serhan et al, 1984). The lipoxins are arachidonic acid metabolites, as shown in Figure 1.2. Synthesis is mostly transcellular, via 5-lipoxygenase (5-LO) in leukocytes and either 15-lipoxygenase (15-LO) in epithelial cells or 12-LO in platelets (Romano & Serhan, 1992). However, 15-LO is also expressed in mast cells (Gulliksson et al., 2007), reticulocytes (Ford-Hutchison, 1991), eosinophils and macrophages (Sigal, et al 1993) raising the possibility of uni-cellular synthesis. There is species-to-species variation in lipoxygenase expression; for example porcine neutrophils can synthesise lipoxin A₄ (LXA₄) without other cell types present, as unlike human neutrophils they express both 5- and 12-lipoxygenase (Stahl, Morse, & Martin, 1997). The lipoxin class comprises lipoxin A₄ to E₄. Lipoxins C₄ to E₄ are synthesised by eosinophils and contain amino acid side chains (Steinhilber & Roth, 1989); they feature very little in the literature. Some pro-resolving actions of lipoxin A₄, the most widely studied of the class, are listed in Table 1.1.

The principal receptor for the lipoxins is the ALX receptor (also known by a number of other names - FPR2, FPRL1, FPRH1, RFP, HM63) a pertussis toxin-sensitive G-protein coupled receptor (GPCR). It is expressed on neutrophils, monocytes, activated T-cells, synovial fibroblasts and epithelial cells, and has been found in rat, mouse, and human tissues, particularly spleen, lung and to a lesser degree heart, placenta and liver

(Takano et al., 1997; Fukunaga et al, 2005; Chiang et al., 2006). Small peptides and proteins also interact with ALX, giving different signals to LXA₄. For example non-formylated MHC binding peptide, thought to be released from mitochondria during lysis of infected cells, can bind to ALX with high affinity and promote neutrophil chemotaxis which is reduced by LXA₄; annexin-1 acts at ALX to down-regulate neutrophil diapedesis (Chiang et al., 2006).

ALX appears to mediate pro-resolving effects via a number of different mechanisms in various cell types. Its activation down-regulates pro-inflammatory genes in epithelial cells, particularly those genes regulated by NF- κ B (Gewirtz et al., 2002). In neutrophils, LXA₄ appears to act via ALX to regulate the polyisoprenyl phosphate signalling pathway (B D Levy et al., 1999) thus inhibiting superoxide anion generation. There is also evidence for the involvement of protein kinase C and the p38 MAPK pathway in signal transduction (Fiore, Romano, Reardon, & Serhan, 1993; Ohira et al., 2004). Increased neutrophil apoptosis by macrophages appears to be coupled to changes in the actin cytoskeleton (Paola Maderna et al., 2002) and a more recent study suggests that protein kinase C-dependent internalisation of ALX is required for this process (P Maderna et al., 2010). In epithelial cells, LXA₄ acts via ALX to stimulate an increase in intracellular calcium (Caroline Bonnans, Mainprice, Chanez, Bousquet, & Urbach, 2003), an effect also seen in neutrophils and monocytes (C Bonnans, Chanez, & Chavis, 2004).

Lipoxins also bind to the CysLT₁ receptor, binding to it with affinity equal to that of leukotriene D₄ (LTD₄) (Gronert et al, 2001); in fact LXA₄ will contract guinea-pig airway smooth muscle at micromolar concentrations (Jacques, Spur, Crea, & Lee, 1988). Machado et al. (2006) showed that LXA₄ activates the AhR receptor on dendritic

cells, triggering expression of the protein Suppressor of Cytokine Signalling-2 (SOCS-2), and thus reducing pro-inflammatory cytokine production. There is also apparent cross-talk between the ALX receptor and the growth factor family of receptors, for example leading to inhibition of vascular endothelial growth factor-stimulated endothelial cell proliferation (Fierro et al, 2002; Chiang et al. 2006).

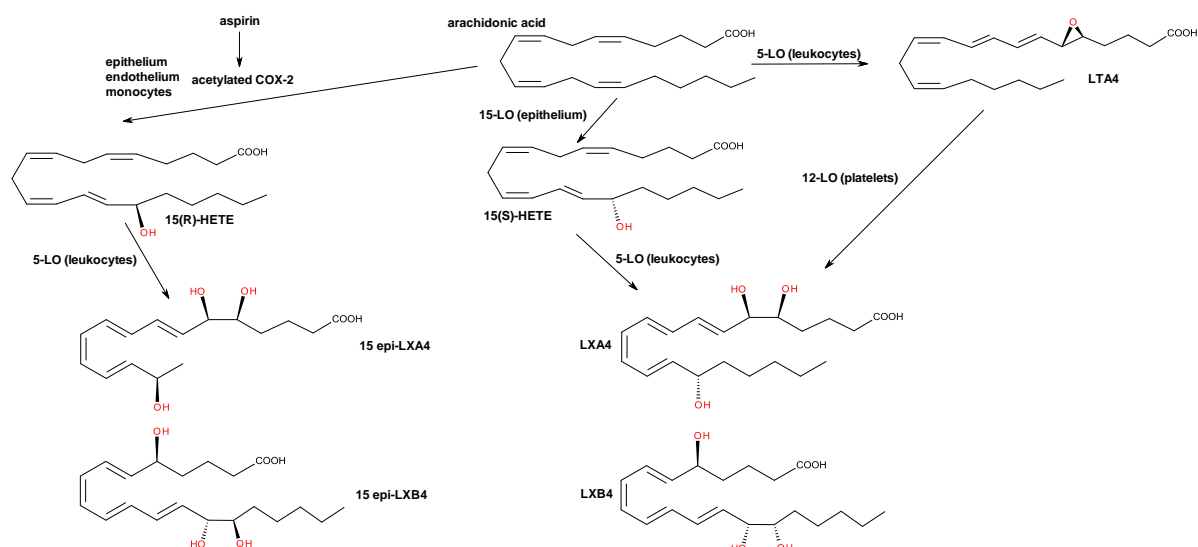


Figure 1.2. Structure and synthesis of lipoxin A₄ and B₄ and aspirin-triggered lipoxins.

Effect	Location	Reference
Reduced neutrophil infiltration	Mouse ear, dorsal air pouch	Takano et al., 1997
Reduced eosinophil infiltration	Mouse ear-skin & airway	Levy et al., 2002
Reduced lymphocyte infiltration	Mouse airway	Levy et al., 2002
Increased macrophage phagocytosis of apoptotic neutrophils	Human macrophages in vitro	Godson et al. 2000
Reduced IL-8 secretion by monocytes	In vitro monocytes from humans with asthma	Bonnans et al., 2002
Reduced Th2 cytokines (IL-5, IL-13), eotaxin, PGE₂, CysLT)	Mouse	Levy et al., 2002

Reduced activation of NF-κB	Mouse expressing human ALX	Devchand et al., 2003
Reduced serum IgE after allergen sensitisation	Mouse	Levy et al., 2002
Blocks LTD₄-initiated airway smooth muscle constriction	In vitro	Dahlen et al., 1988
Inhibited bronchoconstriction to methacholine	Mouse airway	Levy et al., 2002
Inhibits LTC₄-mediated bronchoconstriction in asthma	Human	Christie, Spur, & Lee, 1992

Table 1.1. Some pro-resolving effects of lipoxin A₄.

In vivo, LXA₄ is rapidly metabolised by monocytes to 15-oxo-LXA₄, 13,14-dihydro-15-oxo-LXA₄ and 13,14-dihydro-LXA₄ (C N Serhan, Fiore, Brezinski, & Lynch, 1993); the prostaglandin dehydrogenase and reductase enzymes likely to be responsible were identified by Clish, Levy, Chiang, Tai, & Serhan (2000). There is no published data on the long term stability of lipoxins in aqueous solution. Cayman Chemicals, the principal manufacturer of synthetic lipoxins, recommends that aqueous solutions of LXA₄ are used within 24 hours, but this is precautionary only (personal communication). Where aqueous samples, such as sputum, have been acquired for analysis of lipoxin content, they have been stored at -80°C prior to analysis (for example Vachier et al., 2005).

A relatively large number of studies have examined the relationship between asthma and LXA₄, both *in vivo* and *in vitro*. In the only clinical trial of pro-resolving mediators in asthma to date, Christie et al investigated the effect of inhaled lipoxin in human asthmatics, and found that in concomitant inhalation with leukotriene C₄ (LTC₄) it reduced bronchoconstriction at a given LTC₄ dose compared to LTC₄ alone. Inhaling LXA₄ alone had no bronchodilator effect (Christie et al., 1992). In a murine model of

asthma, LXA₄ and ALX receptor expression were both increased by allergen challenge, and administering a stable LXA₄ analogue reduced levels of pro-inflammatory mediators. Inhibition was selective; interleukin-5 (IL-5) and interleukin 13 (IL-13) were reduced in bronchoalveolar lavage (BAL) fluid, as were levels of eotaxin, PGE₂ and cysLT, but interleukin-12 (IL-12), tumour necrosis factor α (TNF α) and LTB₄ were not. Transgenic expression of human ALX in murine leukocytes reduced pulmonary inflammation and eosinophil infiltration (Bruce D Levy et al., 2002).

A number of studies have measured LXA₄ in the blood or urine of asthmatic subjects. Patients with severe asthma had reduced levels of blood LXA₄ compared to moderate asthmatics in a study of 36 patients (Bruce D Levy et al., 2005). Wu et al (Wu, Yin, Zhang, & Tao, 2010) measured LXA₄ levels and levels of 5-LO and 15-LO mRNA in blood from 106 asthmatic children and 40 controls. They found that mild, moderate and severe asthmatics all had higher LXA₄ levels than the controls, but that levels declined with increasing asthma severity. 5-LO mRNA expression increased with asthma severity, whereas 15-LO expression decreased. Expression in controls was lower than in any of the asthma groups. Tahan et al (Tahan, Saraymen, & Gumus, 2008) compared blood LXA₄ levels in asthmatic children who had positive and negative bronchoconstriction responses to exercise. There was no significant difference in pre-exercise levels in the two groups, but post-exercise the group demonstrating bronchoconstriction showed lower LXA₄ levels than the group which did not constrict. Sanak et al. (2000) measured levels of LXA₄ and 15-epi-LXA₄ in the activated blood of aspirin-intolerant and aspirin-tolerant asthmatics and normal controls. There was no difference in LXA₄ levels between controls and the aspirin-intolerant group; however, the aspirin-tolerant group had a 2-fold greater level of LXA₄. In the aspirin-intolerant group levels of 15-epi-LXA₄ were decreased. This finding was not reproduced in a

study by Celik, Erkeköl, Misirligil, & Melli (2007) who measured LXA₄ in the activated blood of severe asthmatics using a similar method. There was a decreased level of LXA₄ in the severe asthma group compared to mild or moderate disease, but no significant difference between aspirin-tolerant and intolerant groups. Yamaguchi et al. (2011) examined urinary LXA₄ and 15-epi-LXA₄ in aspirin-tolerant and intolerant asthma and healthy controls. HPLC was used to separate the compounds followed by ELISA for quantitation. The LXA₄ concentration was significantly lower than the 15-epi-LXA₄ concentration in the asthmatic subjects; the aspirin-intolerant asthma group had lower 15-epi-LXA₄ levels than the aspirin-tolerant group. 15-epi-LXA₄ concentration was not related to asthma severity.

Of greatest interest are studies measuring LXA₄ in the airway. The first evidence of LXA₄ in human airway is attributed to Lee et al. (1990). Bronchoalveolar lavage (BAL) fluid was collected from patients with a range of respiratory diseases, including two with asthma, and analysed for the presence of LXA₄ by GC-MS. It was present in one sample at a level of 2.8 ng/ml and not detected in the other. None was detected in normal controls. A further study measuring LXA₄ in BAL fluid from asthmatic patients found much lower levels in severe asthma compared to non-severe. Expression of the enzymes 15-LO subtype 2 and COX2 was also down-regulated in blood, BAL fluid, and endobronchial biopsy (EBB) specimens from these patients, though 15-LO subtype 1 expression was significantly increased in EBB samples (Planaguma et al., 2008). In this study ALX receptor expression was reduced in blood in severe and non-severe asthma compared to healthy individuals. In another BAL study, alveolar macrophages from severe asthmatics secreted less LXA₄ than normal controls (Bhavsar et al., 2010). There was differential suppression of LTB₄ and LXA₄ by dexamethasone, increasing the LTB₄:LXA₄ ratio. However, using liquid chromatography Chavis et al found no

LXA₄ secreted from calcium ionophore-stimulated alveolar macrophages or peripheral blood monocytes; it was secreted by stimulated neutrophils from the blood of untreated asthma patients, but not healthy controls (Chavis, Vachier, Godard, Bousquet, & Chanez, 2000). Airway smooth muscle cells from resected lung have also been studied (Parameswaran et al., 2007a). No ALX receptor expression was found in these cells; however, LXA₄ blocked LTE₄-primed migration toward platelet-derived growth factor (PDGF), suggesting that this is mediated via antagonism of the CysLT₁ receptor. Natural killer (NK) cells and Type 2 innate lymphoid cells (ILC2 cells) in human lung and peripheral blood do express ALX in both healthy and asthmatic subjects (Barnig et al., 2013). LXA₄ increases NK cell-mediated eosinophil apoptosis and decreases IL-13 release by ILC2 cells.

There are two literature report of LXA₄ measurement in sputum supernatant (Bonnans et al., 2002; Vachier et al., 2005). In the first study levels were measured by ELISA to be 0.1, 1.3 and 0.8 ng/ml in controls, mild and severe asthma respectively. In the second study LXA₄ levels ranged from 0.2 ng/ml in controls to 1 ng/ml in mild asthma. Again, patients with severe asthma had reduced levels of LXA₄ compared to those with mild disease. LXA₄ has also been measured in exhaled breath condensate of children recovering from status asthmaticus (Hasan, O'Brien, & Mancuso, 2012). Levels were significantly lower compared with control subjects (5.6 vs. 10.5 ng/ml, as measured by ELISA validated by GC-MS). B D Levy et al. (1993) used ELISA to measure LXA₄ in nasal lavage fluid from aspirin sensitive-asthma patients after they each ingested a dose of either aspirin or placebo. Aspirin challenge provoked an increase in LXA₄ level in each patient.

Overall then, there is evidence from both laboratory-based and clinical studies that LXA₄ levels in the lung, and systemically, are elevated in mild asthma compared to

healthy controls, but that this increase is smaller (or levels actually decrease) in more severe disease.

1.3.3 Resolvins

The resolvins are a relatively newly discovered class of lipid pro-resolving mediators; the class consists of D series (RvD 1-4) and E series (RvE 1 and 2) resolvins derived from docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) respectively (Serhan et al., 2002). As with the lipoxins, synthesis is considered to be principally trans-cellular; the biosynthesis of RvD₁ is illustrated in Figure 1.3. An aspirin-triggered form of RvD₁ (AT-RvD₁) exists, biosynthesised via acetylated COX2 and 5-LO. Its structure differs from RvD₁ only in the stereochemistry at carbon 17, and it exhibits pro-resolving activity (Sun et al., 2007). RvD₁ acts at two receptors expressed on leukocytes – the lipoxin receptor ALX and another GPCR, GPR32 (Krishnamoorthy et al., 2010). RvE₁ is also known to act at two receptors – GPCR ChemR23 and the LTB₄ receptor BLT1, for which it is a partial agonist (Arita et al., 2005, Arita et al., 2007). Some of the pro-resolving effects of the resolvins are shown in Table 1.2; this is illustrative only, and by no means an exhaustive list.

Effect	Location	Reference
Resolvin D1		
Stops neutrophil recruitment & transmigration	In vitro human neutrophils Murine peritonitis	(Sun et al., 2007)
Blocks LTB₄-mediated actin re-organisation in neutrophils	In vitro human neutrophils	(Krishnamoorthy et al., 2010)
Resolvin E1		
Enhances macrophage phagocytosis of apoptotic neutrophils	In vitro human cells	(Ohira et al., 2010)
Attenuation of LTB₄-BLT1 pro-inflammatory signalling (NFκB activation)	In vitro human neutrophils	(Arita et al., 2007)
Decreases airway eosinophil and lymphocyte recruitment in response to allergen	Murine asthma model	(Aoki et al., 2008)
Reduces airway hyper-responsiveness to inhaled methacholine	Murine asthma model	(Aoki et al., 2008)

Table 1.2. Some pro-resolving effects of resolvins.

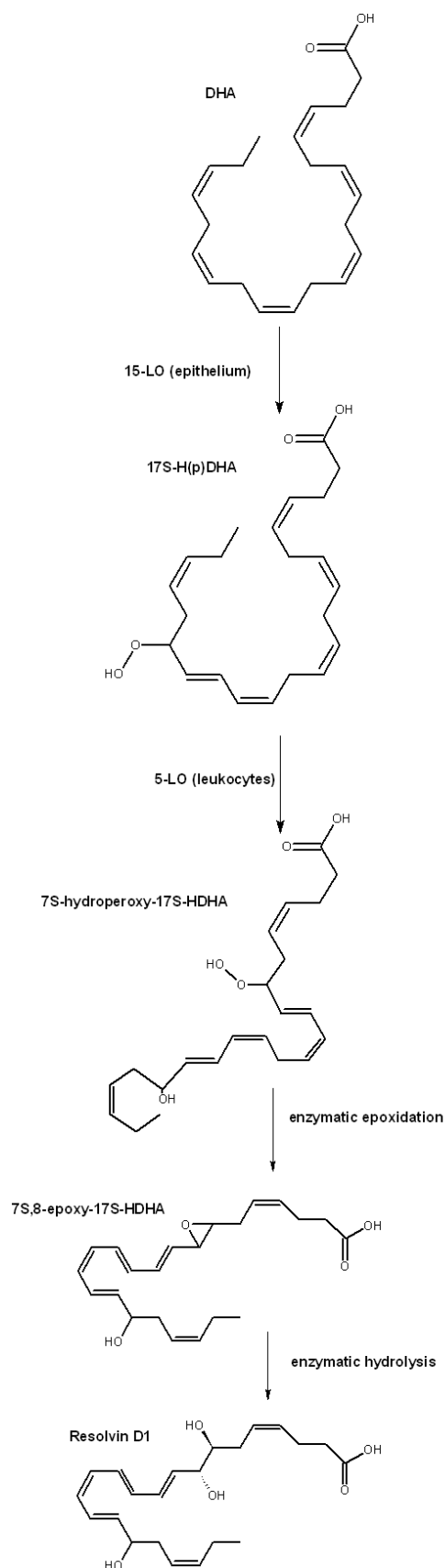


Figure 1.3. Synthesis of resolvin D1 from DHA. The final 3 steps are catalysed by 5-LO (based on Y.-ping Sun et al., 2007).

As with the lipoxins, the metabolism of the resolvins is not fully established; RvE₁ is the most widely studied. Four pathways for RvE₁ metabolism have been identified, via 12-oxo, 18-oxo, 19-hydroxy and 20-hydroxy intermediates; some products retain some pro-resolving activity (Hong et al., 2008). The predominant pathway varies by species, organ and cell type; for example in murine lung, RvE₁ is primarily inactivated by conversion to 18-oxo-RvE₁ by the enzyme 15-prostaglandin dehydrogenase (15-PGDH) whereas ω -oxidation to form 20-hydroxy-RvE₁ is a major route in human peripheral blood neutrophils (Arita et al., 2006). RvD₁ is metabolised by eicosanoid oxidoreductase enzymes to 8 and 17-oxo products; the 17-oxo product is biologically inactive (Sun et al., 2007). As with the lipoxins, there is no published data on the long term stability of resolvins in aqueous solution.

Compared to the lipoxins there has been less investigation of the potential role of resolvins in asthma, and work to date consists of *in vitro* and mouse studies; E-series resolvins have been more extensively studied. Aoki et al. (2008) used an ovalbumin-sensitised murine model of asthma to study the effects of RvE₁ administration. Peritoneal administration provoked decreases in airway hyper-responsiveness, ovalbumin-specific IgE, eosinophil and lymphocyte recruitment, interleukin-13 and respiratory tract mucus secretion. A further study found that RvE₁ administration had anti-inflammatory effects whether given at the time of ovalbumin sensitisation or challenge (Aoki et al., 2010). Again using a mouse model, Haworth, Cernadas, Yang, Serhan, & Levy (2008) found that RvE₁ suppressed BAL fluid levels of IL-23 and IL-6 and increased interferon-gamma levels. Mouse natural killer (NK) cells express receptors for RvE₁; increased numbers of NK cells appear following the end of allergen

exposure, and RvE₁ increases NK cell migration in vivo and increases killing of inflammatory cells (Haworth, Cernadas, & Levy, 2011). Bilal et al. (2011) used a murine asthma model in both wild-type and Fat-1 transgenic mice, which display increased endogenous lung n-3 polyunsaturated fatty acids such as DHA and EPA. Following airway sensitisation and challenge Fat-1 mice had decreased leukocyte accumulation in bronchoalveolar lavage fluid and lung parenchyma, reduced airway hypersensitivity to methacholine and lower BAL fluid concentrations of a range of pro-inflammatory cytokines. Increased levels of RvE₁ and protectin D₁ were found in homogenised lung tissue from the Fat-1 mice.

One study has examined the role of D-series resolvins in asthma – Rogerio et al. (2012) used a mouse model to examine the effects of RvD₁ and AT-RvD₁ administration, and found that RvD₁ decreased airway eosinophilia, IL-5 levels, mucus metaplasia and degradation of the anti-inflammatory nuclear factor IκBα, while AT-RvD₁ had even more potent pro-resolving actions, effecting a decrease in the levels of a range of inflammatory peptide and lipid mediators, increasing the rate of eosinophil clearance, and speeding up resolution of airway hyper-responsiveness. AT-RvD₁ was also more resistant to metabolic inactivation by macrophages.

1.3.4 Protectin D₁

Protectin D₁ (PD₁), also known as Neuroprotectin D₁, is another DHA-derived molecule with a range of pro-resolving effects (structure shown in Figure 1.4 below). For example, at 10 nM concentration it attenuates human neutrophil transmigration, and it is a regulator of neutrophil infiltration in a mouse model of peritonitis (Charles N Serhan et al., 2006). Mouse kidneys produce PD₁ (and RvD₁) in response to

ischaemia/reperfusion injury, and administering PD₁ before inducing ischaemia/reperfusion reduced the degree of injury (Duffield et al., 2006). PD₁ also stimulates murine cardiac and neural stem cell differentiation at nanomolar concentrations (Yanes et al., 2010). When injected intrathecally, PD₁ reduces transient receptor potential subtype V₁ (TRPV₁)-mediated inflammatory pain in the mouse (Park et al., 2011).

PD₁ appears to play a role in the lung. Transgenic mice engineered to convert n-6 polyunsaturated fatty acids to the n-3 acids DHA and EPA exhibit reduced markers of airway inflammation in an ovalbumin-sensitised asthma model – less hypersensitivity to methacholine, reduced airway eosinophils, and increased levels of PD₁ (and RvE₁) post methacholine challenge (Bilal et al., 2011). PD₁ is synthesised by human eosinophils, and suppresses eosinophil chemotaxis though not degranulation; in the presence of DHA eosinophils from patients with severe asthma exhibit reduced synthesis of PD₁ compared to healthy controls (Miyata et al., 2013). PD₁ has been detected by mass spectrometry in exhaled breath condensates, with a small clinical study showing reduced levels in human breath during asthma exacerbation compared to healthy controls (Bruce D Levy et al., 2007).

The receptor by which PD₁ acts has yet to be determined, but its inhibitory effect on TRPV₁ is blocked by pertussis toxin, implying a GPCR-mediated mechanism (Park et al., 2011). As with LXA₄ and RvD₁ an aspirin-triggered form of PD₁ also exists, and has been shown to decrease transendothelial neutrophil migration and enhance efferocytosis of apoptotic neutrophils by macrophages (Charles N Serhan, Fredman, et al., 2012).

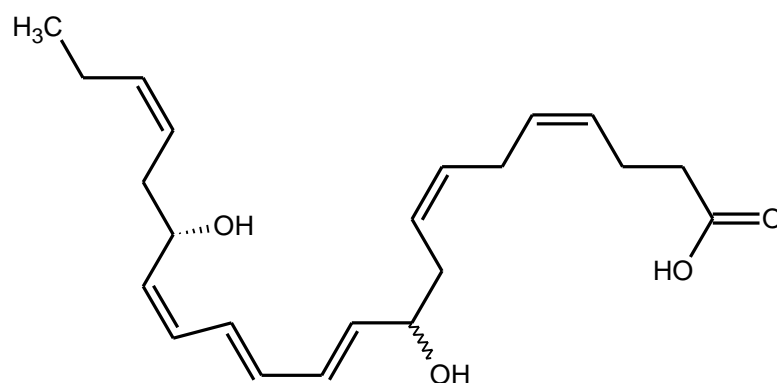


Figure 1.4. Structure of Protectin D₁(based on Charles N Serhan et al., 2006).

1.3.5 Maresin-1

Maresin-1 (MaR1) is a recently discovered DHA-derived pro-resolving lipid synthesised uni-cellularly by macrophages, particularly the M2 subtype; it has been demonstrated to enhance macrophage phagocytosis, reduce neuropathic pain in mice and increase tissue regeneration in *Planaria* flatworms (Serhan et al., 2009; Dalli & Serhan, 2012; Serhan et al., 2012). MaR1 has been detected in human synovial fluid (Giera et al., 2012) and recent work has demonstrated pro-resolving effects of MaR1 in the lung. In a study of bronchial epithelium exposure to organic dust, MaR1 reduced IL-6 and IL-8 production following treatment of a human bronchial epithelial cell line (BEAS-2B) with hog confinement facility-derived organic dust. MaR1 also reduced release of TNF α , IL-6 and CXCL1 in response to organic dust challenge in a mouse lung slice model (Nordgren et al., 2013). Gong et al. (2014) demonstrated inhibition by MaR1 of lipopolysaccharide-stimulated production of TNF α , IL-1 β , IL-6 and pro-inflammatory chemokines, along with reduced pulmonary myeloperoxidase activity and neutrophil infiltration, in a mouse model of acute lung injury. To date there have been no clinical studies of MaR1 in the lung.

Mediator	Receptors	Receptor type	References
LXA₄	ALX	GPCR (G _i)	Bäck et al., 2014
	CysLT ₁	GPCR (G _i /G _q)	Bäck et al., 2014
RvD_x	ALX	GPCR (G _i)	Krishnamoorthy et al., 2010
	GPR32	GPCR	Krishnamoorthy et al., 2010
RvE_x	ChemR23	GPCR	Arita et al., 2005
	BLT ₁	GPCR (G _i /G _q)	Arita et al., 2007
PD₁	unknown	Pertussis toxin-inhibited	Park et al., 2011
MaR1	unknown		
PGE₂	EP ₁₋₄	GPCR (rhodopsin type). EP ₂ and EP ₄ are cAMP mediated, with inhibitory effects; EP ₁ and EP ₃ increase intracellular Ca ²⁺ .	Machado-Carvalho, Roca-Ferrer, & Picado, 2014

Table 1.3. Summary of known pro-resolving mediator receptors.

	Airway cell types known to express receptors	Mediator actions on airway cells	References
LXA₄	ALX: Epithelial cells, neutrophils, activated T-cells, NK cells	Reduced eosinophil & lymphocyte infiltration, reduced serum IgE after allergen sensitisation, inhibits bronchoconstriction to	Bruce D Levy et al., 2002; Barnig et al., 2013; C. Bonnans et al., 2002; Jacques et al., 1988; Parameswaran et al., 2007a; Dahlén et al.,

	<p>CysLT₁:</p> <p>Airway smooth muscle, epithelial cells, bronchial fibroblasts, T_H2 cells</p>	<p>methacholine (mouse).</p> <p>Increases NK cell-mediated eosinophil apoptosis (human cells).</p> <p>Reduces IL-5, IL-13, eotaxin, PGE₂ and cysteinyl-LT in BAL fluid (mouse).</p> <p>Reduces IL-8 secretion by monocytes (asthmatic human cells).</p> <p>Blocks LTE₄-primed ASM cell migration toward PDGF (human cells).</p> <p>Contracts airway smooth muscle strips; blocks LTD₄-initiated airway smooth muscle constriction (guinea-pig in vitro).</p>	1988
RvD_x	<p>GPR32:</p> <p>Monocytes</p> <p>ALX:</p> <p>Epithelial cells, neutrophils, activated T-cells, NK cells</p>	<p>Decreases airway eosinophilia, IL-5 levels, mucus metaplasia and degradation of IκBα (mouse).</p>	Krishnamoorthy et al., 2010; Rogerio et al., 2012
RvE_x	<p>ChemR23:</p> <p>NK cells,</p>	<p>Decreases airway hyper-responsiveness, ovalbumin-specific IgE,</p>	Watanabe et al., 2009; Arita et al., 2007; Haworth et al., 2011;

	<p>monocytes, dendritic cells</p> <p>BLT₁:</p> <p>ASM, neutrophils, eosinophils, T-cells</p>	<p>eosinophil and lymphocyte recruitment, interleukin-13 and respiratory tract mucus secretion (mouse).</p> <p>Suppresses BAL fluid levels of IL-23 and IL-6 and increases interferon-gamma levels (mouse).</p> <p>Increases NK cell migration in vivo and increases killing of inflammatory cells (mouse).</p>	<p>Aoki et al., 2008; Haworth et al., 2008</p>
PD₁	Receptor unknown	<p>Reduces airway eosinophil and T-cell recruitment, mucus, IL-13, cysteinyl-LT, PGD₂ and airway hyper-responsiveness to methacholine (aero-allergen challenged mouse model)</p>	<p>Bruce D Levy et al., 2007</p>
MaR1	Receptor unknown	<p>Reduces IL-6 and IL-8 production following organic dust treatment of epithelial cells (human cells).</p> <p>Reduces release of TNFα, IL-6 and CXCL1 in organic dust-challenged lung slices (mouse lung slice model).</p> <p>Inhibits LPS-stimulated production of TNFα, IL-1β, IL-6 and chemokines; reduces</p>	<p>Nordgren et al., 2013; Gong et al., 2014</p>

		pulmonary myeloperoxidase activity and neutrophil infiltration (mouse model of acute lung injury).	
PGE₂	EP₁₋₄: Epithelial cells, macrophages, eosinophils, neutrophils, mast cells, T-cells, ASM cells	<p>Inhibits mast-cell mediator release via EP₂ (human cells).</p> <p>Inhibits ASM proliferation (human cells).</p> <p>Contractile (EP₁) and relaxant (EP₂) effects on ASM (guinea pig).</p> <p>Inhibits macrophage synthesis of pro-inflammatory chemokines including TNFα and IFNγ via EP₄ (human cells).</p> <p>Reduces cytokine production by CD4⁺ T-cells (allergic mouse model).</p> <p>Inhibits eosinophil trafficking and degranulation via EP₂ (human cells).</p> <p>Decreases secretion of TNFα by dendritic cells (human cells).</p>	Machado-Carvalho et al., 2014; Oliver & Black, 2006; Kay, Yeo, & Peachell, 2006; Takayama et al., 2002; S��fholm et al., 2013; Zaslona et al., 2013; Sturm et al., 2008; Sastre & del Pozo, 2012

Table 1.4. Summary of known effects of pro-resolving mediators in airway cells.

1.4 Pro-resolving mediators and refractoriness

As outlined in Section 1.2.2, a number of theories have been advanced to explain the refractory period. Depletion of bronchoconstricting mediators was one early theory (Edmunds et al., 1978). However, pre- and post-challenge mediator levels have been measured by a number of researchers, and no depletion found – Belcher et al. (1988) measured histamine before and after exercise; Broide et al. (1990) measured mast cell mediators in BAL fluid before and after exercise; Larsson et al. (2011) measured urinary leukotriene and PGD₂ metabolites after repeated mannitol challenges. Another prominent theory is that of down-regulation of receptors for bronchoconstricting mediators (e.g. Kern et al., 1986). However, although a possibility, this does not in itself readily explain the range of agents operating via different mechanisms which can cause refractoriness, or cross-refractoriness between them. For this reason, we favour an epithelium-derived bronchoprotective factor as the mechanism of refractoriness. As discussed in 1.2.3, an EpDRF could act, either directly or *via* a cascade of other mediators, to down-regulate a range of stimulatory receptor types on ASM, or to inhibit a common pathway of ASM contraction. It could also potentially act on other cell types, such as mast cells, to inhibit the bronchoconstriction pathway more proximally.

The pro-resolving mediators are promising candidates for a role in the refractory period in a number of respects. As discussed in detail above, they exhibit widespread anti-inflammatory effects in diverse tissue types including lung. Of particular interest to refractoriness to mast cell-mediated challenges such as allergen and exercise is work on mast cell inhibition conducted in our laboratory. *In vitro* studies have shown inhibition of mast cell histamine release by an epithelium-derived factor unaffected by

indomethacin (Yang, Wardlaw, & Bradding, 2006). The epithelial cell line used in the latter study produces only small amounts of PGE₂, making this an unlikely culprit (Aksoy et al, 1999). Recent work has demonstrated *in vitro* inhibition of mast cell histamine release by pro-resolving mediators. Sensitised human lung mast cells were incubated with varying concentrations of RvD₁, RvD₂ or LXA₄ for 10 minutes, after which they were stimulated with anti-IgE and histamine release measured by radioimmunoassay. The pro-resolving mediators appeared to cause a dose-dependent decrease in histamine release, with each causing an approximate 50% reduction even at a concentration of 10⁻¹² M (N. Martin et al., 2012). This finding is supported by recent work by Karra, Haworth, Levy, & Levi-Schaffer (2013) who found that LXA₄ and LXB₄ reduced IgE-mediated mast cell degranulation by 20-30% *in vitro*. Pro-resolving mediators thus appear to be strong candidates for a role in dampening AHR to mast cell-mediated challenges.

There is also evidence that pro-resolving mediators reduce AHR to direct challenges. RvE₁ appears to reduce airway hyper-responsiveness to methacholine in a mouse model of asthma (Aoki et al., 2008), and a number of studies have implied impaired production of LXA₄ in severe asthma, suggesting it may be involved in airway homeostasis (C. Bonnans et al., 2002; Vachier et al., 2005). LXA₄ also appears to reduce AHR to the ASM agonist LTC₄ in asthmatic humans (Christie et al., 1992).

There is *in vitro* evidence that pro-resolving mediators act on smooth muscle, albeit vascular smooth muscle. Ho et al. (2010) demonstrated the presence of ALX receptors (LXA₄ and RvD₁) and ChemR23 receptors (RvE₁) in human saphenous vein smooth

muscle cells. Miyahara et al. (2013) demonstrated expression of receptors for RvD₁ (both ALX and GPR32) in human and also rabbit vascular smooth muscle cells (VSMC). They further noted that RvD₁ treatment significantly reduced TNF α -induced superoxide production in VSMC; reactive oxygen species such as superoxide can potentiate inflammation and cell-activation signalling. RvD₁ and RvD₂ were also shown to reduce pro-inflammatory gene expression in VSMC, and reduce TNF α -stimulated expression of cell adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) which facilitate inflammatory cell influx. The overall effect is thus one of inhibiting the inflammatory activity of smooth muscle cells. Although there is at present no evidence for expression of pro-resolving mediator receptors in *airway* smooth muscle (Parameswaran et al., 2007a found no expression of ALX in ASM) this has not been extensively investigated and their presence remains a possibility, particularly given their expression in VSMC. Although speculative, it seems highly likely that if present such receptors would mediate an inhibition of pro-inflammatory activity such as AHR.

Given that there is evidence for an inhibitory effect of pro-resolving mediators on mast cells, as well as an anti-AHR action against directly acting challenges, they appear to be a promising group of molecules to investigate as potential bronchoprotective factors inducing a refractory period against challenges operating via a range of mechanisms. In addition to identifying the underlying mechanism, there remain many unanswered clinical questions concerning the refractory period, including how best to measure it, its relationship to the magnitude of the initial response to airway challenge, whether there are common demographic and phenotypic characteristics in the patient group who

demonstrate refractoriness, and whether it is associated with a measurable change in levels of potential bronchoprotective mediators. The overarching aim of this work is to address these questions, clinical and mechanistic, using a clinical study allied to the development of appropriate analytical methods and *in vitro* models. Subsequent sections of this chapter introduce relevant experimental techniques.

1.5 Analytical techniques

1.5.1 Background

In order to analyse samples from our clinical study for the presence of pro-resolving mediators it will be necessary to develop an appropriate assay. The following sections describe the techniques of liquid chromatography (LC) and mass spectrometry (MS) used in our study; the reasoning behind this choice of technique is discussed in Chapter 3.

1.5.2 High performance liquid chromatography

Modern high performance liquid chromatography (HPLC) originates with the work of Horvath, Preiss and Lipsky (1967). The basic experiment is illustrated in Figure 1.6.

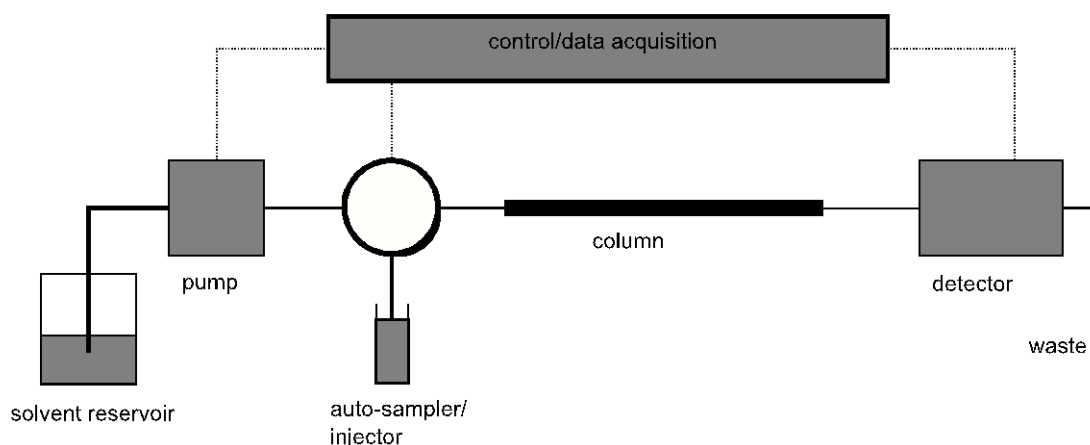


Figure 1.6. Schematic of liquid chromatography apparatus.

A sample is injected into a stream of solvent flowing through a column packed with solid particles. The solvent is known as the mobile phase and the column packing is known as the stationary phase. Analytes within the sample gradually separate as they pass down the column, as they have different affinities for the solid and stationary phases and partition between them. In the most common arrangement, known as “reversed phase” HPLC, the stationary phase is more hydrophobic than the mobile phase and analytes with greater hydrophobicity will therefore be retarded by the column to a greater extent and elute later. In an ideal experiment all analytes will be separated into discrete bands by the time they reach the detector at the end of the column. A wide variety of detector types are used, the most common type being ultraviolet (UV) absorbance. As analytes reach the detector and generate a signal, a plot of signal intensity (e.g. absorbance) against time is generated, a so-called chromatogram. The area under each chromatogram peak will be proportional to the amount of analyte present in the injected sample, and so with appropriate standards the technique is

quantitative. In the current study mass spectrometry is used for detection (a combination known as LC-MS) and this is discussed in detail below.

1.5.3 Mass spectrometry

Mass spectrometry is a technique for analysis of both organic and inorganic molecules with a huge variety of applications. Originating with Thompson (1911) and further developed by Dempster (1918), the basis of the technique is that sample molecules are ionised and transferred into the gas phase. These gas phase ions, now under vacuum, are then separated according to their mass to charge ratio in an electromagnetic field prior to detection. Figure 1.7 shows the basic elements of a mass spectrometer.

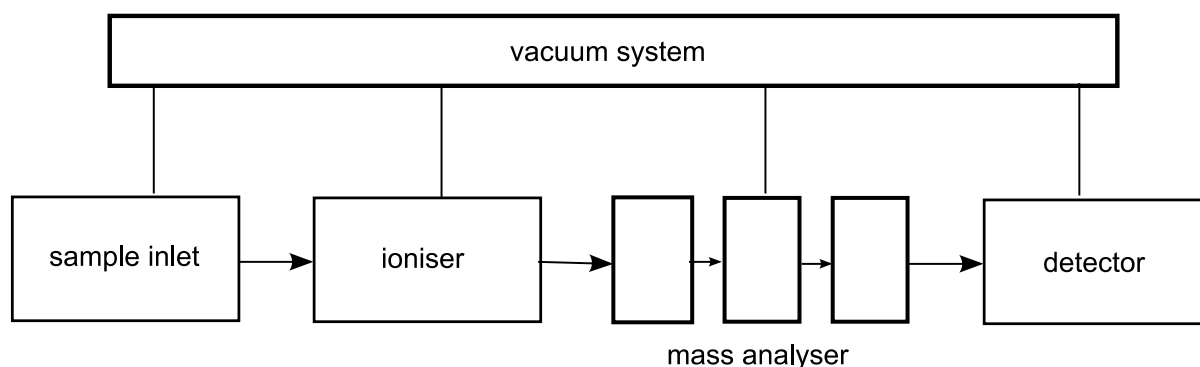


Figure 1.7. Schematic of generic mass spectrometer. The sample inlet and ioniser may be under vacuum or at atmospheric pressure, depending on ioniser type.

In the system used in this study the sample inlet is interfaced to the outlet of the LC system, though a range of sample inlet systems exist for gaseous, liquid and solid samples. A variety of sample ionisation and mass analysis systems exist; here the types used in the current study are outlined.

Sample ionisation – electrospray. The ionisation technique most commonly used in interfacing to LC is electrospray. This is illustrated schematically in Figure 1.8. The technique is based on the work of Dole and subsequently Fenn (Dole et al., 1968; Yamashita & Fenn, 1984). Analyte solution flows down the inlet, either from an LC column or a syringe. The solvent is chosen such that the analyte will be ionised to some extent. Analyte solution exits the needle, which is held at a potential of several kilovolts relative to the cone (The needle is shown at positive potential in Figure 1.8, but it may also be made negative with respect to the cone depending on the analyte of interest). The electrospray chamber is at atmospheric pressure, with a stream of nitrogen gas flowing through it. The cone is connected to the instrument's high vacuum system. Analyte solution, now consisting of charged particles of the same polarity as the needle, exits the needle and is thus directed down both an electric field gradient and a pressure gradient. Solvent continuously evaporates from each particle until its surface tension is no longer sufficient to support its charge, and a so-called coulombic explosion occurs, producing smaller fragment particles (Figure 1.9). These in turn fragment until all solvent has evaporated, leaving only analyte ions which pass through the cone and into the mass analyser.

Electrospray is a so-called “soft” ionisation technique which produces minimal fragmentation – the majority of the ions produced will simply be the ionised form of the analyte molecule as it existed in the sample solution. Some molecules may be further fragmented however.

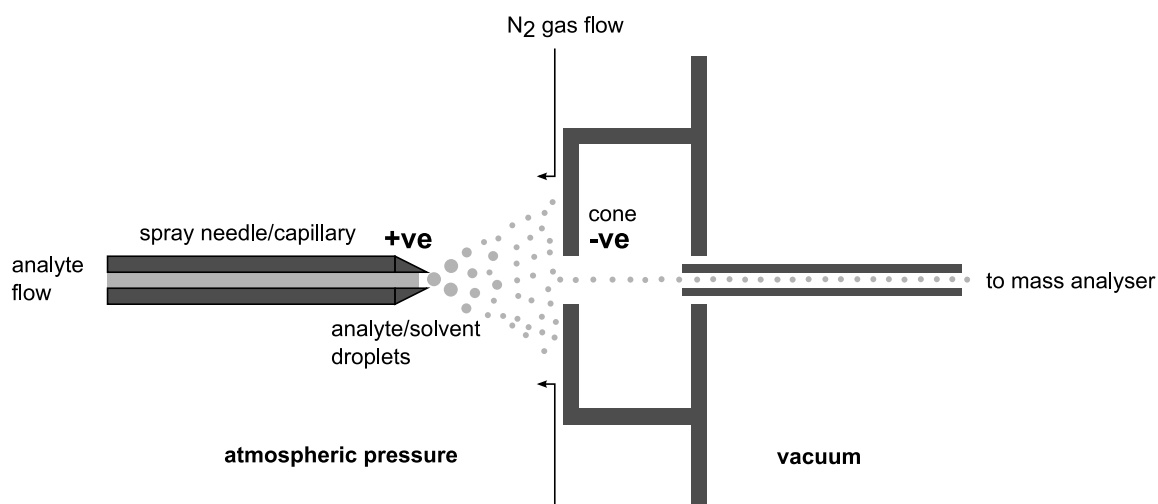


Figure 1.8. Schematic of electrospray ioniser.

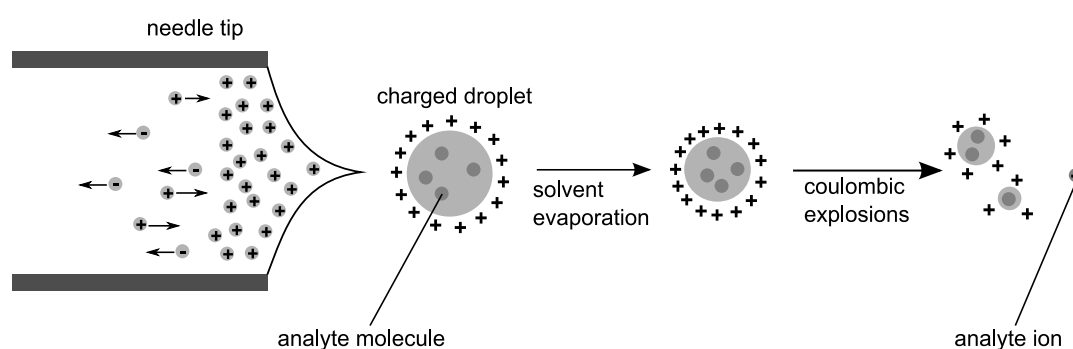


Figure 1.9. The mechanism of ion formation via electrospray.

Mass analysers. All mass analysers use an electromagnetic field to separate ions based on their mass to charge ratio, m/z (the technical details of the many different types – for example quadrupole, time of flight – are not discussed here). By rapidly varying the electromagnetic field within the analyser, and thus allowing ions of varying m/z to pass through to the detector, the relative abundances of the range of masses present in the sample can be plotted as a mass spectrum.

An important technique in modern mass spectrometry is the coupling of two or more mass analysers, known as tandem mass spectrometry (Griffiths et al. 2001); this technique developed from the work of Jennings on collision induced dissociation (Jennings, 1968). The analysers may be coupled spatially, e.g. two quadrupole analysers in series, or temporally, whereby an ion trap is used to store ions and return them for a second pass through a single analyser. The use of multiple analysers allows a variety of new analysis techniques to be used. The most important of these for this study is selected reaction monitoring (SRM) and the basic principle of this technique is illustrated in Figure 1.10. Ions travel from the ioniser into the first analyser, which acts as a filter, allowing one particular mass to pass through while rejecting others. The mass selected is usually that of the unfragmented analyte ion – the precursor ion. These ions then pass into a collision cell, where they collide with inert gas molecules (typically argon) and fragment into product ions. A second analyser is used to select for a second mass, and fragment ions with this mass pass through to the detector. The overall effect is thus to select for a particular “reaction” consisting of a pair of ions of different mass that is characteristic of the analyte under investigation; for example 375.2 → 141.2 is a characteristic SRM ion pair of resolin D₁ and D₂. The key characteristic of SRM is its high sensitivity, which is conferred primarily through the removal of so-called chemical noise (signal due to background ions of similar m/z to the ion of interest). This greatly enhances the signal to noise ratio.

Another tandem MS technique is the product ion scan. Here the first analyser selects for the analyte ion of interest, while the second analyser rapidly scans a range of masses to allow a mass spectrum of product ions to be obtained. This provides structural information about the molecule concerned, aiding identification.

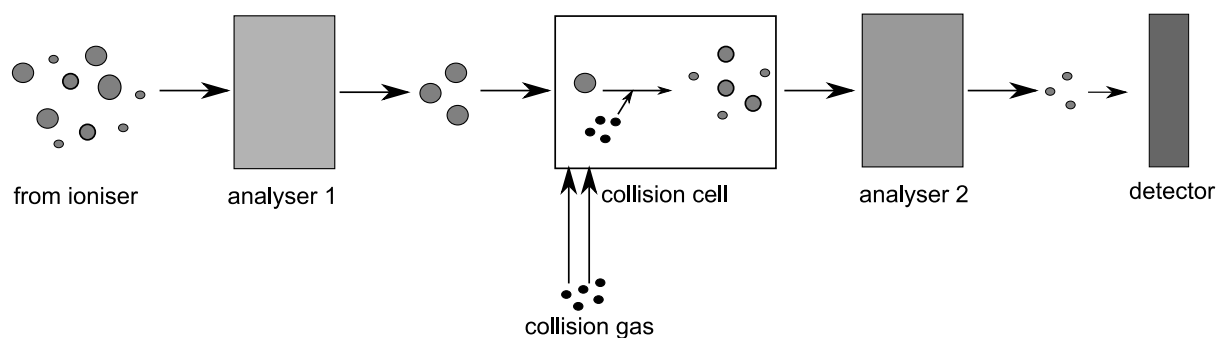


Figure 1.10. Selected reaction monitoring.

1.5.4 Application of LC-MS to pro-resolving lipid mediators

The technique of liquid chromatography separation coupled to mass spectrometric detection and analysis has been applied to pro-resolving mediators by several groups, notably those of Serhan (Masoodi et al, 2008) and Dennis (Blaho et al, 2009) who developed a method for assay of 104 lipid mediators in a single 25 minute run. The published limits of detection for these groups are listed in Table 1.5 as mass injected and molar concentration (calculated from the stated injection volume).

Lipid mediator	Serhan group limit of detection/ pg (nM)	Blaho group limit of detection/ pg (nM)
Lipoxin A ₄	-	1 (0.07)
Protectin D ₁	10 (6)	3 (0.2)
Resolvin D ₁	20 (11)	10 (0.7)
Resolvin E ₁	20 (11)	3 (0.2)
PGE ₂	-	1 (0.07)

Table 1.5. Published limits of detection for lipid mediators by LC-MS.

1.6 In vitro airway models

1.6.1 Background

This section describes two techniques used as *in vitro* models of the airway. Both offer experimental tools that sit between cell culture techniques and *in vivo* experiments, providing a more “real world” environment than cell culture but with greater control and less complexity and expense than human or animal work. Specific details of our application of the techniques, including selection of species, are discussed in Chapters 4 and 5.

1.6.2 Precision cut lung slices

The technique of preparing live, precision cut tissue slices (PCLS) originated with Krumdieck et al (1980) who developed a reciprocating blade instrument for this purpose. Lung slices were first prepared using the agarose filling method by Placke & Fisher (1987) though these slices were 1-2 mm thick. The technique has been subsequently refined and applied to both human lung and a number of animal models, including mouse, rat and horse (for example Bergner & Sanderson, 2002; Dandurand et al 1993; Vietmeier et al., 2007). Specific details of our own method are discussed in Chapter 4, but in general terms a fresh lung or section of lung is filled with a warm solution of agarose, followed by air. The agarose is allowed to cool and set, providing sufficient rigidity to allow tissue to be cut into thin (75 – 250 μm) sections using an oscillating blade tissue slicer. Slices containing airway cross-sections can then be kept alive for days or even weeks in appropriate growth medium, and responses of the slice

to various stimuli can be directly measured and recorded via a microscope and camera. The technique has been utilised in a range of applications including studies of airway contractile response to various stimuli (for example Martin et al, 2001; Bergner & Sanderson, 2002), vascular response (Held et al, 1999), mucociliary function (Kurosawa et al, 1995), allergic responses (Dandurand et al, 1994; Wohlsen et al., 2003) and calcium signalling (Bergner & Sanderson, 2002).

1.6.3 Bronchial ring model

The bronchial ring technique originated with Persson & Ekman (1976) and was further refined by Finney, Karlsson, & Persson (1985). Specific details of our own method are discussed in Chapter 5, but in general terms intact rings of bronchial tissue are dissected out of fresh human or animal lung, and can then be kept alive for several days in appropriate growth medium. Contraction or relaxation of the rings to various stimuli can then be measured by attaching them to a tension-measuring transducer in an organ bath. Larger diameter airways are required than for the PCLS approach, and the airway is not directly visualised, so there is no measure of epithelial integrity; however, sample preparation is much simpler, and the technique provides more direct quantification of bronchial contraction/relaxation. Electric field stimulation of smooth muscle can be used as an alternative to contractile agonists (Watson, Magnussen, & Rabe, 1998). The technique has been used in a wide range of applications, for example constructing dose-response curves for contractile agonists (Finney et al., 1985), measuring the effects of hypoxia on bronchial response (Schindler, Hislop, & Haworth, 2006) and measuring the effects of corticosteroids and bronchodilators on an allergic airway model (Brichetto et al., 2003).

1.7 Summary

In summary then, the primary hypothesis to be tested in this work is that refractoriness in asthma is due to release of bronchoprotective mediator(s) within the airway and that these may be one or more of the pro-resolving lipid mediators (lipoxins, resolvins, protectin or maresin). The resulting study aims are:

1. Development of a sensitive, reliable method for the detection of pro-resolving mediators in sputum and urine. An approach using liquid chromatography-mass spectrometry (LC-MS) will be used, along with immunoassays as appropriate.
2. Development of an *in vitro* model of asthma which will allow direct measurement of the effects of pro-resolving mediators on bronchoconstriction induced by both a mast cell-mediated mechanism and direct airway smooth muscle stimulation.
3. A clinical study of refractoriness in mild asthma, with the aims of a) better understanding the clinical characteristics of the phenomenon and b) measuring levels of inflammatory mediators and potential bronchoprotective factors during the refractory state, to shed light on the underlying mechanisms of refractoriness.

Chapter 2 Methods

2.1 Introduction

This chapter gives detailed descriptions of the experimental methods used for the acquisition of all data reported in this thesis. Briefer method summaries are included in the relevant chapters, along with outlines of method development processes where applicable.

2.2 LC-MS methods

2.2.1 Materials and equipment

For most LC-MS experiments a Waters Acquity liquid chromatography system was used in conjunction with a Waters Xevo TQ triple quadrupole mass spectrometer operating with electrospray ionisation in the negative ion mode. For some experiments a Waters TQ-S StepWave mass spectrometer was used; this is indicated in Chapter 3 where appropriate.

Standards for Resolvin D₁ and D₂, Maresin-1, Lipoxin A₄ and Deuterated-Lipoxin A₄ were obtained from Cayman Chemicals as 100 µg/ml solutions in ethanol. PGE₂ was obtained from Sigma-Aldrich. Solid phase extraction (SPE) cartridges were 1 ml capacity Strata-X, supplied by Phenomenex. The Lipoxin A₄ ELISA kit was manufactured by Oxford Biomedical Research.

2.2.2 LC-MS conditions

The method development process is described in detail in Chapter 3. Optimised instrument parameters for the TQ and TQ-S LC-MS instruments are summarised in Table 2.1 to Table 2.4 below.

Polarity	Negative ion
Capillary voltage	2.6 kV
Cone voltage	25 V
Desolvation temperature	550 °C
Desolvation gas flow	900 l/hr
Collision gas flow	0.2 ml/min

Table 2.1. Optimised Xevo-TQ instrument parameters for detection of pro-resolving lipid mediators.

Polarity	Negative ion
Capillary voltage	2.6 kV
Cone voltage	30 V
Desolvation temperature	550 °C
Desolvation gas flow	1100 l/hr
Collision gas flow	0.15 ml/min

Table 2.2. Optimised Xevo TQ-S instrument parameters for detection of pro-resolving lipid mediators.

Analyte	SRM	SRM source
RvD ₁	375.2->141.2	experiment
RvD ₂	375.2->141.2	experiment
LXA ₄	351.2->115.2	experiment
d5-LXA ₄	356.2->115.2	experiment
PGE ₂	351.0->271.0	experiment
PD ₁	359.2->206.2	literature
RvE ₁	349.0->195.0	literature
RvE ₂	333.2->199.2	literature
MaR ₁	359.2->250.2	experiment

Table 2.3. SRM ion pairs used for LC-MS analysis of pro-resolving mediators.

Column	Waters Acquity BEH C18 1.7 µm stationary phase, 2.1×150 mm		
Flow rate	0.3 ml/min		
Run time	17 minutes		
Column temperature	50 °C		
Injection volume	10 µl		
Solvent	A: 45% acetonitrile in water B: 0.02% acetic acid		
Solvent gradient	Time	%Solvent A	%Solvent B
	0	50	50
	5	20	80
	12	0	100
	14	50	50

Table 2.4. Optimised chromatographic parameters for separation of pro-resolving lipid mediators.

2.2.3 Solid phase extraction

The same solid phase extraction (SPE) procedure was followed with all samples prior to LC-MS analysis, whether cell-culture or sputum supernatant. SPE cartridges were 1 ml capacity Strata-X, supplied by Phenomenex. The SPE cartridge was washed through with 1 ml of methanol followed by 1 ml of water using a vacuum pump. Sample (100 µl to 1 ml in volume, spiked with 10 ng of deuterated lipoxin A₄ for MS assessment of extraction efficiency – see discussion in Chapter 3) was then allowed to pass through the cartridge slowly under low vacuum. The cartridge was then washed with 10% methanol to remove contaminants, before elution of the lipid fraction with 100% methanol. If not used on the day of preparation the samples were stored at -80 °C until use, usually the next day. Prior to LC-MS the methanol was evaporated to dryness in a rotary evaporator and the sample reconstituted in 100 µl of HPLC solvent. In the case of high volume cell culture supernatant, the sample was passed through the same SPE cartridge in 1 ml aliquots before final washing and sample elution, thus allowing concentration of the lipid component.

2.2.4 Cell-culture supernatant preparation

Supernatants were collected from incubation of mast cells alone (human lung mast cells (HLMC) or HMC-1 cell line), epithelial cells alone and co-culture. Co-culture was a 16 hour overnight incubation. Two approaches were used: a) HLMC at 20,000 cells per 400 µl of BEGM growth medium in a 24 well plate coated in a confluent layer of human airway epithelial cells. b) 3 million HMC-1 cells in 10 ml of BEGM in a T-75 flask coated with confluent airway epithelial cells. In each case epithelial and mast cell

monoculture supernatants were similarly prepared by overnight incubation. Some epithelial monoculture supernatants had a longer incubation time of up to 48 hours.

2.2.5 Sputum collection and preparation.

A sputum induction process with nebulised hypertonic saline was used to obtain sputum samples from patients. A complete description is given in the hospital's Sputum Induction and Processing Protocol (University Hospitals of Leicester, 2011); in brief:

1. The procedure is explained to the patient.
2. Baseline FEV₁ is measured three times.
3. A dose of salbutamol is given.
4. After 15 minutes the post-salbutamol FEV₁ is measured 3 times.
5. The subject inhales 3% hypertonic saline from a nebuliser for 5 minutes; they then attempt to expectorate any sputum into a sample pot. FEV₁ is measured.
6. Step 5 is repeated with 4% and then 5% hypertonic saline provided the FEV₁ has not fallen by more than 200 ml or 10% of the post-salbutamol baseline. If the FEV₁ fall is 200-400 ml or 10-20% baseline Step 5 is repeated at the same saline concentration; above these values, or if significant symptoms occur, the procedure is stopped and the patient treated with further salbutamol.

The resulting sputum sample is processed by dispersion of sputum plugs *via* addition of dithiothreitol (DTT) followed by centrifugation to separate out the cellular component (University Hospitals of Leicester, 2011). The resulting cell-free supernatant is stored at -80°C.

2.2.6 Sputum and cell-culture supernatant LC-MS analysis

Sputum or cell-culture supernatant was spiked with 10 ng of d5-LXA₄ internal standard and the lipid fraction extracted by SPE, as described in 2.2.3. The extracted samples were then analysed using the Xevo TQ or TQ-S LC-MS system with our optimised parameters as outlined in 2.2.2.

2.2.7 LXA₄ ELISA analysis

An Oxford Biomedical LXA₄ ELISA kit was used according to the manufacturer's instructions. It is a competitive assay using LXA₄-conjugated horse-radish peroxidase; tetramethylbenzidine is used as the substrate, with spectrophotometric analysis at 650 nm. On the manufacturer's advice sputum and cell-culture supernatant were analysed directly without any extraction process. No sample dilution was used.

2.3 PCLS methods

2.3.1 Materials and equipment

Buffer was made up from x10 concentration stock solution of Hank's buffered salt solution (HBSS) from Sigma-Aldrich with the addition of 2 mM calcium and 1 mM magnesium salts, titrated to pH 7.4. Agarose was low-gelling temperature Type VII from Sigma-Aldrich. Tissue slices were incubated in Dulbecco's modified Eagle medium (DMEM) with added antimicrobial and antimycotic. Carbachol and formoterol were obtained from Sigma-Aldrich and lipid mediators from Cayman Chemicals. High

IgE serum from sheep sensitised with house dust mite was a gift from Dr Ken Snibson, Centre for Animal Biotechnology, University of Melbourne, Australia. Allergopharma Soluprick House dust mite skin-prick test solution was obtained from Diagenics. Two oscillating blade tissue slicers were used; a semi-automated Electron Microscopy Sciences EMS5000 model and a Campden Instruments manual slicer. A Zeiss Axiovert 25 inverted microscope with a heated stage held at 37 °C and a Q Imaging Retinga 1300 digital camera attached to an Apple Macintosh computer were used for imaging of tissue slices.

2.3.2 Mouse lung harvesting

Mice were male Black 657 wild-type. Immediately following culling of the animal by cervical traction, the animal was cleaned with triclosan and the anterior chest wall removed by dissection with scissors. The trachea was cannulated using a 20-gauge intravenous cannula and the lungs inflated with approximately 1.2 ml of air from a syringe to verify cannula position. Once this was satisfactory, the lungs were deflated and approximately 1.2 ml of warm low-gelling agarose solution (1.5 - 2% in buffer as above) was flushed in, followed by approximately 0.5 ml of air. Ice was packed around the chest for approximately 10 minutes to facilitate gelling of the agarose. The lungs were then dissected out of the animal and kept in cold buffer until ready for slicing, which was carried out as soon as possible after harvesting.

2.3.3 Sheep lung preparation

Sheep lungs were obtained fresh from a local abattoir, transported on ice and processed immediately on arrival in the laboratory. At least one intact lobe was required to enable the agarose-filling procedure to be used, and abattoir staff co-operated in providing suitable lungs. To prepare lung for slicing, a small lobe was identified and a length of ca. 3 mm diameter plastic intravenous therapy tubing inserted into its main bronchus, as illustrated in Figure 2.1. Approximately 25 – 50 ml of warm 2% agarose solution was flushed in from a 50 ml syringe until the tissue was firm, followed by a similar volume of air. Agarose was melted by heating in a microwave oven and then cooled to below 40 °C prior to filling, to minimise the risk of tissue damage. After filling with agarose, the bronchus and tubing were clamped shut and the lobe kept on ice for 10 minutes to allow the agarose to set prior to cutting.

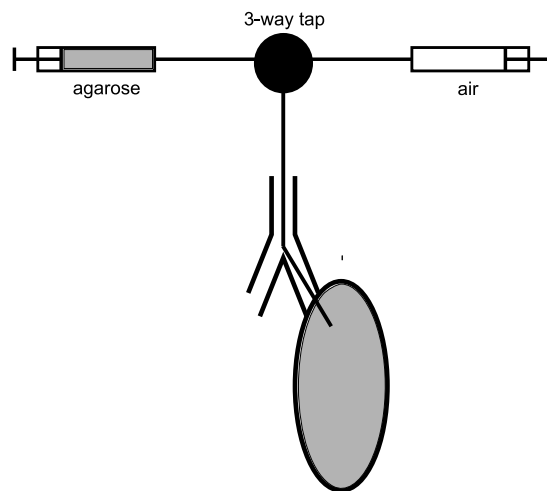


Figure 2.1. Experimental set up for agarose-filling of sheep lung.

2.3.4 Lung slice preparation

Small mouse lung lobes were positioned on the slicer platform without further preparation; larger lobes and sheep lung were sectioned into 0.5 – 1 cm diameter pieces using a scalpel blade. The cutting plane was oriented so as to maximise the number of airways cut in cross-section. The automated EMS instrument was used for slicing mouse tissue; however, this proved inadequate for cutting sheep lung and the Campden Instruments manually-advanced blade device was used for this. A slice thickness of 180 or 200 μm was selected on the tissue slicer, with high oscillation speed and low advance speed (with the manual tissue slicer the advance speed is controlled by the operator turning a handle). The resulting slices were incubated at 37.5 °C in DMEM in a 6 well plate, with 5 – 10 slices in 4 ml of medium per well. The medium was changed 3-4 times in the first 24 hours and the slices were left for a minimum of 24 hours before use, to allow recovery of the epithelium as assessed by the presence of beating cilia. Slices were assessed using an inverted microscope for the presence of: 1. at least one airway seen in cross-section; 2. beating cilia visible on the epithelium for >50% of the airway circumference. Only those slices meeting these criteria were used for experiments.

2.3.5 Passive sensitisation of sheep PCLS

Sheep PCLS were passively sensitised to house dust-mite (HDM) by overnight incubation in 1 ml per slice of high IgE sensitised serum (a gift from Dr Ken Snibson, Centre for Animal Biotechnology, University of Melbourne, Australia) at 10% concentration in DMEM.

2.3.6 Sample analysis

A single lung slice was placed in 1 ml of fresh DMEM in a well of a 24-well plate. A small U-shaped piece of stainless steel wire (Figure 2.2) was used to hold the slice on the bottom of the well. The plate was positioned on the microscope stage which was maintained at 37.5 °C. A x10 objective was used to focus on an airway, and images recorded as appropriate (Figure 2.3). The field of view was calibrated using a haemocytometer scale. Mediators of interest were added to the well via a pipette, and then mixed by repeatedly withdrawing and replacing 150 µl volumes of media using a second pipette. If constructing a dose-response curve the slice was allowed to equilibrate for 5 minutes after addition of each dose of mediator, prior to capturing the next image. Images were analysed using ImageJ software to draw round the airway lumen circumference and calculate the airway cross-sectional area. Airways were included in analysis only if their maximum contraction to carbachol was $\geq 10\%$.



Figure 2.2. Stainless steel U-piece used to hold a lung slice to the bottom of its well.



Figure 2.3. Heated microscope stage with a 24-well plate containing a precision-cut lung slice *in situ*. The slice is imaged by the camera *via* a x10 objective and recorded on a computer situated to the right of the picture.

2.3.7 Statistical analysis

EC₅₀ values for dose-response curves were calculated by curve fitting using the *R* statistics package (the *drc* add-on package is required). A 4 parameter logistic model was used:

$$f(x, (b, c, d, e)) = c + \frac{d - c}{1 + \exp\{b(\log(x) - \log(e))\}}$$

Where e is the EC_{50} , c and d are the upper and lower limits respectively and b is the slope around e .

2.4 Bronchial ring methods

2.4.1 Materials and equipment

The organ bath used in these experiments was a proprietary design as shown in Figure 2.4 and acquired from Astra-Zeneca; the tension transducers were interfaced to an AD Instruments Octal bridge and amplifier connected to a computer running AD Instruments Chart version 5 software. Krebs-Henseleit buffer was prepared in 1 l quantities, with the following composition: sodium chloride 118 mM, potassium chloride 4.7 mM, potassium dihydrogen phosphate 1.2 mM, dextrose 11.1 mM, magnesium sulphate 1.2 mM, calcium chloride 2.8 mM and sodium bicarbonate 25 mM. Carbachol and histamine were obtained from Sigma-Aldrich (solutions made up in phosphate buffered saline) and lipid mediators from Cayman Chemicals (diluted as required with ethanol). Lipid mediator stock solutions were stored at -80°C . Cetirizine was obtained from R&D Systems, montelukast and PGD_2 from Cambridge Bioscience and mannitol from Sigma-Aldrich. Mannitol solutions were prepared in Krebs-Henseleit buffer; montelukast and PGD_2 solutions were prepared in ethanol and cetirizine in phosphate buffered saline. Bronchial rings were incubated in Dulbecco's modified Eagle medium (DMEM) with added antimicrobial and antimycotic at 1% v/v. Rabbit antibody against human $F_{\epsilon}R_1$ was obtained from Upstate Cell Signalling Solutions.

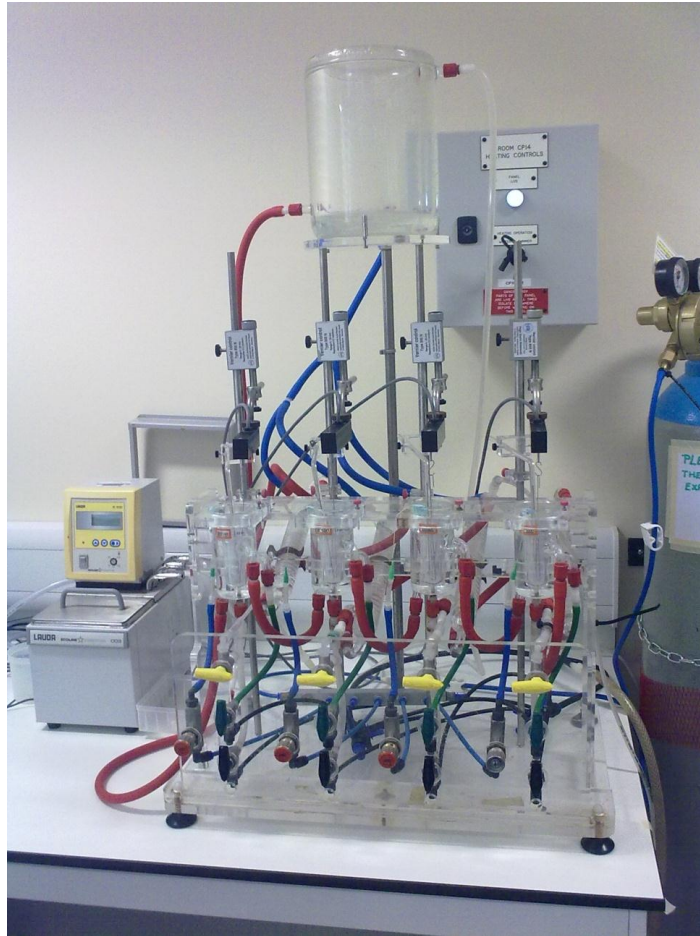


Figure 2.4. Organ bath used in bronchial ring experiments.

2.4.2 Pig lung sample preparation

Pig lungs were obtained from male and female animals fresh from a local abattoir, transported on ice and processed immediately on arrival in the laboratory. A minimum of 16 bronchial rings with internal diameter 1.5 – 3 mm and length 2 – 3 mm were dissected from a lung using scissors and forceps; a typical ring is shown in Figure 2.5. Following dissection rings were incubated at 37.5 °C in DMEM with added 1% v/v antimicrobial and antimycotic in a 24 well plate, with 1 ring per 1.5 ml of medium per well. The medium was changed 3-4 times in the first 2 hours and the rings were left for

a minimum of 24 hours before use. Rings were used for experiments for up to 4 days following the day of preparation, with daily changes of growth medium.



Figure 2.5. Porcine bronchial ring.

2.4.3 Human lung sample preparation

Small pieces of human lung were intermittently obtained from the thoracic surgeons following resection for lung cancer, and small airways dissected out in a similar manner to that described above. The rings thus obtained were very small, typically ca. 1 mm internal diameter with no or very little cartilage, and often difficult to visually distinguish from blood vessels. In uncertain cases a subsequent strong contractile response to carbachol and antibody to $F_{\epsilon}R_1$ (see below) was used to confirm their status as bronchi. Note that some structures which appear macroscopically to be blood vessels may also contract weakly to carbachol, presumably because they no longer have sufficient intact endothelium to synthesise the nitric oxide required for a dilatory

response (Furchgott & Zawadzki, 1980). Once dissected, airway rings were washed and incubated in DMEM as described above for porcine rings.

2.4.4 Bronchial ring contraction measurements

Contraction of the bronchial rings was measured using the organ bath shown in Figure 2.4. Up to 4 rings can be assessed simultaneously. Each well of the bath contains two wire hangers on which a ring can be suspended; the lower of the wires is fixed, the upper is attached to a tension-measuring transducer (Figure 2.6). Baseline tension can be applied to the ring by adjusting the position of the upper wire via a Vernier control. The transducers are connected to an amplifier interfaced to a computer with recording software as detailed above, allowing continuous recording of tension. Prior to each set of experiments the apparatus was calibrated by applying tension to each transducer using a known weight; the gain of the amplifier was set to 2 mV or 5 mV full scale, which following calibration corresponded to a maximum measurable tension of approximately 12.5 g or 31.5 g respectively. The usual data acquisition rate was 1000 samples/s. At the start of an experiment, a ring was suspended in each well in 5 – 10 ml of Krebs-Henseleit buffer. 95% O₂/5% CO₂ gas was bubbled into the buffer from a cylinder. 0.6 g of baseline tension was applied to each ring, and the rings left to stabilise for a minimum of 45 minutes with change of buffer every 15 minutes and regular adjustment of the tension to maintain 0.6 g. Once a stable baseline was achieved, reagents were added to the wells with a pipette as appropriate for each experiment.

For experiments in which rings were contracted using high concentration mannitol, each well of the organ bath was emptied and immediately re-filled with mannitol solution (\pm any required mediator) using a 10 ml pipette.



Figure 2.6. Porcine bronchial ring suspended in organ bath.

2.5 Clinical study of the refractory period

2.5.1 Materials and equipment

Mannitol challenge tests were administered using Osmohale kits obtained from Pharmaxis; methacholine and salbutamol were obtained from the hospital pharmacy. Spirometry was conducted using a Vitalograph Gold Standard spirometer.

2.5.2 Consent

All volunteers received oral and written information about the nature, purpose, possible risk and benefit of the study; the Patient Information Sheet is shown in the Appendix. Volunteers were notified that they were free to withdraw from the study at any time. Potential subjects were given the opportunity to ask questions and allowed time (>24 hours) to consider the information provided. Written consent was obtained from all volunteers before conducting any procedure for the study.

2.5.3 Recruitment

Recruitment was by invitation, based on the presence of mild asthma controlled by short-acting bronchodilators with or without low dose inhaled steroids. Recruitment methods included posters placed in public places (see Appendix), invitations to volunteers who had failed screening for other studies and invitations to well-characterised patients already known to the clinical study team (see Appendix for invitation letter).

The aim was to recruit 20 patients to the study. There is a scarcity of data on which to base a sample size calculation. However, analysis of a previous study in our centre of 13 patients with asthma, in which Refractory Index (RI) was measured on two separate occasions, found a mean RI of 53%, with an intra-subject standard deviation of 13.3. In the current study, in each subject we compare RI measured using cumulative and bolus dose mannitol tests. We use a paired t-test for this purpose. A sample size calculation based on this method with the above standard deviation gives a value of 19 subjects necessary to demonstrate a mean absolute difference in RI of 10%, with $\alpha = 0.05$ and $\beta = 0.1$. (Sample size calculated using the equation $n = ((z_{\alpha} + z_{\beta}) s / \Delta)^2$, where n = sample

size, z_α and z_β are z-scores corresponding to α and β errors of 0.05 and 0.1 respectively, s is the standard deviation of 13.3 and $\Delta = 10$, the target difference in mean RI between the two groups of measurements).

Inclusion and exclusion criteria for the study were:

Inclusion

- Age 18 or over
- Diagnosis of asthma
- Treatment with short-acting beta agonist +/- low dose steroids (400 μg per day beclomethasone or equivalent)
- Symptoms well controlled on treatment (never hospitalised with asthma, no more than one course of oral steroids in the past 12 months)
- Non-smoking (ex smoker >1 year, <10 pack year history)

Exclusion

- Major co-morbidity
- Pregnancy
- Previous hypersensitivity to methacholine or mannitol

Withdrawal

- If a volunteer develops any of the exclusion criteria during the trial
- Volunteer requests withdrawal
- Discretion of the investigator

If a subject withdraws consent to the use of donated biological samples then the samples should be disposed of or destroyed, if not already analysed and documented. In

the event that analysis has already been performed the results can be retained but no further analysis should be performed and the sample must be disposed of.

2.5.4 Study visits

Volunteers made a screening visit followed, if recruited to the study, by four further visits. The visits are described below and summarised in the flow chart in Figure 2.7. Informed consent was obtained at the screening visit.

Volunteers received the information and consent form at least 24 hours before the screening visit. If the investigator or their delegate was satisfied that the volunteer had read and understood these and if the volunteer gave their written consent, they were then assigned a unique volunteer number and their details were entered into an enrolment log.

The screening visit included medical history and brief physical examination, measurement of height and weight, baseline spirometry, a pregnancy test if appropriate and a bronchial challenge test with methacholine to establish that the subject demonstrated airway hyper-responsiveness. If recruited to the study, volunteers were advised of the need to withhold their short-acting beta agonist for 12 hours prior to the next visit (Visit 2). It was emphasised that they should not withhold medication if they felt that it was essential for symptom control.

At Visit 2, initial assessment included a detailed questionnaire on level of physical activity, respiratory symptoms, asthma treatment and asthma control using the validated Juniper Asthma Control Questionnaire (ACQ) (Juniper, O'Byrne, Guyatt, Ferrie, &

King, 1999). Induced sputum and urine were obtained to determine a baseline for cell and molecular markers of airway inflammation and potential bronchoprotective factors.

On the third visit volunteers received a cumulative dose of inhaled mannitol until a 15% reduction in FEV₁ was achieved (the PD15 dose). This cumulative dose was then repeated once FEV₁ was >95% of the original baseline, and further FEV₁ measurements made to determine if the subject was now refractory. Induced sputum and urine were obtained following this to determine cell and molecular markers of airway inflammation and potential bronchoprotective factors. If a subject did not demonstrate a 15% FEV₁ reduction at the maximum cumulative mannitol dose of 635 mg then they did not proceed to the second challenge.

On the fourth visit the volunteer received the PD15 dose of mannitol as a single bolus (in practice mannitol capsules were administered in sequence as rapidly as possible, without the post-dose waiting period and spirometry used in the cumulative challenge). FEV₁ was measured 2, 5, 7, 10, 15, 20 and 30 minutes after. This bolus dose was then repeated when FEV₁ was >95% of the original baseline and further serial FEV₁ measurements made. Induced sputum and urine were again obtained. Those subjects at Visit 3 who did not develop a 15% fall in FEV₁ may have developed refractoriness during the challenge procedure, before a 15% fall was achieved. Therefore these subjects received a single bolus dose of 635 mg mannitol at Visit 4 to assess whether a bolus dose was able to induce a greater fall in FEV₁ than cumulative challenge.

On the fifth visit the subject received a bolus dose of mannitol previously shown in Visit 3 to cause a 5% reduction in FEV₁. This dose does not correlate to clinically significant bronchoconstriction. A bolus PD15 dose of mannitol was then given after 30

minutes to determine if the subject had become refractory. Induced sputum and urine were again obtained.

Sputum samples from the baseline visits were analysed by microscopy for sputum eosinophilia, a marker of atopic airway inflammation, in the local research laboratory at Glenfield Hospital, Leicester. Urine and sputum supernatant from all visits was retained to test for markers of inflammation and possible bronchoprotective factors.

Study sputum samples were analysed using the LC-MS methods of 2.2 above and the immunoassays described in 2.6 below. Urine samples were analysed by immunoassay.

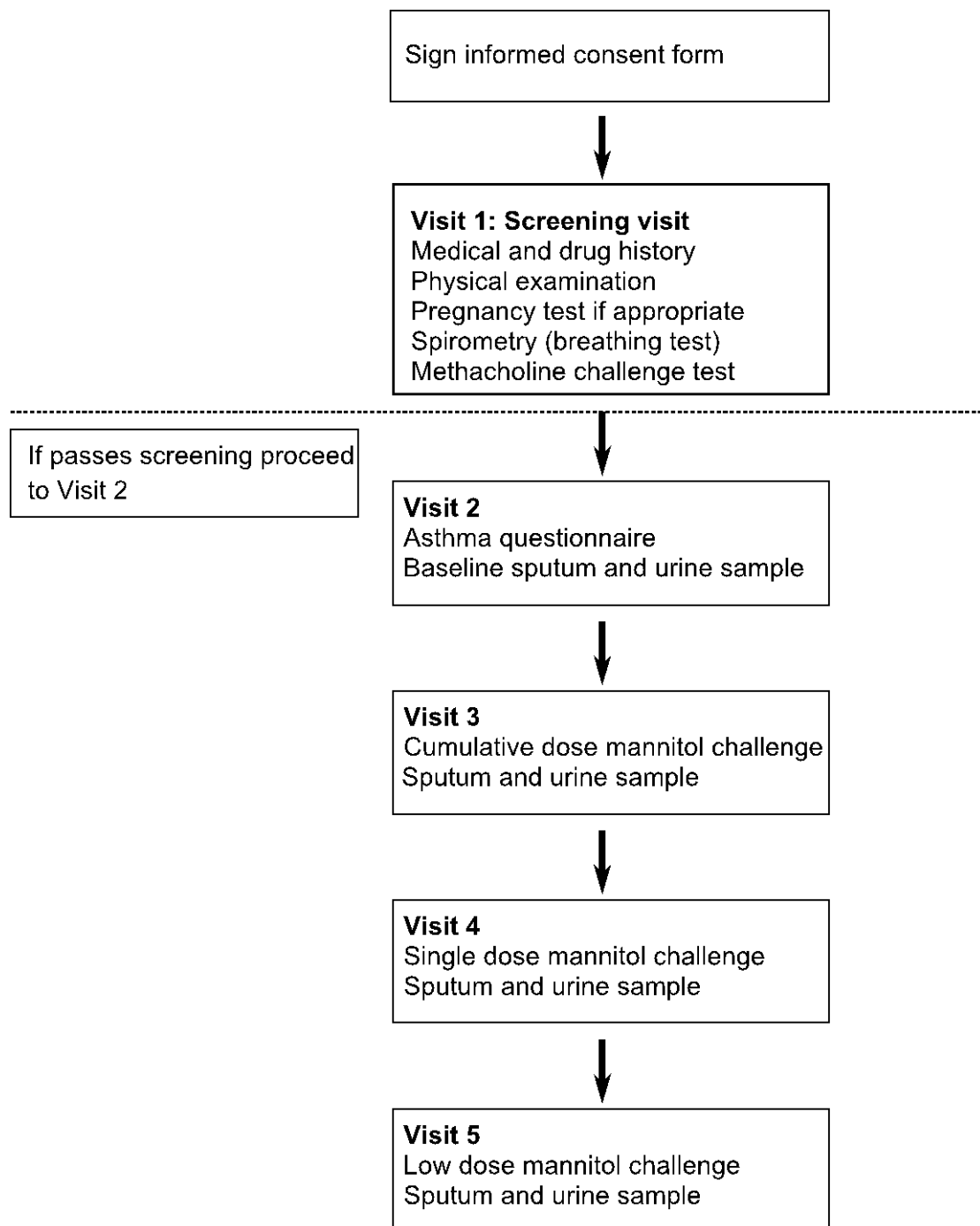


Figure 2.7. Summary flow chart of study visits.

2.5.5 Details of protocol tests

Spirometry

Forced expiratory volume in one second and forced vital capacity are recorded in all subjects, with the best value of attempts being taken. Results are also expressed as percentage of predicted.

Methacholine Challenge Test

Methacholine challenge testing is performed using a tidal breathing method. Methacholine is delivered using a Wrights nebuliser in doubling concentrations from 0.03 mg/ml – 16 mg/ml or until at least a 20% decrease in FEV₁ compared with baseline is reached (University Hospitals of Leicester, 2010).

Mannitol challenge test

Bronchial provocation to mannitol is performed using a dry powder preparation contained in capsules and administered from an Osmohaler. For cumulative dose testing mannitol is administered up to a cumulative dose of 635mg or until a 15% decrease in FEV₁ compared with baseline is reached. Full details of the procedure are given in the Osmohale Procedure Guidelines (Pharmaxis Pharmaceuticals, 2010). For bolus dosing, the dose previously shown to give a 15% FEV₁ decrease is used and capsules administered as quickly as tolerated without the intermediate pauses and spirometry.

Induced sputum

Sputum induction follows a standardised technique compliant with the European Respiratory Society guidelines, using pre-treatment with inhaled bronchodilator to offset the potential constrictive effect of nebulised hypertonic saline. It uses an ultrasonic nebuliser with 3-5% hypertonic saline and is performed under strict infection control procedures (University Hospitals of Leicester, 2011). The procedure is described in 2.2.5 above.

Urine collection

Volunteers are asked to provide a specimen of urine into a suitable container.

2.5.6 Recording of adverse events

Only serious adverse events related to study procedures were to be recorded in the volunteers' medical notes. In fact no serious adverse events occurred during the study.

2.5.7 Data storage & protection

Study data for the patients is stored electronically and/or on paper and information on this is filed in the study master file.

The informed consent document incorporates wording that complies with relevant data protection and privacy legislation. Pursuant to this wording, volunteers authorise the collection, use and disclosure of their study data by the investigator and by those persons who need it for the purposes of the study.

2.6 Immunoassays

2.6.1 LXA₄ ELISA

An Oxford Biomedical LXA₄ ELISA kit was used to analyse cell-culture supernatant and/or sputum samples in the work described in Chapters 3 and 6. It is a competitive assay using LXA₄-conjugated horse-radish peroxidase; tetramethylbenzidine is used as the substrate, with spectrophotometric analysis at 650 nm. In both cases it was used according to the manufacturer's instructions. On the manufacturer's advice sputum and cell-culture supernatant were analysed directly without any extraction process. Sample dilution is described in the text of the appropriate chapter.

2.6.2 RvD₁ ELISA

A Cayman Chemical RvD₁ ELISA kit was used to analyse sputum samples from the clinical study described in Chapter 6. It is a competitive assay using RvD₁-conjugated acetylcholinesterase, with Ellman's reagent as the substrate. The assay was used according to the manufacturer's instructions. No sample extraction process was used; samples were diluted 3-fold in the kit EIA buffer prior to analysis.

2.6.3 11 β -prostaglandin F_{2 α} (PGD₂ metabolite) ELISA

A Cayman Chemical 11 β -PGF_{2 α} (PGD₂ metabolite) ELISA kit was used to analyse urine samples from the clinical study described in Chapter 6. It is a competitive assay using 11 β -PGF_{2 α} -conjugated acetylcholinesterase, with Ellman's reagent as the

substrate. The assay was used according to the manufacturer's instructions. No sample extraction process was used; samples were diluted 20-fold in the kit EIA buffer prior to analysis.

2.6.4 Prostaglandin E₂ metabolite ELISA

A Cayman Chemical PGE₂ metabolite ELISA kit was used to analyse urine samples from the clinical study described in Chapter 6. It is a competitive assay that converts the PGE₂ metabolites 13,14-dihydro-15-keto-PGA₂ and 13,14-dihydro-15-keto-PGE₂ into a single, stable PGE₂ metabolite (PGEM). The competitor is a PGEM-conjugated acetylcholinesterase, with Ellman's reagent as the substrate. The assay was used according to the manufacturer's instructions. No sample extraction process was used; samples were diluted 20-fold in the kit EIA buffer prior to analysis.

2.6.5 Urinary creatinine analysis

A Cayman Chemicals urinary creatinine assay was used to analyse urine samples from the clinical study described in Chapter 6. Urinary creatinine concentration was used to normalise the PGD₂ and PGE₂ metabolite concentrations. It is a chemical assay based on the Jaffe reaction; urine was diluted 10-fold prior to analysis, and the kit was used according to the manufacturer's instructions.

Chapter 3 Sputum and cell supernatant analysis by LC-MS

3.1 Introduction

This chapter outlines the development of a LC-MS assay for pro-resolving lipid mediators, and its application to sputum and cell supernatant samples.

The development of such an assay is clearly important for a number of reasons. As outlined in Chapter 1, there is already tentative evidence for the involvement of pro-resolving mediators in airways disease, and in order to test our hypotheses it is essential that we are able to detect these molecules both *in vitro* and in clinical samples. This would allow analysis of sputum to assess variability of mediator levels with clinical parameters such as asthma severity or degree of eosinophilia, and importantly would also allow analysis of sputum from patients in our clinical study for any change in mediator levels between baseline and refractory period.

Enzyme-linked immunosorbent assays (ELISAs) are commercially available for a number of pro-resolving mediators with good sensitivity, as shown in Table 3.1. However, an LC-MS assay offers a number of advantages. Firstly it has a low per-analysis cost – although the instrumentation is expensive, once available the same column and instrument can be used for analysis of a large number of samples with little additional cost. LC-MS is also versatile, in that it can analyse a small or very large number of samples in one run, with no time-expiring reagents. As long as the structure of the lipid of interest is known, it can be assayed in the sample without the need for specific antibodies to be available. Crucially, large numbers of analytes can be measured in one run, rather than requiring a separate experiment on a new sample for each analyte, as with ELISA. Blaho et al. (2009) for example, used LC-MS to analyse 104 lipids per experiment in synovial fluid samples. This feature is particularly important in analysis of sputum, where only small sample volumes may be available.

Sample preparation is simple and quick, as outlined below. Another key advantage of LC-MS relates to its ability to provide structural information about analytes. This gives the possibility of obtaining confirmatory evidence for the identity of an analyte via a product ion scan. Accurate quantification is possible by calibration using standards of known concentration; RvD₁, RvD₂, LXA₄ and MaR₁ are commercially available at the present time but not E-series resolvins or PD₁. Table 3.2 below lists published limits of detection for pro-resolving mediators measured by LC-MS. PGE₂ is also of interest in our study as it is known to have some bronchoprotective properties.

Mediator	Manufacturer	Quoted limit of detection
Lipoxin A ₄	Oxford Biomedical Research	0.02 pg/μl
Resolvin D ₁	Cayman Chemical	0.01 – 0.02 pg/μl
Resolvin E ₁	MyBioSource	100 pg/μl
Prostaglandin E ₂	Cayman Chemical	0.015 pg/μl

Table 3.1. Commercially available ELISAs for assay of pro-resolving lipid mediators.

Lipid mediator	Serhan group limit of detection/ pg (nM)	Blaho group limit of detection/ pg (nM)
Lipoxin A ₄	-	1 (0.07)
Protectin D1	10 (6)	3 (0.2)
Resolvin D1	20 (11)	10 (0.7)
Resolvin E1	20 (11)	3 (0.2)
PGE ₂	-	1 (0.07)

Table 3.2. Published limits of detection for pro-resolving lipid mediators by LC-MS. Molar concentrations were calculated from the stated injection volume.

3.2 Methods

3.2.1 Materials and equipment

For most experiments described here, a Waters Acquity liquid chromatography system was used in conjunction with a Waters Xevo TQ triple quadrupole mass spectrometer operating with electrospray ionisation in the negative ion mode. For some experiments a Waters Xevo TQ-S (StepWave) mass spectrometer was used; this is indicated in the results section where appropriate.

Standards for resolvin D₁ and D₂, maresin-1, lipoxin A₄ and deuterated-lipoxin A₄ were obtained from Cayman Chemicals as 100 µg/ml solutions in ethanol. PGE₂ was obtained from Sigma-Aldrich. Solid phase extraction (SPE) cartridges were 1 ml capacity Strata-X, supplied by Phenomenex. The lipoxin A₄ ELISA kit was manufactured by Oxford Biomedical Research.

3.2.2 LC-MS Method optimisation

Mass spectrometry optimisation: Xevo TQ. Negative ion mode was used. For mediators for which standards were available (RvD₁, RvD₂, LXA₄, MaR₁, PGE₂) a concentrated solution of the standard was flushed into the spectrometer via a syringe driver or the spectrometer's fluidics system, and the mass of the de-protonated molecule selected in the first mass analyser. A product ion scan was then performed to find the most prominent fragment ion peak and the signal optimised by systematic variation of instrument parameters. The most prominent fragment was then used as the product ion in the SRM for that analyte. For analytes for which no standard is commercially available (resolvin E₁ and E₂, protectin D₁) SRM pairs were taken from the literature. Optimised instrument parameters are listed in Table 3.3; SRM pairs are listed in Table 3.4.

Polarity	Negative ion
Capillary voltage	2.6 kV
Cone voltage	25 V
Desolvation temperature	550 °C
Desolvation gas flow	900 l/hr
Collision gas flow	0.2 ml/min

Table 3.3. Optimised Xevo-TQ instrument parameters for detection of pro-resolving lipid mediators.

Analyte	SRM	SRM source
RvD ₁	375.2->141.2	experiment
RvD ₂	375.2->141.2	experiment
LXA ₄	351.2->115.2	experiment
d5-LXA ₄	356.2->115.2	experiment
PGE ₂	351.0->271.0	experiment
PD ₁	359.2->206.2	literature
RvE ₁	349.0->195.0	literature
RvE ₂	333.2->199.2	literature
MaR ₁	359.2->250.2	experiment

Table 3.4. SRM ion pairs used for LC-MS analysis of pro-resolving mediators.

Chromatography optimisation. The column was a Waters Acquity BEH C18 1.7 μ m stationary phase, 2.1×150 mm, with a binary solvent system using 45% acetonitrile in water as Solvent A and 0.02% acetic acid as Solvent B. Injection volume was 10 μ l. Column temperature, flow rate and solvent gradient were adjusted to optimise chromatographic resolution of the available lipid mediator standards. Good resolution of PGE₂ and RvD₂ was obtained; RvD₁ and LXA₄ elute as a single peak but are easily distinguished by mass spectrometry. Interestingly maresin-1 could not be detected on column, despite being visible in the MS-optimisation process. The optimised chromatographic parameters are given in Table 3.5 below. Total run time was 17 minutes.

Flow rate	0.3 ml/min		
Column temperature	50 °C		
Solvent gradient	Time	%Solvent A	%Solvent B
	0	50	50
	5	20	80
	12	0	100
	14	50	50

Table 3.5. Optimised chromatographic parameters for separation of pro-resolving lipid mediators.

Xevo TQ-S optimisation. A single set of samples was analysed on a Xevo TQ-S (StepWave) mass spectrometer. This is a more advanced model than the TQ incorporating new ion guide technology with the potential for greatly increased sensitivity. A process of optimisation using lipid mediator standards was carried out in a similar manner to that described above. The chromatographic conditions were unchanged. Optimised MS parameters are listed in Table 3.6.

Polarity	Negative ion
Capillary voltage	2.6 kV
Cone voltage	30 V
Desolvation temperature	550 °C
Desolvation gas flow	1100 l/hr
Collision gas flow	0.15 ml/min

Table 3.6. Optimised Xevo TQ-S instrument parameters for detection of pro-resolving lipid mediators.

3.2.3 Sample preparation

Solid phase extraction. Full details of the solid phase extraction procedure used are given in Chapter 2 above. The same solid phase extraction procedure was followed with all samples, whether cell-culture or sputum supernatant. In summary:

The Phenomenex Strata-X SPE cartridge was washed with methanol followed by water. Sample (100 µl to 1 ml, spiked with 10 ng of deuterated lipoxin A₄) was then allowed to pass through the cartridge slowly. The cartridge was then washed with 10% methanol to remove contaminants before elution of the lipid fraction with 100% methanol. Samples were stored at -80 °C until use, usually the same or next day. Prior to LC-MS

the methanol was evaporated to dryness in a rotary evaporator and the sample reconstituted in 100 µl of HPLC solvent. In the case of high volume cell culture supernatant, the sample was passed through the same SPE cartridge in 1 ml aliquots before final washing and sample elution, thus allowing concentration of the lipid component.

Sample spiking. Deuterated lipoxin A₄ (d5-LXA₄) was used as an internal standard in all experiments with sputum or cell-culture supernatant. 10 ng (10 µl of 1 ng/µl solution in methanol) was added to the sample prior to SPE. Analysing a d5-LXA₄ standard of known concentration as well as measuring it in the extracted sample then allowed calculation of the extraction efficiency of the d5-LXA₄ spike from the supernatant. For example, if extracted samples were reconstituted in 100 µl of solvent then with 100% extraction efficiency the d5-LXA₄ concentration would be 100 pg/µl. Extraction efficiency can then be calculated from:

$$E = \frac{A_{sample}}{A_{standard}} \times 100$$

Where E is the %extraction efficiency, A_{sample} is the d5-LXA₄ peak area for the sample and $A_{standard}$ is the d5-LXA₄ peak area for a 100 pg/µl standard. The ideal procedure would be to use deuterated analogues of all compounds of interest as internal standards but this is prohibitively expensive; we are making the assumption that the d5-LXA₄ extraction efficiency will be reasonably representative of the extraction efficiency for our range of lipid mediators. All deuterated internal standards will contain small quantities of non-deuterated material which could be confused with material genuinely present in the sample; however our d5-LXA₄ has a quoted purity of 99% (Cayman

Chemical) and analysis with the Xevo TQ shows no detectable LXA₄ present even at a d5-LXA₄ concentration of 10 ng/μl (Figure 3.1), giving a measured purity of at least 99.99% assuming the limit of detection for LXA₄ is 0.5 pg/μl (see Section 3.3.1).

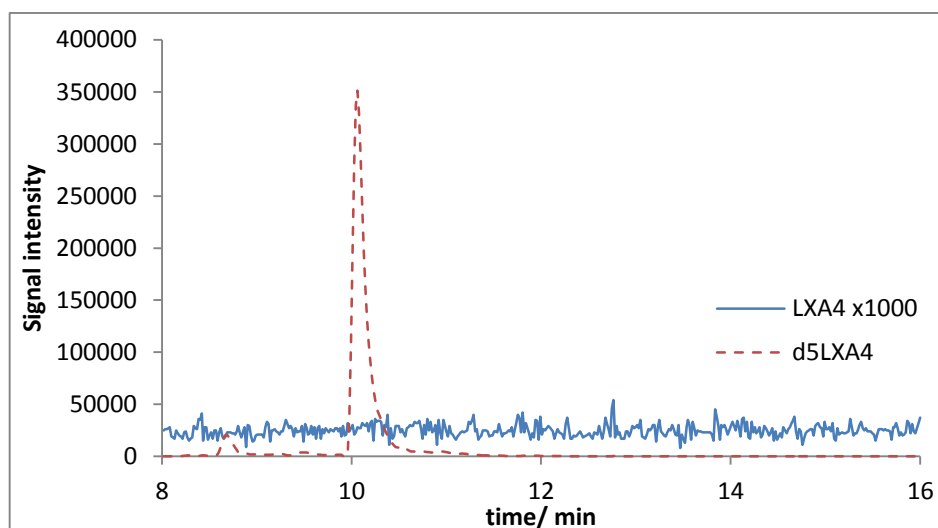


Figure 3.1. Xevo TQ mass chromatogram of 10 ng/μl d5-LXA₄ (dashed trace) showing the absence of any detectable signal on the LXA₄ channel (solid trace, signal intensity multiplied by 1000 for clarity).

Cell-culture supernatant preparation. Supernatants were collected from incubation of mast cells alone (human lung mast cells (HLMC) or HMC-1 cell line), epithelial cells alone and co-culture. Co-culture was a 16 hour overnight incubation. Two approaches were used: a) HLMC at 20,000 cells per 400 μl of BEGM growth medium in a 24 well plate coated in a confluent layer of human airway epithelial cells. b) 3 million HMC-1 cells in 10 ml of BEGM in a T-75 flask coated with confluent airway epithelial cells. In each case epithelial and mast cell monoculture supernatants were similarly prepared by overnight incubation. Some epithelial monoculture supernatants had a longer incubation time of up to 48 hours.

Sputum collection and preparation. Details are given in Chapter 2. A sputum induction process with nebulised hypertonic saline is used to obtain sputum samples from patients. These are then processed by dispersion of sputum plugs *via* addition of dithiothreitol (DTT) followed by centrifugation to separate out the cellular component. The resulting cell-free supernatant is stored at -80°C.

3.2.4 Sputum and cell-culture supernatant analysis

LC-MS analysis. Sputum or cell-culture supernatant was spiked with d5-LXA₄ internal standard and the lipid fraction extracted by SPE, as described above. The extracted samples were then analysed using the Xevo TQ or TQ-S LC-MS system with our optimised parameters as outlined above.

LXA₄ ELISA analysis. An Oxford Biomedical LXA₄ ELISA was used according to the manufacturer's instructions. On the manufacturer's advice sputum and cell-culture supernatant were analysed directly without any extraction process. No sample dilution was used.

3.3 Results

3.3.1 Limits of detection using Xevo-TQ

Figure 3.2 shows a mass chromatogram for the separation of a mixture of pro-resolving mediator standards. RvD₁, LXA₄ and d5-LXA₄ elute as a single peak but are easily

distinguished by mass spectrometry; other analytes show good chromatographic resolution. Calibration curves were constructed for each standard using solutions of known concentration, and these are shown in Figure 3.3 to Figure 3.6. The limits of detection (LOD – defined as the amount of sample present at a signal-to-noise ratio of 3) are listed in Table 3.7. Unfortunately instrument performance appeared to deteriorate somewhat over the course of the project, and these LOD were probably not achievable in all experiments.

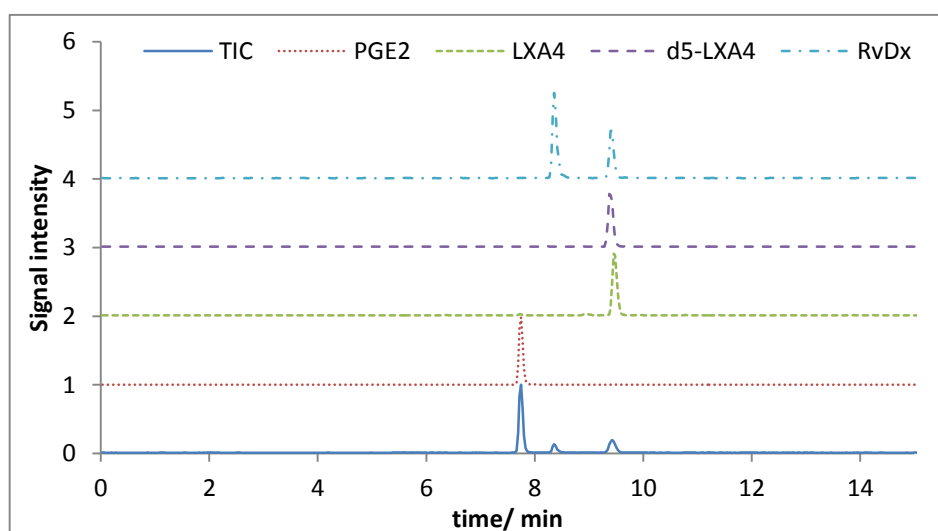


Figure 3.2. Mass chromatogram of mix of 5 standards at $125 \text{ pg } \mu\text{l}^{-1}$ (PGE_2) and $94 \text{ pg } \mu\text{l}^{-1}$ (RvD_1 , RvD_2 , LXA_4 , d5-LXA_4) on 4 SRM channels. Bottom trace shows combined total ion current for all channels. Signal intensity is in arbitrary units. Traces have been offset and RvD_x , LXA_4 and d5-LXA_4 signal intensity have been multiplied by 10 for clarity. Experimental conditions as described in 3.2.2.

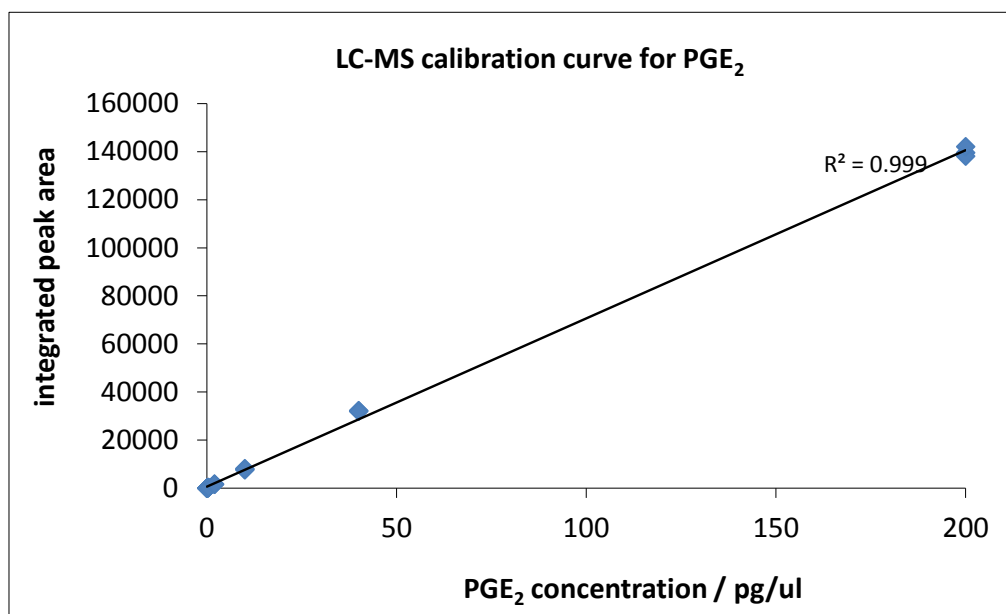


Figure 3.3. Calibration curve for detection of PGE₂ by LC-MS using optimised Xevo TQ protocol.

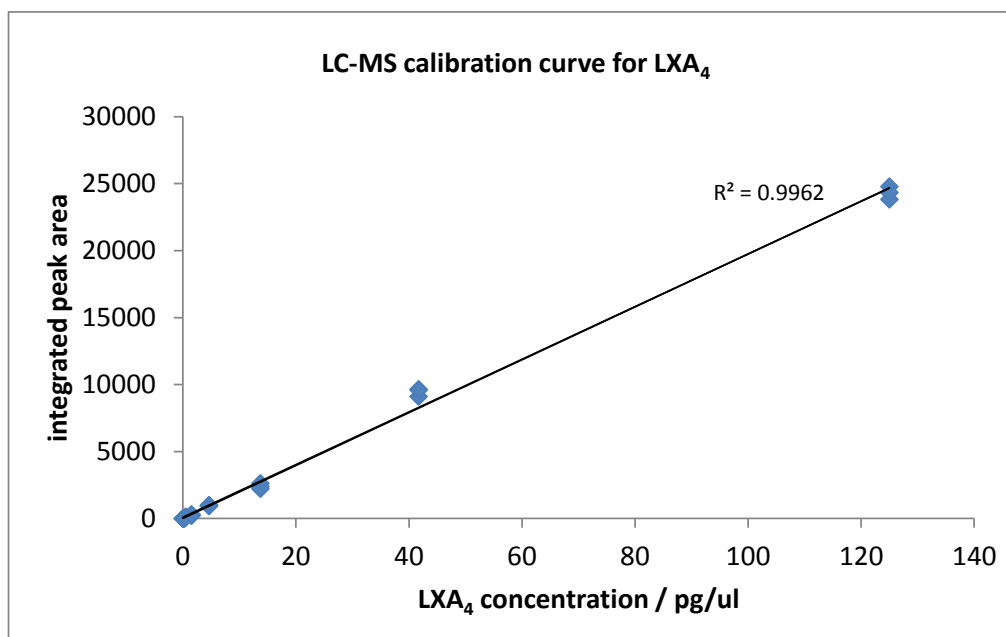


Figure 3.4. Calibration curve for detection of LXA₄ by LC-MS using optimised Xevo TQ protocol.

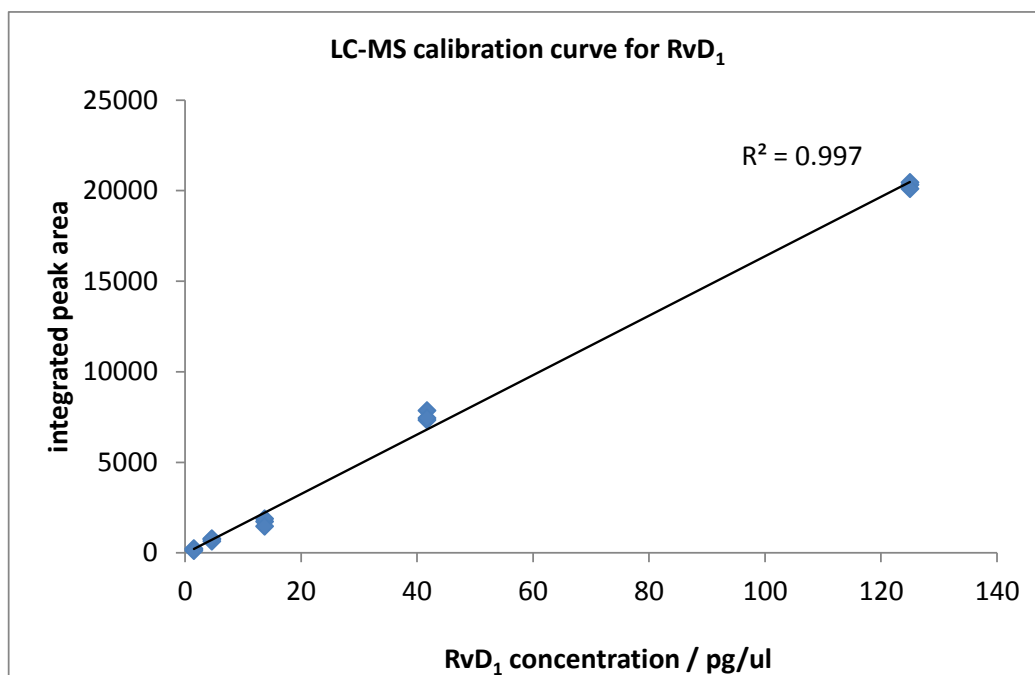


Figure 3.5. Calibration curve for detection of RvD₁ by LC-MS using optimised Xevo TQ protocol.

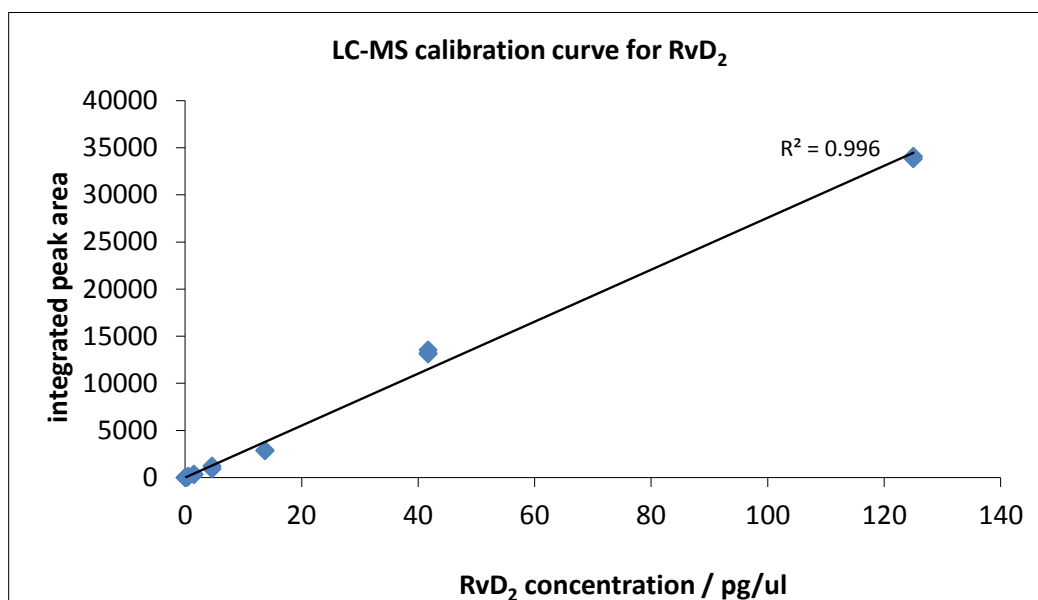


Figure 3.6. Calibration curve for detection of RvD₂ by LC-MS using optimised Xevo TQ protocol.

Mediator	Limit of detection		
	Mass/ pg	Conc/ pg/ μ l	Conc/ nM
PGE ₂	4	0.4	1.1
LXA ₄	5	0.5	1.4
RvD ₁	10	1	2.7
RvD ₂	5	0.5	1.3

Table 3.7. Limits of detection for lipid mediators by LC-MS using the Waters Xevo-TQ.

3.3.2 Limits of detection using Xevo TQ-S

Due to the limited time available on the newer Stepwave instrument it was not possible to determine complete calibration curves for each mediator. However, very approximate values can be estimated from the signal to noise ratios in the data obtained during optimisation with 10 pg/ μ l standards, assuming signal scales linearly with sample concentration and the limit of detection lies at a signal to RMS noise ratio (S/N) of 3. Measured S/N at 10 pg/ μ l is approximately 18000 for PGE₂ and 4000 for LXA₄, suggesting that the TQ-S is vastly more sensitive than the TQ; approximate limits of detection are 0.002 pg/ μ l and 0.01 pg/ μ l for PGE₂ and LXA₄ respectively. Limits of detection for RvD₁ and RvD₂ were difficult to estimate due to poor chromatographic resolution of the two compounds (likely to be the result of time-constraints reducing the time allowed for column equilibration). With a 10 pg/ μ l concentration of each standard injected, the signal to noise ratio for the Resolvin D-series SRM was approximately 2500, suggesting that the LOD for these compounds would be of the order of 0.02-0.04 pg/ μ l (assuming the contribution to the peak area from RvD₂ is twice that of RvD₁, as with the TQ, and that the LOD is at S/N = 3). These results are summarised in Table 3.8.

Mediator	Limit of detection/pg (nM)		
	Mass/ pg	Conc/ pg/ μ l	Conc/ nM
PGE ₂	0.02	0.002	0.007
LXA ₄	0.1	0.01	0.03
RvD ₁	0.4	0.04	0.1
RvD ₂	0.2	0.02	0.05

Table 3.8. Estimated limits of detection for lipid mediators by LC-MS using the Waters Xevo-TQ-S.

3.3.3 Pro-resolving mediators in sputum and cell culture supernatant

To investigate whether our method can detect pro-resolving mediators in asthmatic sputum supernatant, ten samples obtained from patients at the Difficult Asthma Clinic at Glenfield Hospital were processed by SPE as described above and analysed using the Xevo TQ. Sputum supernatant samples from this clinic are routinely stored for future research use with appropriate Ethics Committee approval and the informed consent of patients. Details of sputum collection and processing are given in Chapter 2. Samples were randomly selected from recent clinic visits and ranged in age from 1 – 6 months at time of analysis.

In order to investigate the applicability of our method to *in vitro* airway models, three freshly-prepared high-volume cell-culture supernatant samples (human airway epithelial cell, human lung mast cell and co-culture as described in Section 3.2.3 above) were similarly processed and analysed. In each case 10 ml of supernatant was passed through a single SPE cartridge. The lipid fraction was eluted with methanol, dried and reconstituted in 100 μ l of LC-MS solvent to give a 100-fold increase in concentration.

Results for both sputum and cell-culture supernatant were disappointing. Sample spiking with d5-LXA₄ showed good extraction efficiency for our SPE method at an average of 84%. However, no lipid mediators were detected in any of the sputum or cell-culture supernatant samples.

A LXA₄ ELISA (see Section 3.2.4 above) with a LOD of 0.02 pg/μl was also used to analyse cell-culture and sputum supernatant. Six cell-culture supernatant samples were used, 3 mast cell, 3 airway epithelial cell and 3 co-culture, prepare as described in Section 3.2.3 above. The sputum samples included the ten analysed by LC-MS described above and a further ten, again selected from recent Difficult Asthma Clinic samples. The ELISA calibration curve is shown in Figure 3.7. Again, no LXA₄ was detected in any of the cell-culture supernatant samples. However, the ELISA did detect LXA₄ in all 20 sputum samples at a level of >2 pg/μl (the assay's upper limit) despite no LXA₄ peak being seen in the samples analysed by LC-MS. 2 pg/μl is a higher level of LXA₄ than that previously detected in sputum from even mild asthmatics (see Section 1.3.2 above) and it is likely that this result is spurious; possible reasons for this are discussed in Section 3.4 below.

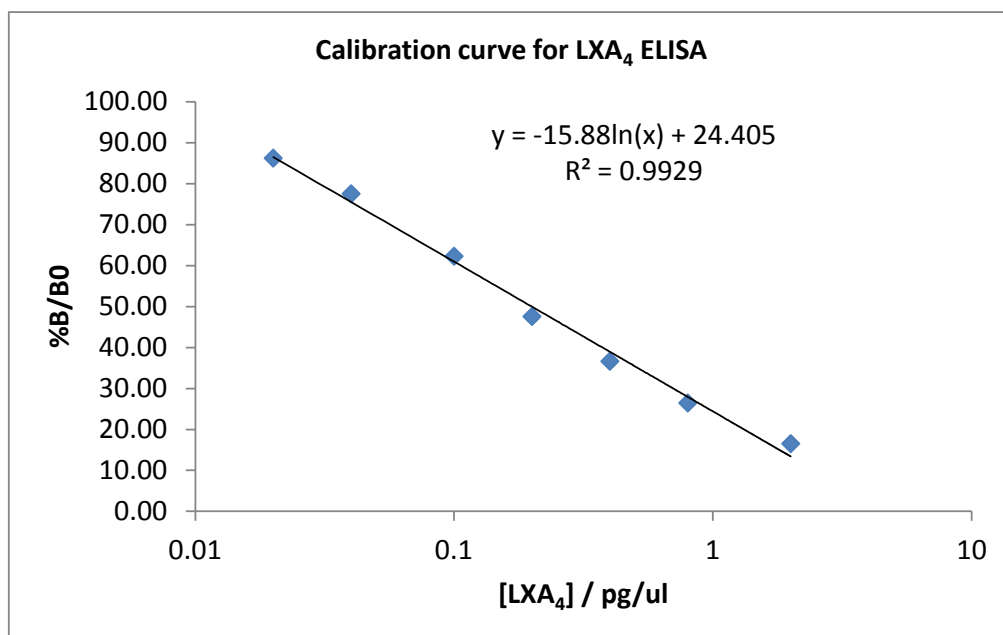


Figure 3.7. Calibration curve for Oxford Biomedical LXA₄ ELISA for sputum and cell-culture supernatant analysis.

Given the greatly increased sensitivity of the TQ-S instrument as discussed in Section 3.3.2 above, a further set of 10 asthmatic sputum samples was analysed with the more advanced instrument. Sputum samples were again all from attendees at the Difficult Asthma Clinic at Glenfield Hospital, ranged in age from 3 to 17 months from time of donation and had been stored at -80°C. Sample spiking with d5-LXA₄ gave a calculated mean extraction efficiency of 107% - this is likely to be a result of the limited column equilibration time leading to an improvement in instrument performance between measurement of standards and samples. All samples show the presence of PGE₂, at a level undetectable with the less sensitive TQ instrument (note: the pro-inflammatory mediator PGD₂, also found in sputum, is isomeric with PGE₂ and is detectable on the same SRM channel; given that chromatographic separation was poor in this experiment it is possible that these peaks represent co-eluting PGE₂ and PGD₂). An example mass chromatogram is shown in Figure 3.8. In the absence of calibration curves it is not

possible to accurately quantify the levels detected, but by extrapolation from data obtained during optimisation with 10 pg/μl standards, levels range from an estimated 0.13 to 1.9 pg on column, corresponding to a concentration of 13 to 190 pg/ml or 0.02 to 0.3 nM in the original sputum supernatant, assuming 100% extraction efficiency. The majority of samples again showed no resolving mediator present; however Figure 3.9 shows the mass chromatogram of one of the 10 samples (13 months old at analysis) revealing a very low level of LXA₄ (approximately 0.11 pg on column, concentration 11 fg/μl or 0.015 nM in the sputum supernatant assuming 100% extraction efficiency). This is close to the limit of detection, and a number of other peaks of similar height are visible above the noise. The marked peak is identifiable as a genuine analyte peak as it is present in triplicate injections of the sample and its retention time exactly matches that of the d5-LXA₄ standard with which the sample was spiked. Unfortunately the TQ-S instrument is no longer available for analysing further samples, and the much less sensitive TQ instrument will be used for analysis of samples from our clinical study as described in Chapter 6.

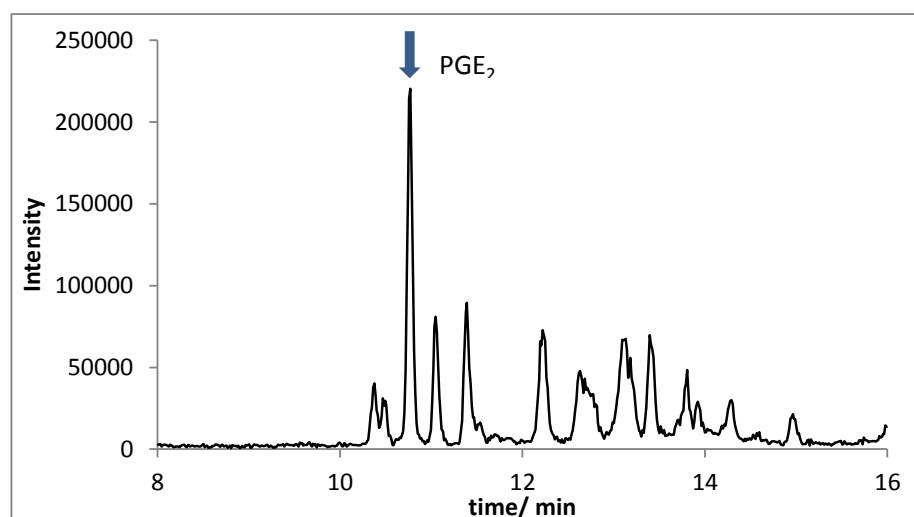


Figure 3.8. PGE₂ SRM channel of mass chromatogram of an asthmatic sputum sample following solid phase extraction. The marked peak is identifiable as PGE₂ *via* its retention time in comparison to a PGE₂ standard and its appearance at identical time on the 351.3->189.3 channel, which also represents a PGE₂ fragmentation. A number of smaller peaks of unknown origin are also seen. Conditions as in 3.2.2.

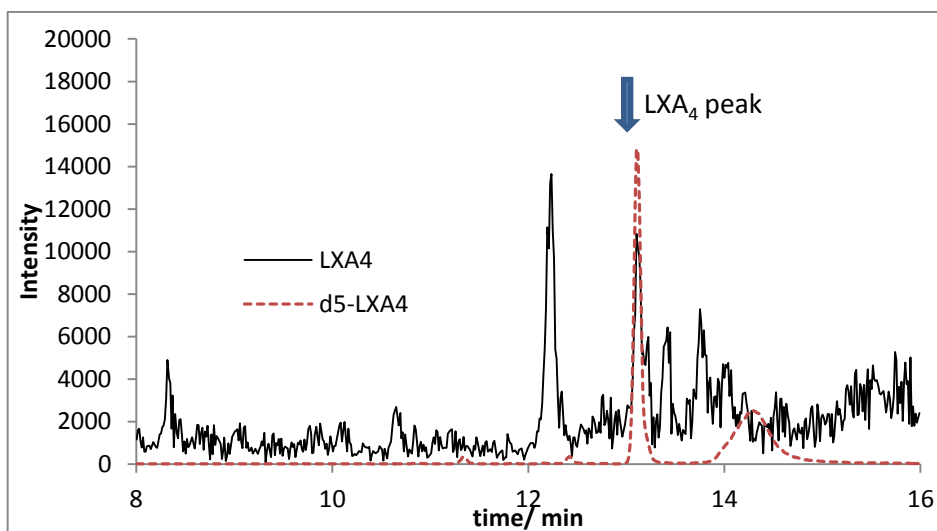


Figure 3.9. LXA₄ and d5-LXA₄ SRM channels of mass chromatogram of an asthmatic sputum sample following solid phase extraction. The marked peak is identifiable as lipoxin A₄ due to its reproducibility across triplicate injections and its retention time being identical to the deuterated lipoxin A₄ internal standard. The signal intensity of the d5-LXA₄ channel has been reduced 1000-fold in order to display both traces on the same axes. Conditions as in 3.2.2.

3.3.4 Stability of lipid mediators in sputum

It is possible that our failure to unambiguously detect significant quantities of pro-resolving mediators in any of our sputum samples is a result of the lipids rapidly deteriorating during sample storage (all samples not used within 24 hours of preparation were stored at -80°C). To establish whether or not this could be the case, a series of sample stability tests was conducted. Samples for stability testing were created by spiking sputum samples obtained from the Difficult Asthma Clinic with synthetic lipid mediators. The composition of the samples is listed in Table 3.9. High initial concentrations were used to ensure that they would be easily detectable using the Xevo TQ MS. As stock solutions of mediators are made up in ethanol there is inevitably a

small amount of organic solvent in each sample, which may affect mediator stability; the ethanol content of the samples is also included in Table 3.9.

Samples	[PGE ₂]/ pg/μl	[RvD ₁]/ pg/μl	[LXA ₄]/ pg/μl	%EtOH
1 - 3	23	88	81	7
4 - 6	248	248	248	0.7
7 - 9	246	47	246	2

Table 3.9. Composition of samples used for stability testing.

In each case the solid phase extraction process outlined above was followed prior to analysis; 100 μl of sputum was used in each analysis, with samples reconstituted in 100 μl of LC-MS solvent following extraction. Samples 7-9 were analysed prior to and immediately following a freeze-thaw cycle on Day 1, and then again after 1 month. Samples 1 – 6 were analysed post-freeze-thaw on Day 1 and again after 1 month and 5 months. 5 month samples were extracted to methanol 5 weeks prior to drying, reconstitution and analysis due to persistent instrument problems. They were stored in methanol at -80°C. Each sample was run in triplicate and the peak areas averaged. In each case the peak area for each analyte was corrected for extraction efficiency via d5-LXA₄ internal standard and for varying instrument sensitivity by analysis of a set of lipid mediator standards prior to the sputum analysis. The following equation was used to calculate corrected analyte peak area for Day *x* as a percentage of the Day 1 peak area:

$$A_{corrected\%} = \frac{A_x}{A_0} \times \frac{\%recovery_0}{\%recovery_x} \times \frac{A_{standard(0)}}{A_{standard(x)}} \times 100$$

Where *A_x* is the Day *x* peak area, *%recovery_x* is the Day *x* extraction efficiency as determined by d5-LXA₄ spiking, *A_{standard(x)}* is the Day *x* peak area for the standard for

that lipid, and A_0 , $\%recovery_0$ and $A_{standard(0)}$ are the Day 1 equivalents. Results are shown in Table 3.10.

Some corrected peak areas are greater than 100% of the Day 1 values; this probably reflects a combination of variability in instrument sensitivity between analysis of standards and samples and pipetting errors.

Mediator	Sample	%Peak Area Day 1 pre- freeze	%Peak Area Day 1 post- freeze	%Peak Area 1 month	%Peak Area 5 months
PGE₂	1-3	-	100	119 ± 39	51 ± 1.5
	4-6	-	100	91 ± 7.4	48 ± 15
	7-9	100	113 ± 7	119 ± 20	-
RvD₁	1-3	-	100	109 ± 39	70 ± 12
	4-6	-	100	79 ± 12	79 ± 6.5
	7-8*	100*	127 ± 6*	102 ± 13*	-
LXA₄	1-3	-	100	72 ± 10	84 ± 9
	4-6	-	100	97 ± 12	95 ± 5.6
	7-9	100	124 ± 28	122 ± 23	-

Table 3.10. Corrected peak areas for pro-resolving mediators in sputum measured over 5 months. Each of samples 1 to 9 was measured in triplicate; data expressed as peak area ± standard error for the 3 repeats. *Sample 9 excluded as only one of its Day 1 repeats gave a measurable RvD₁ peak area.

The most striking finding is the loss of 50% of the PGE₂ signal after 5 months. This is surprising given that samples were stored at -80°C; however, manufacturer product information states a rate of degradation of 10% in less than 48 hours for PGE₂ in aqueous solution at 25°C and physiological pH (Sigma-Aldrich, 2002). A slower rate in frozen storage is therefore consistent with this. LXA₄ and RvD₁ appear to be more stable at -80°C; the LXA₄ corrected peak area at 5 months is not significantly different

from the Day 1 level at 95% confidence; RvD₁ shows a loss of 20-30% over the 5 months.

3.4 Discussion

3.4.1 Limits of detection for pro-resolving mediators

Table 3.11 summarises our limits of detection for pro-resolving mediators versus other published methods. It can be seen that while our RvD₁ LOD compares well with the earlier work of Serhan's group, using the Xevo TQ we do not achieve detection at the level of Blaho *et al.* (2009) for this or other mediators. With the more advanced Xevo TQ-S however, estimated LOD are considerably better than Blaho *et al.* and at least comparable to if not better than ELISA values. Given continuing advances in instrumentation and the other advantages of LC-MS over ELISA (summarised in Table 3.12) it seems likely that LC-MS will gradually supplant ELISA as tool of choice for analysing pro-resolving mediators in clinical samples. It is unfortunate that we have not had access to the more powerful LC-MS instrument for analysis of samples from our clinical study.

Method	LOD in pg/μl				
	ELISA	LC-MS (Serhan*)	LC-MS (Blaho [†])	LC-MS (Xevo TQ)	LC-MS (Xevo TQ-S [§])
PGE ₂	0.015	-	0.02	0.4	0.002
LXA ₄	0.02	-	0.02	0.5	0.01
RvD ₁	0.01-0.02	4	0.2	1	0.04
RvD ₂	-	-	-	0.5	0.02

Table 3.11. Summary of limits of detection of pro-resolving mediators analysed by different methods. ELISA LOD for PGE₂, LXA₄ and RvD₁ are the quoted LOD for kits from Cayman Chemical, Oxford Biomedical and Cayman Chemical respectively. *Masoodi *et al.*, 2008. [†]Blaho *et al.*, 2009. [§]Xevo TQ-S LOD are estimated from the S/N of 10 pg/μl standards.

- Low analysis cost after initial instrument outlay
- Flexibility to analyse large or small numbers of samples in one run
- No time-expiring reagents
- No need for specific antibodies
- Multiple mediators can be measured in one experiment – important with small sample volumes
- Potential to provide structural information about analytes
- Good linearity allows accurate quantification possible by calibration using standards of known concentration
- Can use internal standard to determine efficiency of any sample extraction process
- Excellent limits of detection with newer instrumentation

Table 3.12. Advantages of LC-MS for detection of pro-resolving lipid mediators.

3.4.2 Pro-resolving mediators in sputum

The failure to see pro-resolving mediators in most of our sputum samples, even with the high sensitivity TQ-S instrument, may of course reflect either that they are not present, or that they are present in concentrations below our limit of detection. There is evidence from cell culture work that pro-resolving mediators can have biological effects even down to femtomolar concentrations (N. Martin et al., 2012) in which case detection of biologically active levels of these mediators would not realistically be achievable by currently available methods. However, there are two literature report of LXA₄ measurement in sputum supernatant (Bonnans et al., 2002; Vachier et al., 2005). In the first study levels were measured by ELISA to be 0.1, 1.3 and 0.8 pg/μl in controls, mild and severe asthma respectively. In the second study LXA₄ levels ranged from 0.2 pg/μl

in controls to 1 pg/μl in mild asthma. Again, patients with severe asthma had reduced levels of LXA₄ compared to those with mild disease. Levels in these studies are approximately an order of magnitude higher than the level detected in our sole LXA₄-positive sputum sample. It is possible that the LXA₄ in our samples had deteriorated over time, although the sputum had been stored at -80°C, and PGE₂ was readily detectable with the higher sensitivity instrument. As detailed in 3.3.4 above, LXA₄ appears to be stable in sputum over at least 5 months, more stable in fact than PGE₂. The sample in which a low level of LXA₄ was detected was one of the older samples at 13 months.

It is interesting that the set of ten sputum samples in which no LXA₄ was detected when analysed with the Xevo TQ mass spectrometer (LOD 0.5 pg/μl for LXA₄) showed levels of >2 pg/μl when analysed by ELISA. Several of these sputum samples were included in the set analysed on the high sensitivity TQ-S instrument, and again no LXA₄ was detected with the one exception detailed in 3.3.3 above. One possible reason for our failure to detect any LXA₄ by LC-MS is that the ELISA is giving falsely high levels, perhaps as a result of the dithiothreitol (DTT) used in sputum processing interfering with antibody-substrate binding; DTT has been shown to increase apparent detected levels of leukotriene B₄ standard in a competitive ELISA (Woolhouse, Bayley, & Stockley, 2002). LXA₄ has also been measured in asthmatic bronchoalveolar lavage (BAL) fluid, apparently by both ELISA and LC-MS (Planaguma et al., 2008). Levels were found to be 0.011 pg/μl in severe asthma and 0.15 pg/μl in mild disease, more in keeping with the levels from our study than the Bonnans and Vachier sputum studies. It is not entirely clear whether these values refer to ELISA or LC-MS data. Sputum supernatant samples from our current clinical study have a DTT-free fraction stored for future analysis of inflammatory and resolving mediators, in order to avoid the

possibility of DTT interference occurring. It may be wise to treat the results of ELISA-based measures of sputum LXA₄ or other pro-resolving mediators with caution unless it is clear that samples are free of DTT and other potential interfering substances.

3.4.3 Stability of pro-resolving mediators

The finding of 50% loss in the PGE₂ signal in sputum after 5 months storage at -80°C is surprising, but not inconsistent with manufacturer data showing a rate of degradation of 10% per 43 hours for PGE₂ in aqueous solution at 25°C (Sigma-Aldrich, 2002). This clearly has implications for measurement of PGE₂ in clinical samples; for accurate quantification measurement should occur within a month of acquisition, a period which our study shows does not result in statistically significant loss of signal. This may be difficult over the course of a project in which samples are collected over a period of many months, with limited instrument availability or the need to accumulate samples to run on a single ELISA plate. An added limitation to measuring sputum PGE₂ is that it is rapidly metabolised *in vivo*, with more than 90% of circulating PGE₂ cleared by a single passage through the lungs (Cayman Chemical, 2013a). This makes the use of sputum or urine PGE₂ metabolites a more attractive option for assessing lung PGE₂ production, and this will be the approach used in our clinical study.

LXA₄ and RvD₁ in sputum appear to be more stable than PGE₂ when stored at -80°C, with the LXA₄ signal at 5 months not significantly different from the Day 1 level at 95% confidence, and RvD₁ showing a loss of 20-30% over 5 months. However, RvD₁ in particular still requires prompt analysis for accurate quantification; the most recent sputum samples analysed using the Xevo TQ-S in 3.3.3 above were 3 months old, and should therefore have still contained the vast majority of any initial RvD₁ present.

Chapter 4 Precision-cut lung slice model

4.1 Introduction

4.1.1 Background

The precision-cut lung slice technique is outlined in Chapter 1. The purpose of using this technique, and also the bronchial ring method discussed in the next chapter, was to develop an *in vitro* model of the allergic airway which could be used to investigate the effects of pro-resolving mediators on bronchoconstriction. There are two questions to answer with this model. Firstly, do the pro-resolving mediators directly affect airway smooth muscle to attenuate the action of directly-acting bronchoconstrictors such as histamine? Secondly, do they have an effect earlier in the pathway of mast cell-mediated bronchoconstriction by acting on mast cells themselves? To answer both of these questions, and thus assess the likelihood that pro-resolving mediators play a role in refractoriness, it is clearly necessary to develop a model in which bronchoconstriction is provoked by allergen, or another agent provoking mast cell degranulation. The first step is to select the appropriate species for the model, and this is discussed in the next section.

4.1.2 Species selection

For initial training in the PCLS technique mice were used, as they have been widely used with this technique and the preparation and cutting process is relatively straightforward (see Chapter 2). However, mice are not necessarily the best choice for development of this model. Clearly human lung would be the ideal, and small pieces are sometimes available following resection of lung cancers, but improvements in lung-sparing surgical technique mean that complete pneumonectomy is now very rare at our

centre, and it is very unusual to have even a complete lobe available for research. Preparation of PCLS via the technique outlined in Chapter 2 is therefore not feasible. For development of our model we need to select a species which closely models human lung. Mice are widely used in asthma research – there is wide availability of probes and transgenic animals, they are easy to sensitise using ovalbumin and produce a Th2 response in a similar manner to humans; however, as well as the obvious disparity in airway size they have scanty airway smooth muscle, mast cell degranulation releases serotonin which is not found in human mast cells and they have no late-phase allergic response (Zosky & Sly, 2007). No animal spontaneously develops a disease entirely like human asthma; therefore any animal model will involve some compromise. In this project we were constrained by the need to use an animal from which lung is readily obtainable fresh from a local abattoir, as facilities and funding are not available for breeding animals specifically for the study. The sheep appears to offer a number of advantages. Lungs are readily available; they are capable of generating a response to inhaled allergen with an early and late phase response and a mediator pattern similar to human asthmatics; the resulting inflammation responds to corticosteroid therapy (Zosky & Sly, 2007). Moreover, a gift of house dust mite-sensitised sheep serum was available, giving the potential for passive sensitisation of lung slices. The only study of sheep PCLS in the scientific literature is a doctoral thesis describing the relative effects of bronchoconstricting mediators in pre-term and adult sheep (Dassow, 2010). This further adds to the novelty of our approach.

4.2 Method summary

4.2.1 Preparation of sheep PCLS

The method of preparing sheep PCLS is described more fully in Chapter 2. In brief, lungs were obtained fresh from a local abattoir; a small lobe was filled with warm 2% agarose solution and kept on ice until the agarose solidified. Lung was then sectioned into 0.5 – 1 cm diameter pieces using a scalpel blade and 200 μ m slices cut using an oscillating blade tissue slicer. The resulting slices were then incubated at 37.5 °C in DMEM with frequent media changes in the first 24 hours and daily changes thereafter. Slices were assessed using an inverted microscope for the presence of an airway with beating cilia visible on the epithelium for >50% of the airway circumference prior to use. In experiments involving attempted passive sensitisation to house dust-mite, slices were incubated overnight in high IgE sensitised serum at 10% concentration in DMEM.

4.2.2 Sample analysis

More detail is given in Chapter 2; in summary: A single lung slice was placed in 1 ml of fresh DMEM in a well of a 24-well plate. A small U-shaped piece of stainless steel wire was used to hold the slice on the bottom of the well. The plate was positioned on the microscope stage which was maintained at 37.5 °C; mediators of interest were added to the well via a pipette and images of an airway lumen recorded as appropriate. Images were analysed using ImageJ software, and dose-response best fit curves calculated using a 4 parameter logistic model in R. Slices showing a maximal carbachol-induced contraction of less than 10% initial lumen area were not analysed.

4.3 Results

The purpose of initial experiments was to establish the technique and characterise the sheep airway contractile response prior to development of the sensitised model. Figure 4.1 shows an airway in a sheep lung slice. Addition of increasing concentrations of the muscarinic agonist carbachol causes gradual contraction of the airway and allows a cumulative dose response curve to be constructed; an example is shown in Figure 4.2. For each dose-response curve a best fit line can be determined, allowing the carbachol EC_{50} value of the airway (dose of carbachol giving 50% of maximum contraction) to be estimated. The model used to determine best fit parameters is described in Chapter 2. Figure 4.3 shows the best fit line for the dose-response curve of Figure 4.2; the estimated carbachol EC_{50} with standard error for this airway is $(3.2 \pm 0.34) \times 10^{-6}$.

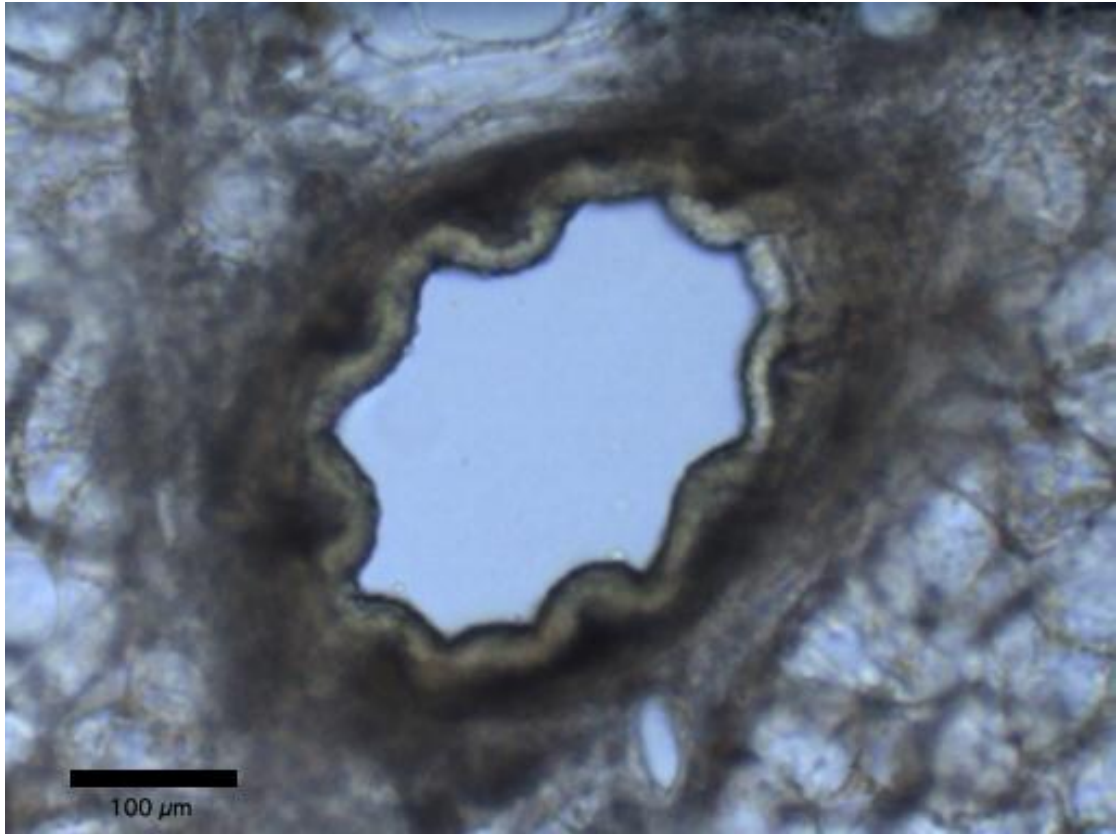


Figure 4.1. Sheep precision-cut lung slice in DMEM, imaged on inverting microscope via x10 objective, showing approx 200 μm diameter airway in cross-section.

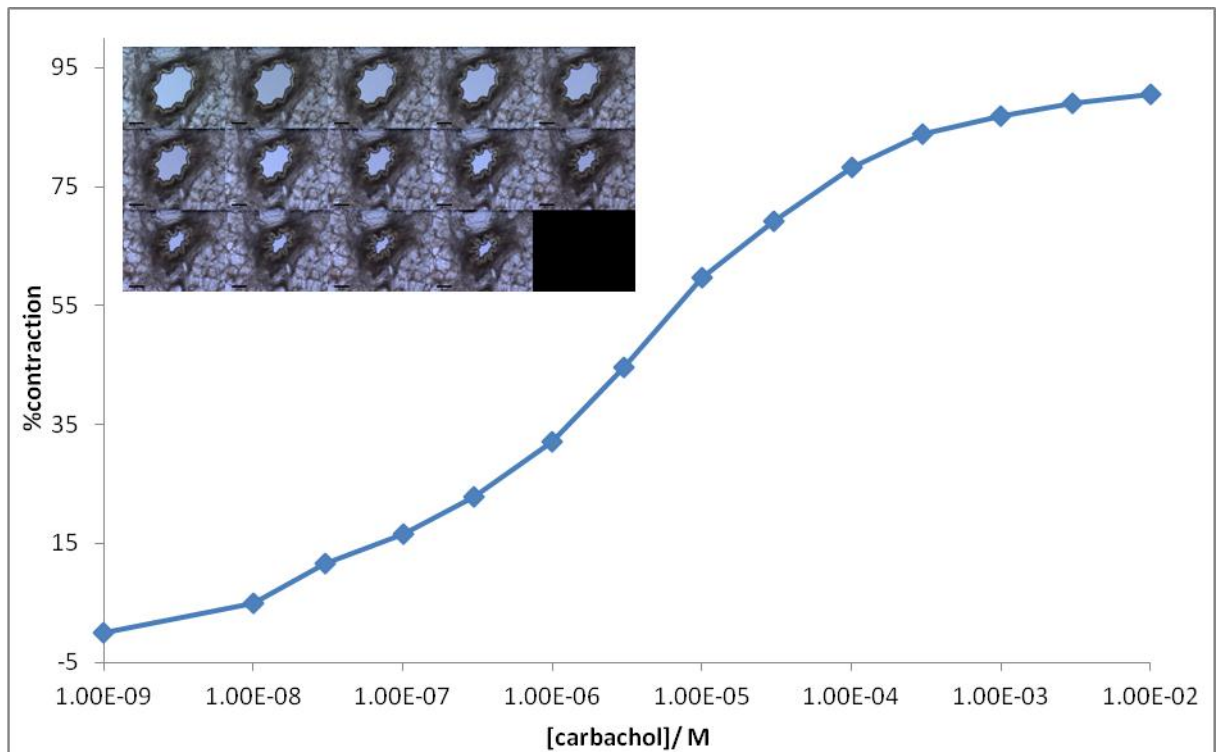


Figure 4.2. Cumulative carbachol dose response curve for a single airway in a sheep precision-cut lung slice. %contraction calculated as $100 - (\text{lumen cross-sectional area} / \text{initial cross-sectional area} \times 100)$. The inset shows images of the airway lumen with increasing carbachol concentration.

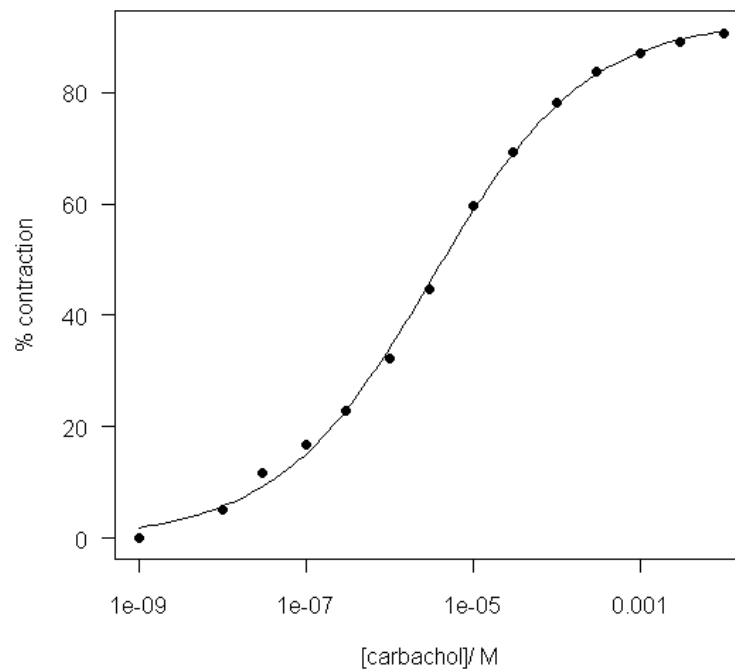


Figure 4.3. Best fit line for the carbachol dose-response curve of Figure 4.2. calculated using 4 parameter logistic model. Estimated EC_{50} with standard error is $(3.2 \pm 0.34) \times 10^{-6}$.

Figure 4.4 shows the mean carbachol dose-response curve for 11 airways from 7 animals, ranging in diameter from 75 to 310 μm ; Figure 4.5 shows the best fit curve. The estimated EC_{50} is $(5.2 \pm 0.87) \times 10^{-6}$. No comparator EC_{50} values are available for the sheep; the study conducted by Dassow (2010) used the related compound methacholine rather than carbachol. Reported carbachol EC_{50} values in humans, measured using the bronchial ring technique, range from $0.4 - 1.1 \times 10^{-6}$ (Finney et al., 1985; Finney, Anderson, & Black, 1987; Ben-Jebria, Marthan, & Rossetti, 1994). Cooper & Panettieri (2008) reported a value of 0.7×10^{-6} in the mouse using PCLS. Sheep lung thus appears relatively insensitive to carbachol. Airways were included in the analysis only if the maximum contraction to carbachol was $\geq 10\%$. This is essentially an arbitrary figure but was chosen empirically to ensure that a genuine change in measured airway cross-sectional area was clearly distinguishable from variation due to measurement error.

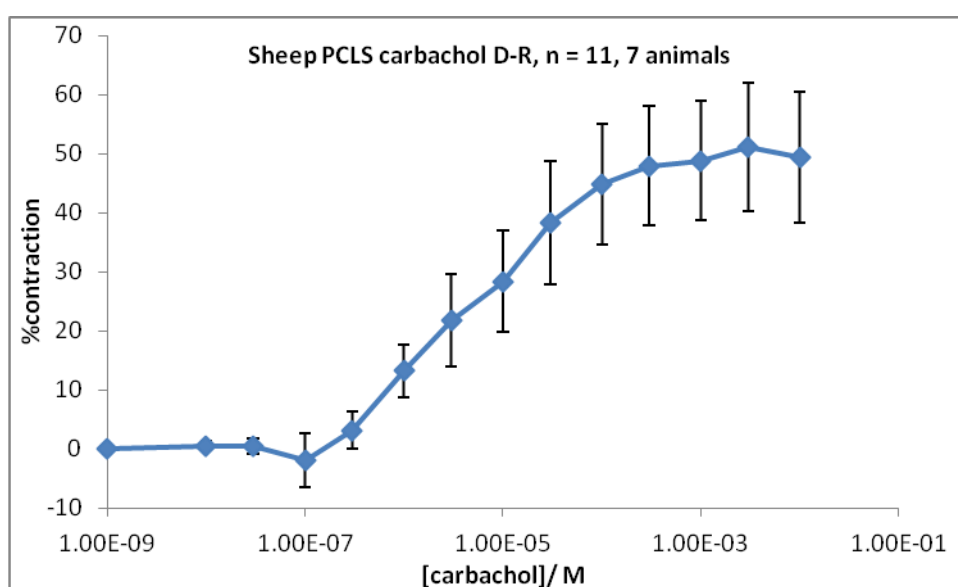


Figure 4.4. Mean carbachol dose response curve for 11 sheep PCLS from 7 animals. Error bars show the standard error.

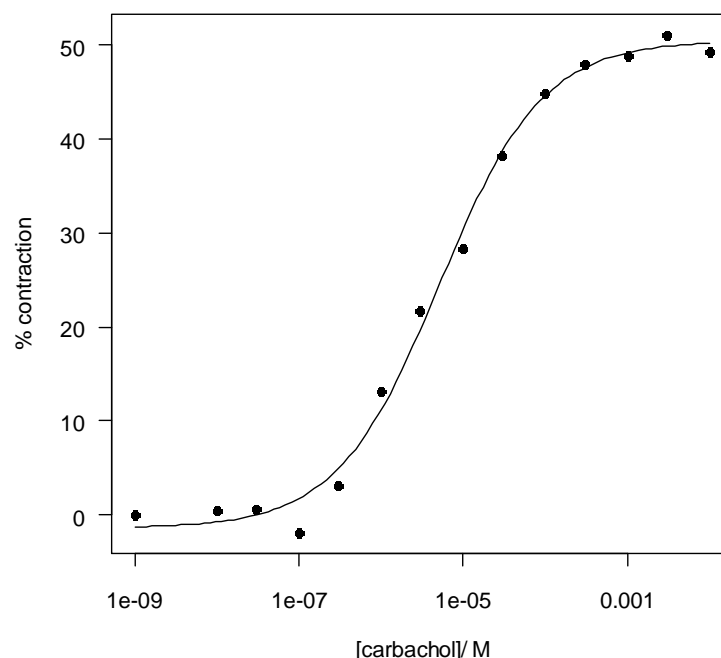


Figure 4.5. Best fit line for the carbachol dose-response curve of Figure 4.4. calculated using a 4 parameter logistic model. Estimated EC_{50} with standard error is $(5.2 \pm 0.87) \times 10^{-6}$.

Following establishment of the basic sheep PCLS technique, development of a passively-sensitised model of allergic sheep airway was attempted using serum from HDM-sensitised animals, via the method described in Chapter 2. Slices were challenged with 1000 units of HDM protein by addition of 20 μ l of Allergopharma Soluprick HDM skin-prick test solution to the well. Initially this approach appeared promising. Figure 4.6 shows a sensitised airway contracting following addition of HDM extract; it is then allowed to relax back to baseline before carbachol is used to determine its maximum contraction. Unfortunately non-sensitised control slices show a similar response to HDM extract. Figure 4.7 shows a comparison of the response to HDM extract normalised to maximum carbachol response for 9 HDM-sensitised and 4 non-sensitised

control slices. The sensitisation process has made no statistically significant difference to the response to HDM extract, and the contraction is therefore unlikely to be an allergic response.

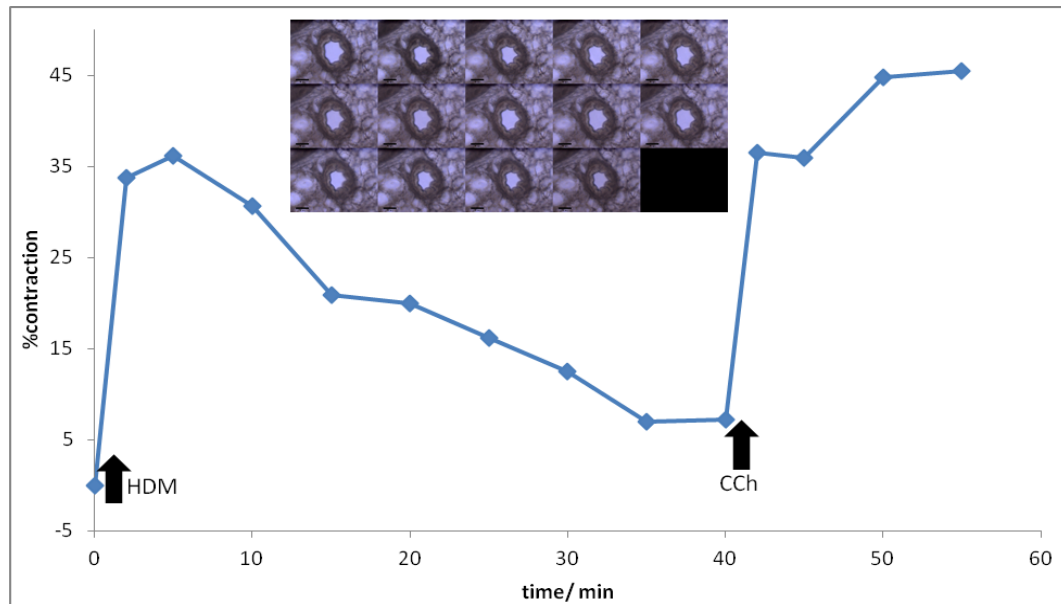


Figure 4.6. Contraction of an HDM-sensitised slice with house dust mite extract, followed by maximal contraction with carbachol.

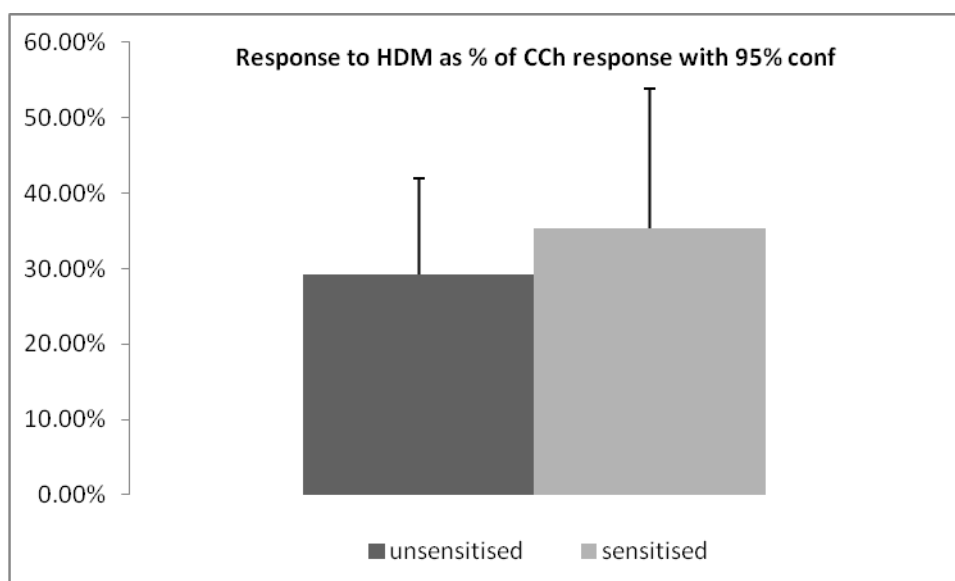


Figure 4.7. Response to house dust mite extract of sheep PCLS treated with HDM-sensitised sheep serum and non-sensitised controls. n = 4 (2 animals) for controls, n = 9 (3 animals) for sensitised slices.

Although sheep PCLS showed early promise as a method for developing an allergic airway model, and the method has novelty as a new application of the PCLS technique, it was considered unviable for a number of reasons:

1. The bronchial responsiveness of the slices was very variable. Out of 18 animals from which slices were made, 8 produced no slices in which the maximum response to carbachol was $\geq 10\%$ and were thus not used at all.
2. Slice preparation was unreliable given the tissue slicer available and the lack of control of tissue supply. Although reliability improved with experience, it was not unusual to find that no slices with healthy epithelium could be prepared.
3. There was no evidence of passive sensitisation of slices using HDM-sensitised serum.
4. An organ bath became available, allowing use of the bronchial ring technique discussed in the next chapter.

Attempts to develop a model via this route were therefore abandoned. These issues are discussed in more detail in Section 4.4 below.

4.4 Discussion

4.4.1 The sheep PCLS technique

Development of this technique was technically challenging. The key issues for successful PCLS preparation were:

1. *A supply of good quality lung.* Breeding animals specifically for research would be ideal but with larger mammals this is prohibitively expensive and requires specialised facilities, aside from the ethical concerns. It was therefore necessary to use lung from an abattoir, the nearest of which was approximately 30 minutes away. Obtaining fresh, “still warm” lung was important, as was the integrity of the lung. Usual abattoir practice is to cut into organs during the butchering process in order to inspect them internally for disease. A cut to a lung lobe renders it impossible to fill with agarose for slicing. It was therefore important that the abattoir staff were willing to leave a small lobe uncut.

2. *Agarose temperature.* Agarose exhibits hysteresis in its heating/cooling curves, i.e. it melts on heating at a much higher temperature than the temperature at which it solidifies on cooling. The agarose must be warmed to ca. 60°C to melt it and then cooled to < 40°C prior to lung filling, in order to avoid damaging the tissue.

3. *The tissue slicer.* Sheep tissue is more resistant to slicing than mouse tissue, and the Electron Microscopy Sciences EMS-5000 instrument used for initial PCLS training experiments using mice was unable to slice it. The acquisition of a Campden Instruments slicer with manual sample advance allowed slicing to proceed; even so

successful slicing required well-filled lung, frequent changes of blade and good cooling of tissue.

4. *Washing of slices.* On the day of slice preparation, to maintain health the freshly cut slices required several changes of growth medium in the hours following preparation, presumably to wash away mediators released during the period between animal death and slice incubation.

Even with optimisation of the above parameters, there was variability in the quality of the slices obtained (as assessed by the presence of beating cilia on bronchial epithelium) and their bronchoconstrictor response for reasons which are not entirely clear. One reason is probably the lack of control over the lung supply – lungs were always obtained on the morning of slaughter with as short a delay as possible, but nevertheless there will be variability in tissue viability which will be reflected in the variable slice quality. The variation in the magnitude of the bronchoconstriction response is probably more fundamental – there is a natural animal to animal variability in bronchial responsiveness to challenge, as with humans (Zosky & Sly, 2007). Similarly, the degree of variation in the carbachol EC₅₀ value is not surprising; for example in the rat there is a 1000-fold airway to airway variation in methacholine EC₅₀, or 100-fold if slices are stratified for airway size (C. Martin, Uhlig, & Ullrich, 1996).

4.4.2 Attempt at sensitisation of sheep PCLS

Although the basic sheep PCLS model was modestly successful, attempts at passive sensitisation of slices failed; although HDM-sensitised slices responded to HDM extract, non-sensitised slices also did so to the same extent. The reason for this is

uncertain. It is highly unlikely that the slice-donor animals were exposed to house dust mite and became naturally sensitised. It is possible that an excipient of the HDM extract is responsible; the principal excipient in the solution used is glycerol at 50%, which will be at a final concentration of 1% after addition to the solution containing the slice. It could thus be hypothesised that it caused bronchoconstriction via an osmotic effect. However, treatment of 3 slices with the appropriate concentration of glycerol gave a mean contraction of only 2%, equating to 5% of the maximal carbachol response. A more likely possibility is that the HDM extract causes bronchoconstriction via a non-specific protease effect. It is known that the house dust mite protein Der p 1 can act as a cysteine protease and potentially cause bronchoconstriction by non-IgE receptor-dependent degranulation of mast cells (D. C. Machado, Horton, Harrop, Peachell, & Helm, 1996). It is therefore possible that the mechanism of contraction is indeed mast cell-mediated, but not allergic. Due to the uncertainty surrounding this however, and for other reasons, the model was not pursued further, as discussed below.

4.4.3 Problems with sheep PCLS approach

As alluded to above, despite its early promise the PCLS approach to developing an in vitro model of allergic asthma was abandoned due to a number of problems. Firstly, although the basic sheep PCLS approach worked reasonably well if attention was paid to the factors outlined in 4.4.1, preparation of slices remained technically challenging, and even with experience poor quality slices were sometimes obtained. Even when slices appeared to be of good quality as assessed by cilia activity, maximal contraction to carbachol varied between 0 and 100%, presumably as a result of natural animal to animal and airway to airway variation. Eight out of 18 animals with slices meeting our

quality criteria did not yield any airways which were more than minimally contractile to carbachol. Furthermore, it was not possible to demonstrate an allergic effect in the sensitised model, or to determine with certainty what the mechanism of contraction in the sensitised-slice experiments was. It is possible that further investigation may have yielded improved results; however, C Martin (University of Konstanz) has previously attempted passive sensitisation of sheep PCLS and abandoned the method as unreliable (personal communication). There is known to be a high degree of animal to animal variability to bronchial challenge in sheep (Zosky & Sly, 2007).

One possible approach to creation of an *in vitro* allergic sheep model which to our knowledge has not been previously attempted would be to make use of the natural sensitisation of some sheep to the roundworm *Ascaris suum*. Naturally *Ascaris*-sensitised sheep have been used to study allergic airway responses in live animals (Abraham, Delehunt, Yerger, & Marchette, 1983) and it may be that this approach could be applied to a PCLS model using abattoir-acquired lung, although this would be dependent on the frequency of sensitisation in locally farmed animals; for example Ahmed, Krainson, & Yerger (1983) found 13 sensitised sheep out of 32 in their study group.

As a result of the doubtful prospect of further progress in the light of the above factors, and the new acquisition of an organ bath, the sheep PCLS model was abandoned in favour of a bronchial ring approach. This has the advantage of simpler, more reliable sample preparation and measurement and is the subject of the next chapter.

Chapter 5 Bronchial ring model

5.1 Introduction

5.1.1 Background

The previous chapter outlines an attempt to develop a precision-cut lung slice model of the asthmatic airway in order to investigate our hypothesis that pro-resolving mediators have a role in the refractory period *via* an effect on direct or mast cell-mediated bronchoconstriction. The problems with this approach, and the need for an alternative, have been outlined. In this chapter a new approach using the bronchial ring technique will be discussed.

An overview of the bronchial ring technique is given in Chapter 1; the potential advantages of this method over PCLS include much simpler sample preparation, simpler quantification of bronchoconstriction, and importantly the possibility of using human lung without the requirement for an intact lobe. Small quantities of human lung obtained from the thoracic surgeons at Glenfield Hospital are available, with appropriate Research Ethics Committee approval and patient consent to use for research purposes. However, increasing use of lung-conserving surgical techniques means that supply is intermittent and the pieces of tissue obtained are small, often containing only very small, distal airways. The small number of human bronchial rings obtained therefore needs to be supplemented with an animal model, and the need to use a species readily available from an abattoir thus remains.

5.1.2 Species

Initial development work for the bronchial ring model continued to use sheep lung. However, early experiments with directly-acting bronchoconstrictors prompted a

change. Although a human-like dose-response was seen to carbachol, as with the PCLS model, the response to histamine was complex. Sheep bronchial rings did not contract in response to histamine challenge, and in fact rings partially-contracted with carbachol would relax to histamine (data not shown), an effect presumably due to the particular balance of bronchoconstricting H₁ receptors and bronchodilating H₂ receptors in the sheep airway (Ahmed et al., 1983). Many of the published clinical studies of the refractory period have involved bronchial challenges acting via mechanisms involving histamine, with evidence of refractoriness to acetylcholine and its analogues being weak. Our plan is to investigate the impact of pro-resolving mediators on a mast cell-mediated mechanism at the mast cell and airway smooth muscle level, so it is important that our animal model has a human-like response to histamine.

As we are limited to animals available at an abattoir, once sheep are ruled out the choice of species is essentially between pig and cow, neither of which have been extensively used for *in vitro* airway models. Both bovine and porcine airway smooth muscle contract in response to histamine (Chand, Eyre, & DeRoth, 1979; Chand & DeRoth, 1978); pig was chosen for our study as there is a good local supply available.

5.1.3 Mast cell-mediated bronchoconstriction

Degranulation of human mast cells can be stimulated using a commercially available antibody against the mast cell FcεR₁ receptor, as described in Methods. Although the pig lung is known to contain mast cells (Chen et al., 2000) antibodies against the human FcεR₁ receptor were not effective in the pig (data not shown). As a pig-specific antibody is not available to induce mast cell degranulation, in the absence of sensitised tissue an alternative method must be used. Mast cells are known to degranulate in response to

osmotic stimuli and high concentrations of mannitol (0.1 – 1 M) have been shown to cause degranulation *in vitro* (Eggleston, Kagey-Sobotka, & Lichtenstein, 1987; Peachell & Morcos, 1998). Mannitol-induced bronchial ring contraction would also simulate the stimulus used to induce bronchoconstriction in our clinical study (Chapter 6) potentially allowing correlation between any mediators detected in post-challenge sputum supernatant and their effect on mannitol induced contraction – do they cause refractoriness to mannitol *in vitro*? Previous clinical studies of the effect of antihistamine on mannitol inhalation have strongly suggested that mannitol induces bronchoconstriction via mast cell degranulation (Brannan et al., 2001; J.D. Brannan, Gulliksson, Anderson, Chew, & Kumlin, 2003; Currie et al., 2003).

5.2 Methods summary

5.2.1 Materials and equipment

The organ bath used in these experiments was a proprietary design as shown in Chapter 2, Figure 2.4 and acquired from Astra-Zeneca. Krebs-Henseleit buffer was prepared in 1 l quantities. Mannitol solutions were prepared in Krebs-Henseleit buffer; montelukast and PGD₂ solutions were prepared in ethanol and cetirizine in phosphate buffered saline. Bronchial rings were incubated in Dulbecco's modified Eagle medium (DMEM) with added antimicrobial and antimycotic at 1% v/v.

5.2.2 Bronchial ring preparation

Pig lungs were obtained from male and female animals fresh from a local abattoir; bronchial rings with internal diameter 1.5 – 3 mm and length 2 – 3 mm were dissected

from a lung using scissors and forceps, and a typical ring is shown in Chapter 2, Figure 2.5. Following dissection rings were incubated at 37.5 °C in DMEM. Rings were used for experiments for up to 4 days following the day of preparation.

Small pieces of human lung were intermittently obtained from the thoracic surgeons following resection for lung cancer, and small airways dissected out in a similar manner to that described above. The rings obtained were typically ca. 1 mm internal diameter with no or very little cartilage; a strong contractile response to carbachol and anti-F_cεR₁ was used to confirm their status as bronchi. Once dissected, rings were washed and incubated in DMEM.

5.2.3 Bronchial ring contraction measurements

Contraction of the bronchial rings was measured using the organ bath described in Chapter 2 and illustrated in Figure 2.4. Up to 4 rings were assessed simultaneously. At the start of an experiment, a ring was suspended in each well in 5 – 10 ml of Krebs-Henseleit buffer, with 95% O₂/5% CO₂ gas bubbled into the buffer from a cylinder. 0.6 g of baseline tension was applied to each ring, and the rings allowed to equilibrate. Once a stable baseline was achieved, reagents were added to the wells as appropriate for each experiment.

For experiments in which rings were contracted using high concentration mannitol, each well of the organ bath was emptied and immediately re-filled with mannitol solution (± any required mediator) using a pipette.

5.3 Results

5.3.1 Porcine bronchial rings: direct stimulation of ASM

Figure 5.1 shows the basic experiment; a bronchial ring suspended in the organ bath is stimulated with a cumulative dose of histamine, the contraction being allowed to reach a plateau before addition of the next dose. This approach allows carbachol and histamine dose-response curves to be plotted; examples are shown in Figure 5.2 and Figure 5.3. As previously described for the PCLS approach, a 4-parameter logistic model can be used to obtain EC_{50} values if desired; the best fit curves for the data of Figure 5.2 and Figure 5.3 are illustrated in Figure 5.4, and the EC_{50} values obtained are $(4.5 \pm 2.2) \times 10^{-7}$ and $(2.1 \pm 0.8) \times 10^{-5}$ for carbachol and histamine respectively. As stated in Chapter 4, reported human carbachol EC_{50} values are of the order of $0.4 - 1.1 \times 10^{-6}$, so the pig value is comparable to this, and somewhat smaller than our sheep value of 5.2×10^{-6} . The human histamine EC_{50} has been reported as 6.3×10^{-7} (Finney et al., 1985) so the pig airway appears to be somewhat less sensitive to histamine. The calculated standard error for the pig carbachol EC_{50} is somewhat larger than the corresponding value obtained by PCLS for the sheep (49% versus 17% of the mean value, $n = 12$ and $n = 11$ respectively). However, it is not clear whether this is due to the difference in technique or whether it reflects differences in airway to airway variability between species.

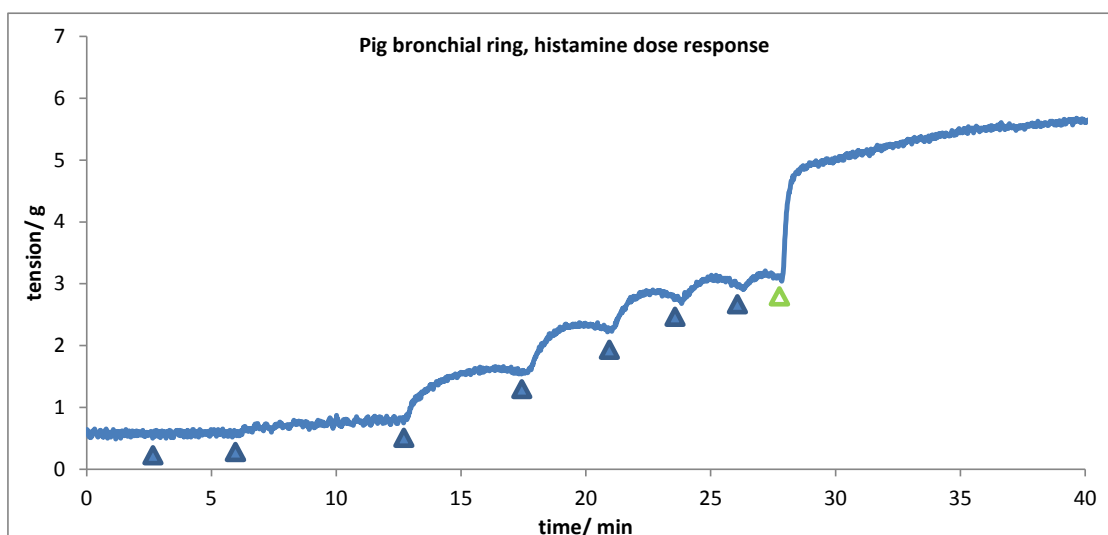


Figure 5.1. Contractile response of a single porcine bronchial ring to tripling doses of histamine. Each filled triangle represents the addition of a histamine dose; the open triangle indicates the addition of 3×10^{-4} M carbachol, shown to cause maximal contraction.

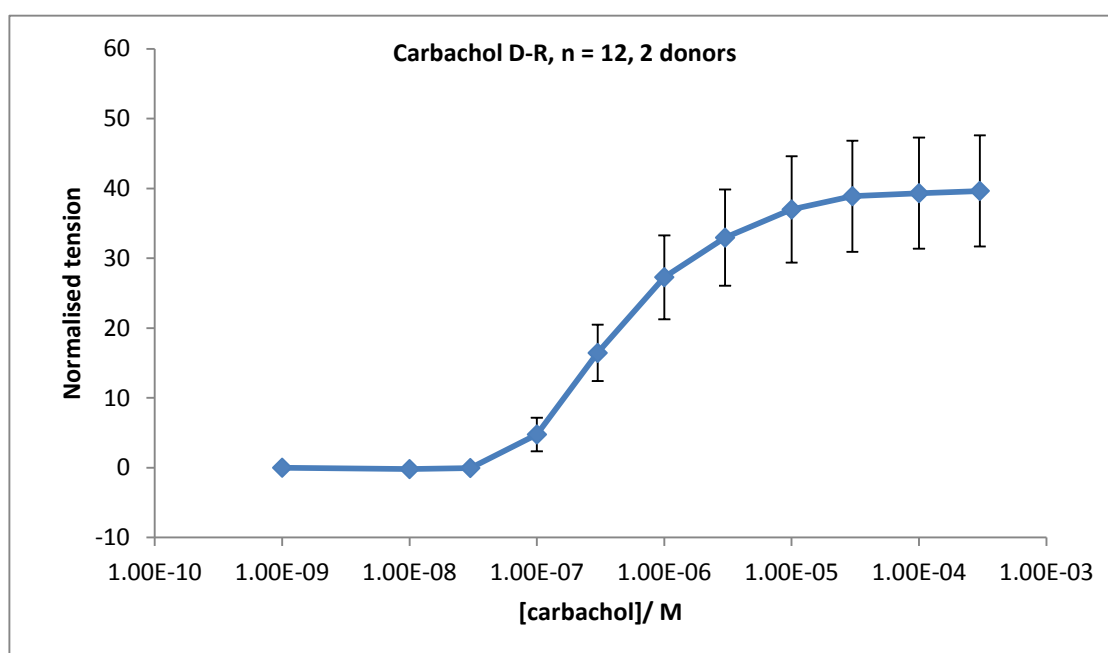


Figure 5.2. Mean carbachol dose response curve for 12 porcine bronchial rings obtained from 2 donor animals. Error bars indicate standard error.

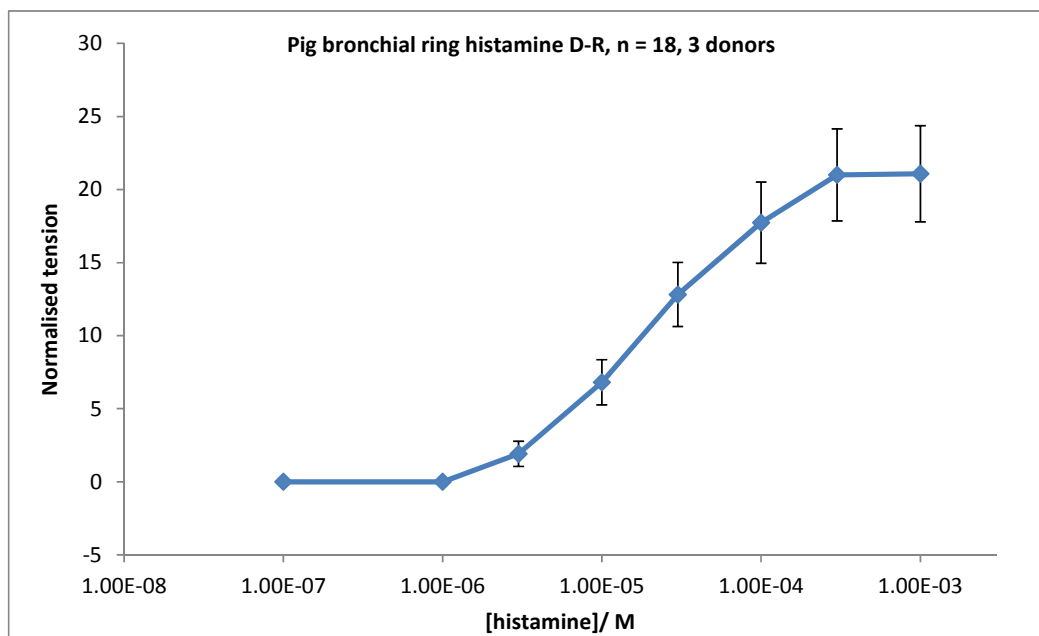


Figure 5.3. Mean histamine dose response curve for 18 porcine bronchial rings obtained from 3 donor animals. Error bars indicate standard error.

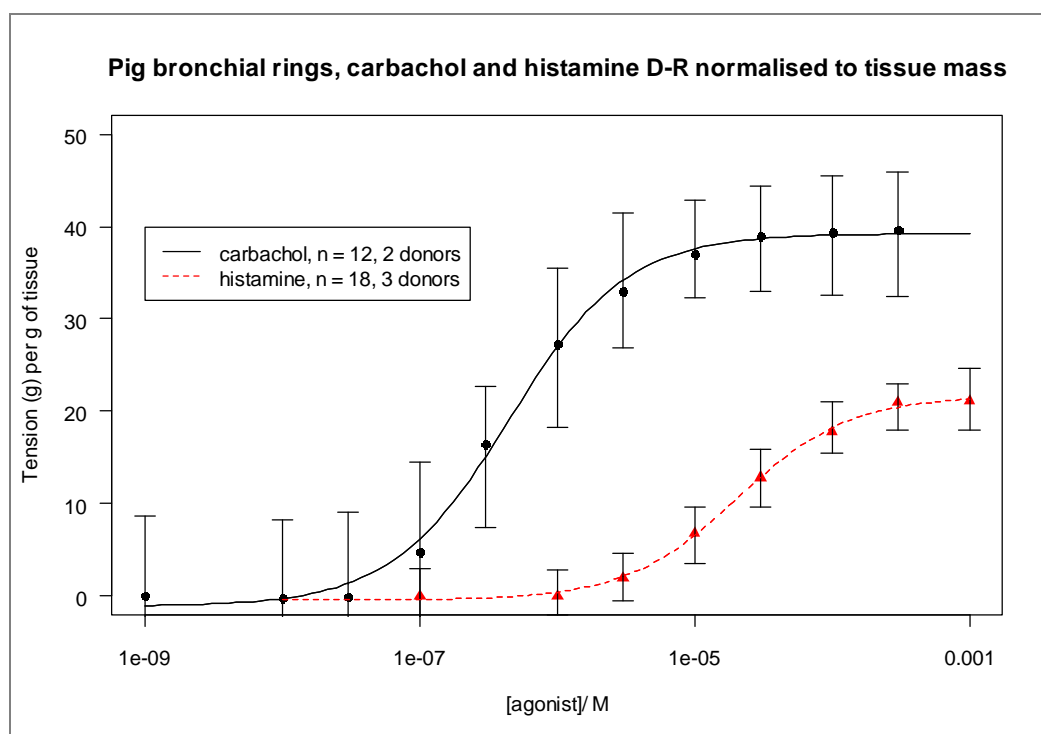


Figure 5.4. Carbachol and histamine dose response curves for porcine bronchial rings fitted using a 4-parameter logistic model. Error bars indicate the standard error in the best fit values.

To investigate the effect of pro-resolving mediators on histamine-induced contraction rings were used in sets of four, with two being incubated for 20 minutes with mediator prior to addition of histamine and two being incubated with 0.1% ethanol as a control. Contraction was normalised to the mass of the bronchial ring. The ideal normalisation method would be to express contraction as a percentage of the maximal contraction to carbachol, but the extremely long relaxation time of porcine rings following carbachol-induced contraction made determining a maximal contraction and then washing out the carbachol prior to mediator incubation impractical.

Selection of resolving mediator concentration was based on levels which had been shown to be biologically active in the literature: LXA₄ inhibits interleukin-8 release from human peripheral blood mast cells over the concentration range 0.1 – 100 nM (C. Bonnans et al., 2002); a 15 minute incubation with MaR₁ increases human macrophage phagocytosis over a similar concentration range (Charles N Serhan et al., 2009). A 15 minute incubation with 10 nM RvD₁ decreases actin polymerisation in human neutrophils (Krishnamoorthy et al., 2010).

Figure 5.5 shows the histamine dose response curve following a 20 minute incubation with 10 nM LXA₄ or 0.1% ethanol control. The LXA₄ clearly has no effect on histamine-stimulated smooth muscle contraction at this concentration and incubation time. Figure 5.6 and Figure 5.7 show similar experiments with 10 nM RvD₁ and MaR₁ respectively.

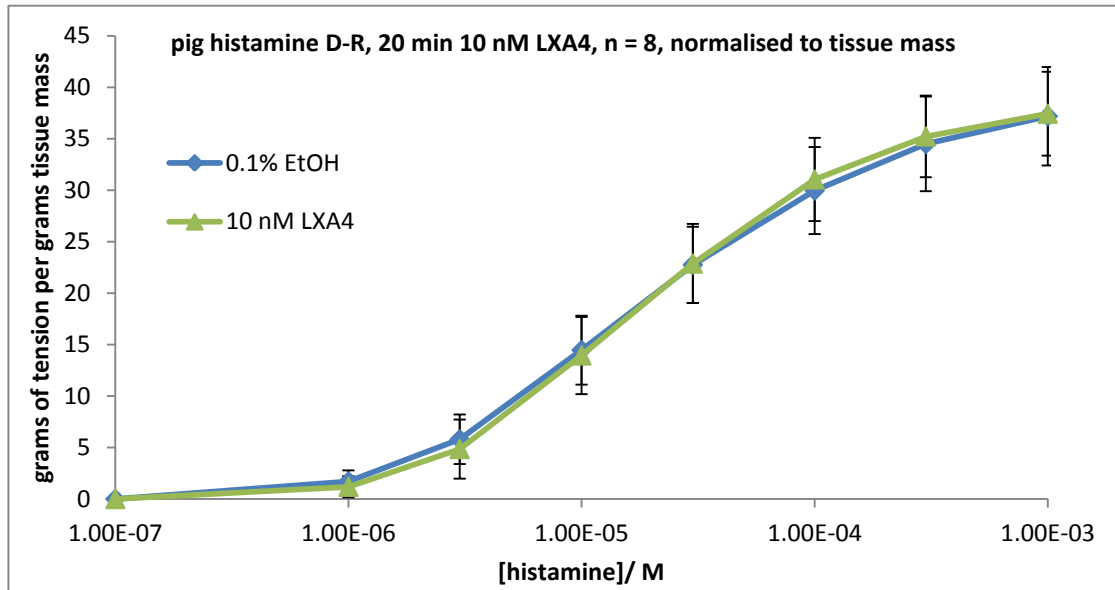


Figure 5.5. Histamine dose-response curve in porcine bronchial rings, following 20 minute incubation with 10 nM LXA₄ or 0.1% ethanol. Contraction is normalised to ring tissue mass. Error bars indicate standard error.

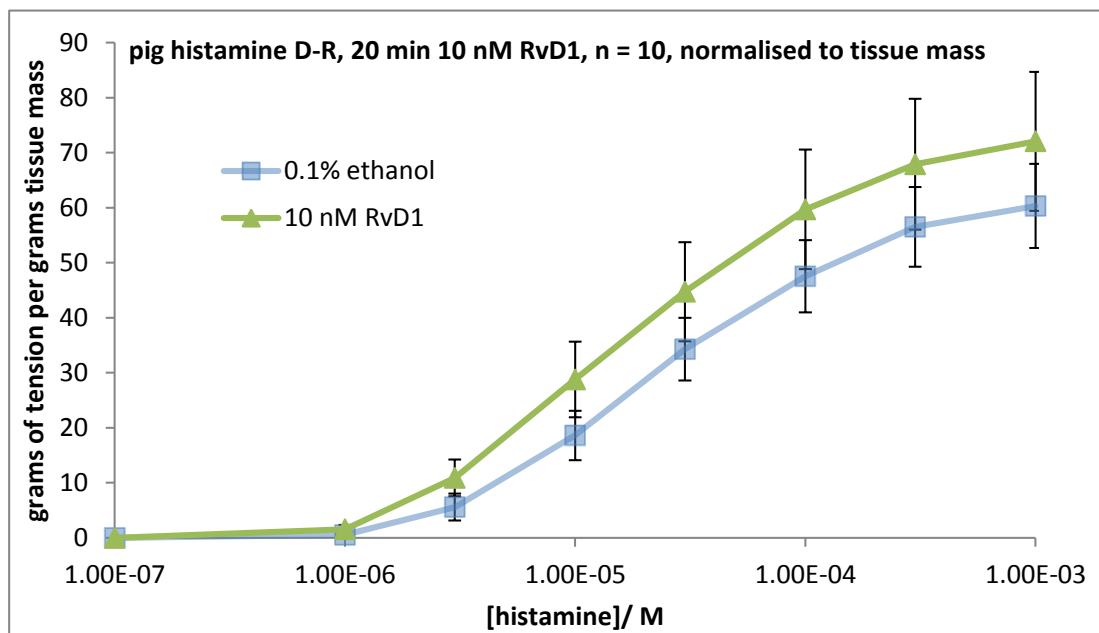


Figure 5.6. Histamine dose-response curve in porcine bronchial rings, following 20 minute incubation with 10 nM RvD₁ or 0.1% ethanol. Contraction is normalised to ring tissue mass. Error bars indicate standard error.

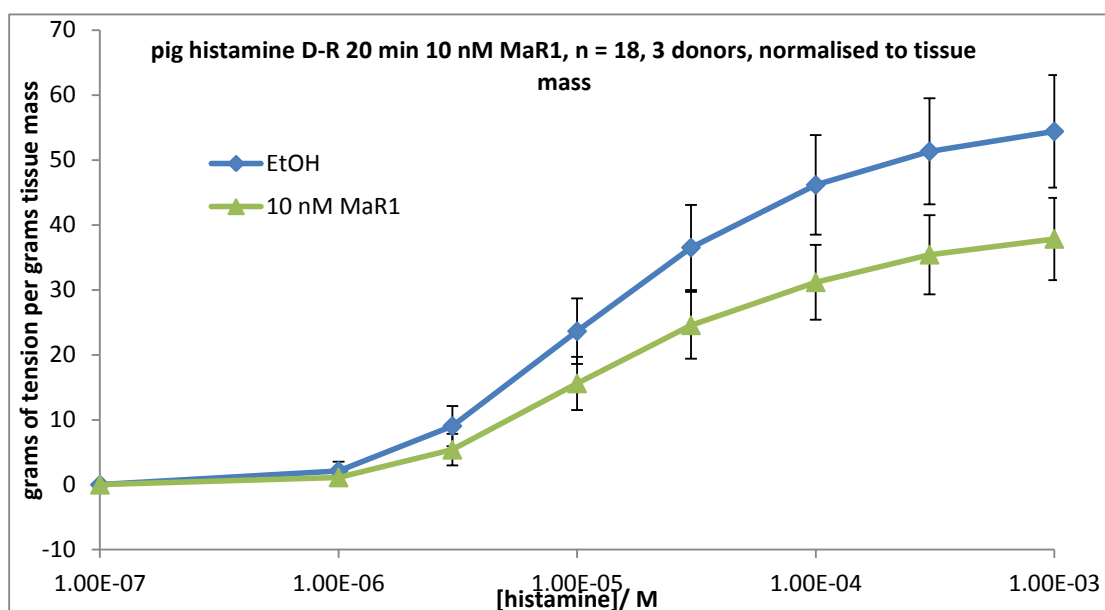


Figure 5.7. Histamine dose-response curve in porcine bronchial rings, following 20 minute incubation with 10 nM MaR₁ or 0.1% ethanol. Contraction is normalised to ring tissue mass. Error bars indicate standard error.

RvD₁ gives a similar negative result to LXA₄; MaR₁ however, appears to significantly reduce the maximal contraction of the rings over 3 donors. The majority of the effect is contributed by one donor, but the other two do show a smaller effect in the same direction. It is difficult to test the statistical significance of a difference between two non-linear curves. However, transforming the data using a $(\log_{10}x)^2$ function produces approximately linear dose-response curves, allowing linear regression analysis; the difference in the intercepts of the two transformed curves, corresponding to a reduction in maximum contraction of 30% with 10 nM MaR₁, is statistically significant with $p = 0.01$.

In view of the small effect seen with 10 nM MaR₁, the experiment was repeated with 1 nM and 100 nM concentrations of the mediator and the results are shown in Figure 5.8 and Figure 5.9 respectively. No effect of MaR₁ is seen.

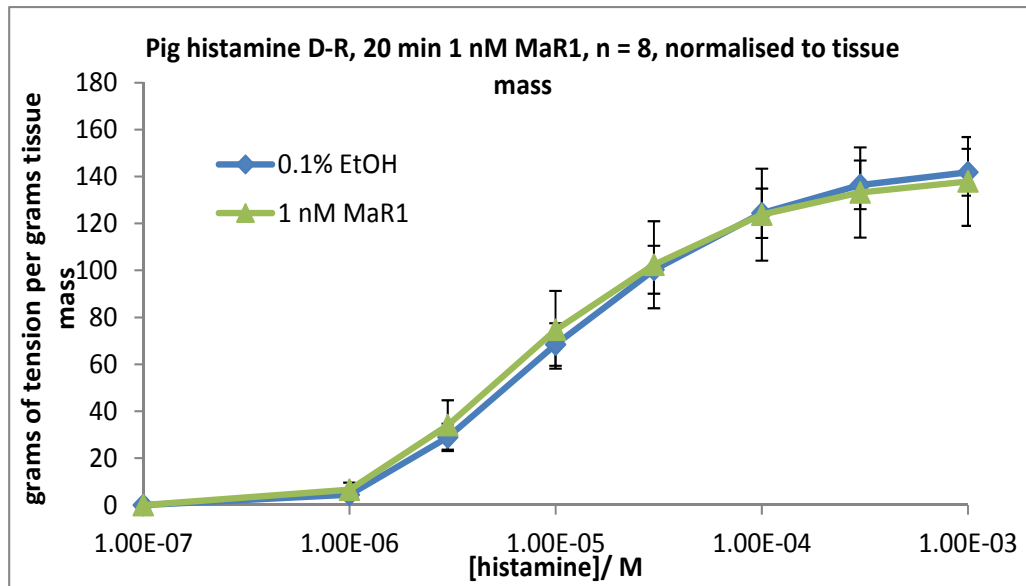


Figure 5.8. Histamine dose-response curve in porcine bronchial rings, following 20 minute incubation with 1 nM MaR₁ or 0.1% ethanol. Contraction is normalised to ring tissue mass. Error bars indicate standard error.

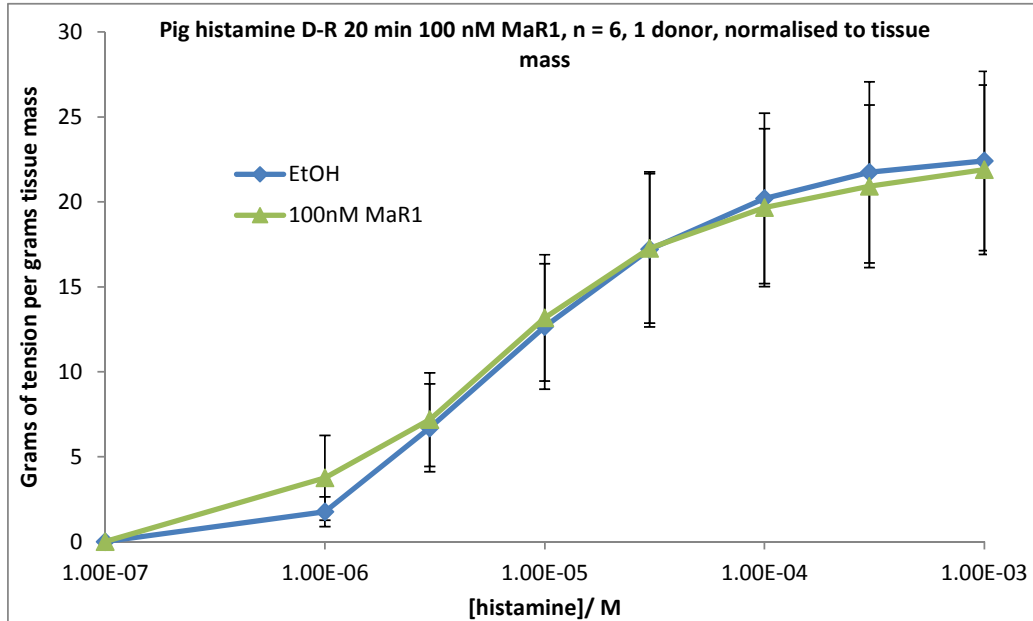


Figure 5.9. Histamine dose-response curve in porcine bronchial rings, following 20 minute incubation with 100 nM MaR₁ or 0.1% ethanol. Contraction is normalised to ring tissue mass. Error bars indicate standard error.

In summary then, incubation of bronchial rings with LXA₄ or RvD₁ has no effect on their response to histamine. Incubation with 10nM MaR₁ appears to produce a small attenuation in the histamine response, but this is not repeatable with either 1 nM or 100 nM MaR₁. It is therefore unlikely that this observation represents a true biological effect.

5.3.2 Development of indirect stimulation model in pig

As discussed in 5.1.3, the lack of antibodies to pig mast cell receptors led us to propose the use of mannitol as an osmotic stimulant to mast cell degranulation and consequent bronchoconstriction. Figure 5.10 shows the effect of high concentration mannitol on a set of porcine bronchial rings; after a delay of approximately 2 minutes all four rings slowly contract, in a manner similar to the α F ϵ R₁ induced contraction of human bronchial rings. A dose-response curve for mannitol is shown in Figure 5.11; an effect is seen for mannitol concentrations ≥ 250 mM, and is essentially maximal at 500 mM. For a given ring, the effect is repeatable without diminution – in 6 pig bronchial rings contracted with mannitol, washed and challenged with mannitol again the magnitude of the second contraction was (100 ± 1.6) % of the initial contraction (mean \pm standard error).

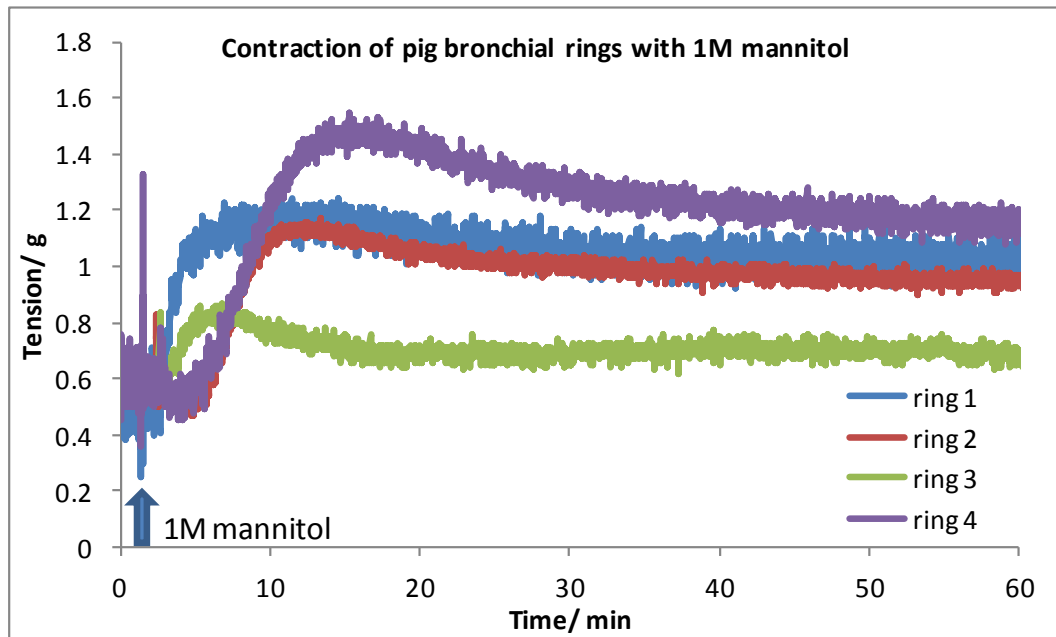


Figure 5.10. Contraction of four porcine bronchial rings induced by 1M mannitol.

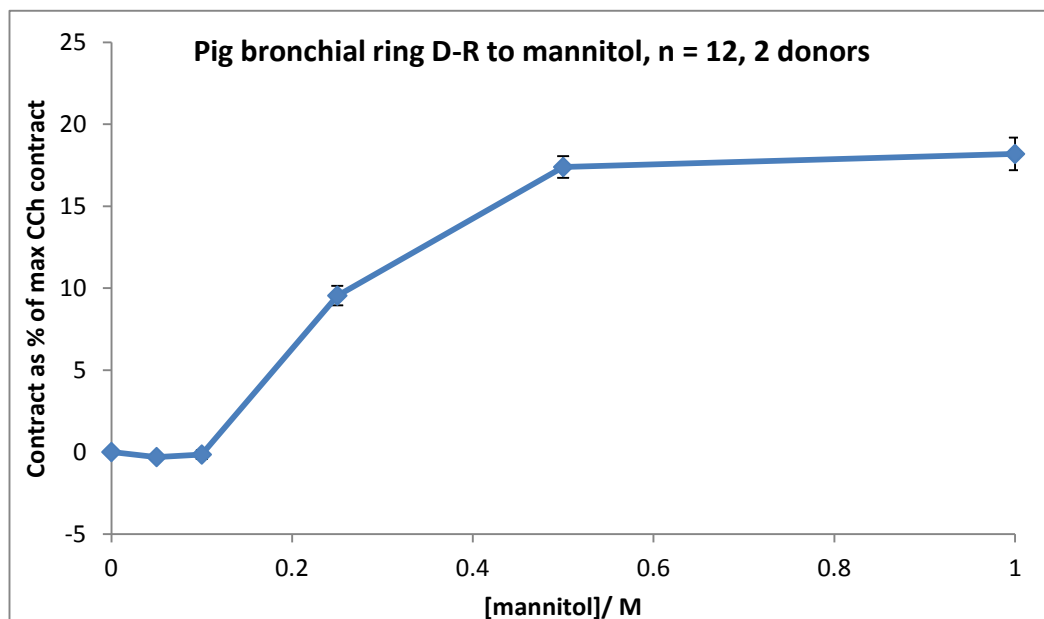


Figure 5.11. Mannitol dose-response curve in porcine bronchial rings. Contraction is normalised to the maximal carbachol response for each ring. Error bars indicate standard error.

In order to have confidence in our model it is necessary to show that the bronchoconstriction induced by mannitol is indeed mast cell-mediated, and not a direct

effect of increased osmolarity on ASM. In order to do this we attempted to investigate the effect of antagonists of the mast cell mediators histamine, LTC₄ and PGD₂ on mannitol-induced bronchoconstriction. Interestingly, concentrations of up to 30 nM LTC₄ and 10 μM PGD₂ induced no contraction in porcine bronchial rings which had shown a good response to carbachol (n = 6 and n = 4 respectively). No literature data for the pig was available, so concentration ranges were based on those effective in the guinea-pig (Dahlén et al., 1988; McKenniff, Norman, Cuthbert, & Gardiner, 1991). Our result suggests that LTC₄ and PGD₂ do not play a significant role in mast cell-mediated bronchoconstriction in the pig airway. The effect of the H₁-antagonist cetirizine on mannitol-induced bronchoconstriction is shown in Figure 5.12; there is no evidence that antagonism of histamine H₁ receptors attenuates bronchoconstriction. As illustrated in Figure 5.13, cetirizine does almost completely ablate the direct bronchoconstriction response to histamine in pig bronchial rings; we therefore conclude that mannitol does not cause bronchoconstriction *via* a PGD₂, LTC₄, or histamine-mediated mechanism.

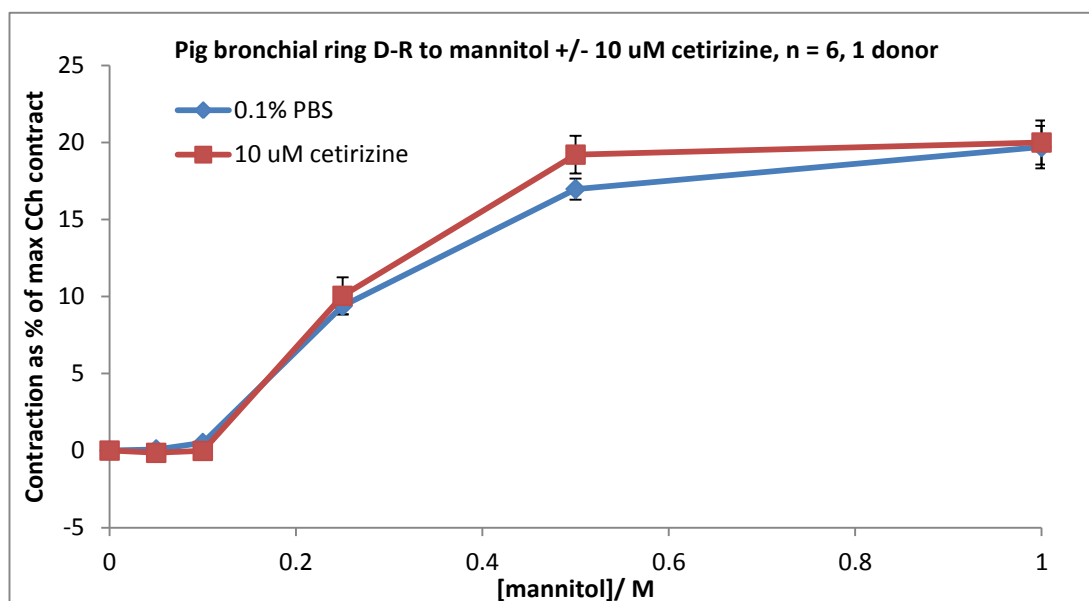


Figure 5.12. Mannitol dose-response curve in porcine bronchial rings in the presence and absence of 10 μ M cetirizine. Contraction is normalised to the maximal carbachol response for each ring. Error bars indicate standard error.

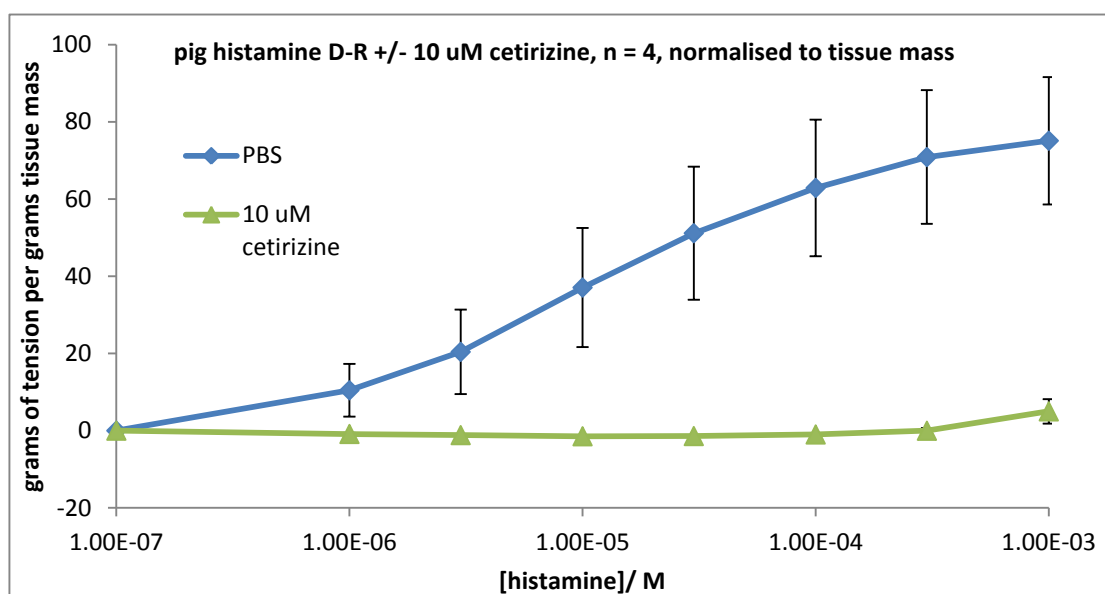


Figure 5.13. Histamine dose-response curve in porcine bronchial rings in the presence and absence of 10 μ M cetirizine. Contraction is normalised to ring tissue mass. Error bars indicate standard error.

Although we were unable to confirm that mannitol-induced bronchoconstriction is mast cell-mediated *in vitro* (discussed in 5.4.2 below) an interesting property of our model was noted, which may have relevance to the refractory period - once a ring has been partially contracted using mannitol its response to further contraction with carbachol is inhibited. In Figure 5.14 the top trace shows a bronchial ring which has been maximally contracted with carbachol (300 μ M). The carbachol is then washed out over a period of hours by repeated changes of buffer, until the ring is back to its baseline tension. In the bottom trace, 0.5 M mannitol is now added to cause contraction (first triangle); adding carbachol following this (second triangle) induces a maximal contraction with less than a third of the magnitude of the original contraction. The effect is dose dependent, as shown in Figure 5.15.

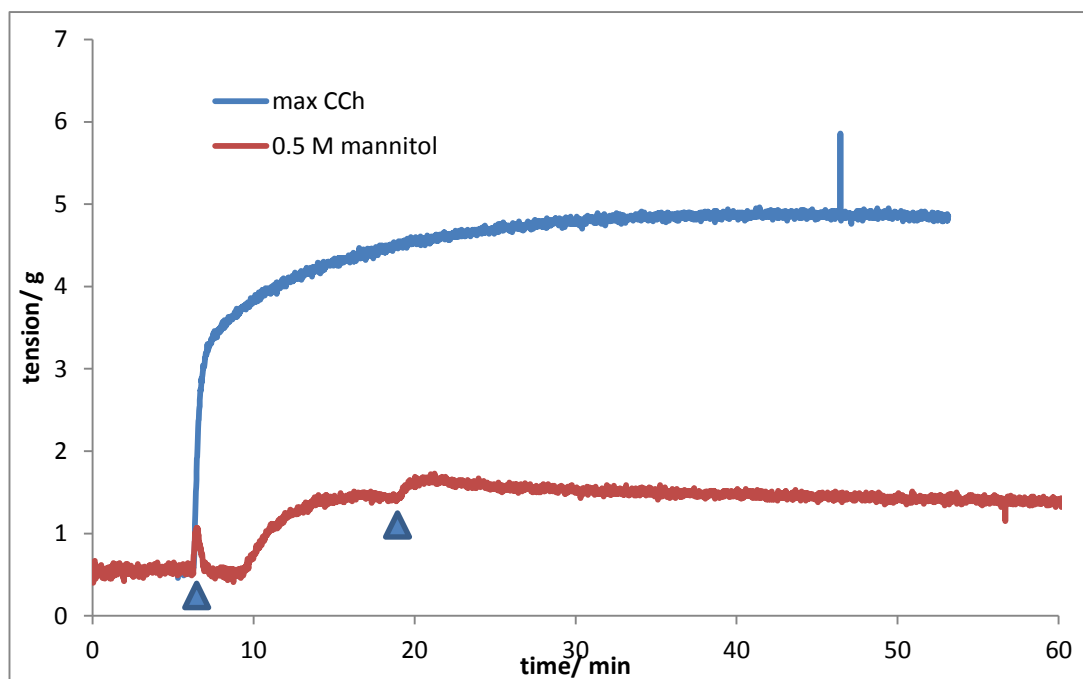


Figure 5.14. Pig bronchial ring maximal response to carbachol (upper trace) followed by wash out and challenge with 0.5 M mannitol (first triangle) and then re-challenge with carbachol (second triangle).

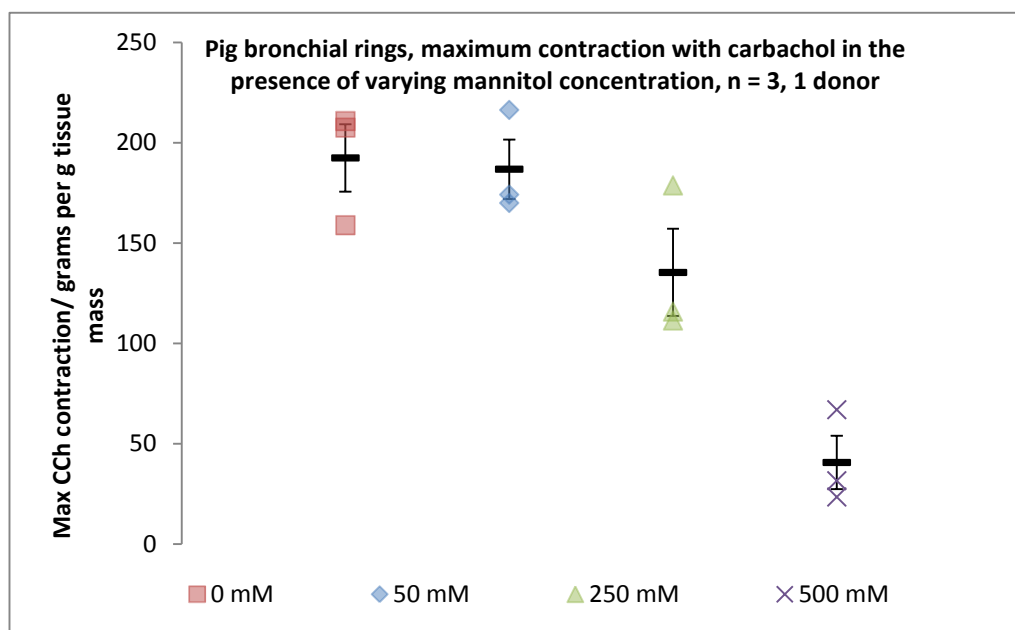


Figure 5.15. Dose-dependent inhibition of maximal carbachol bronchoconstriction by mannitol in porcine bronchial rings. Contraction is normalised to ring tissue mass. Error bars indicate standard error.

The inhibitory effect of mannitol on carbachol-induced contraction is easily reversible. Figure 5.16 shows 3 traces. The first shows a pig bronchial ring contracted with 1 M mannitol (first triangle) followed by the addition of carbachol (second triangle) which causes no further contraction. The mannitol is then washed out; trace 2 shows the immediate effect of replacing the mannitol solution with fresh buffer; the ring immediately contracts strongly, presumably as there is still carbachol bound to smooth muscle muscarinic receptors but no longer any mannitol present. Continued washing over a period of hours will return the ring to a baseline level of contraction (trace 3). Addition of carbachol now induces a strong contraction, as expected. As illustrated in Figure 5.17, the maximal carbachol response following mannitol challenge and wash out is $(99 \pm 2.5)\%$ of the baseline carbachol response, not significantly different to controls in which no mannitol is added. This suggests that the suppressive effect of

mannitol on carbachol-induced bronchoconstriction is not due to a permanent deleterious effect of high concentration mannitol on the bronchial rings.

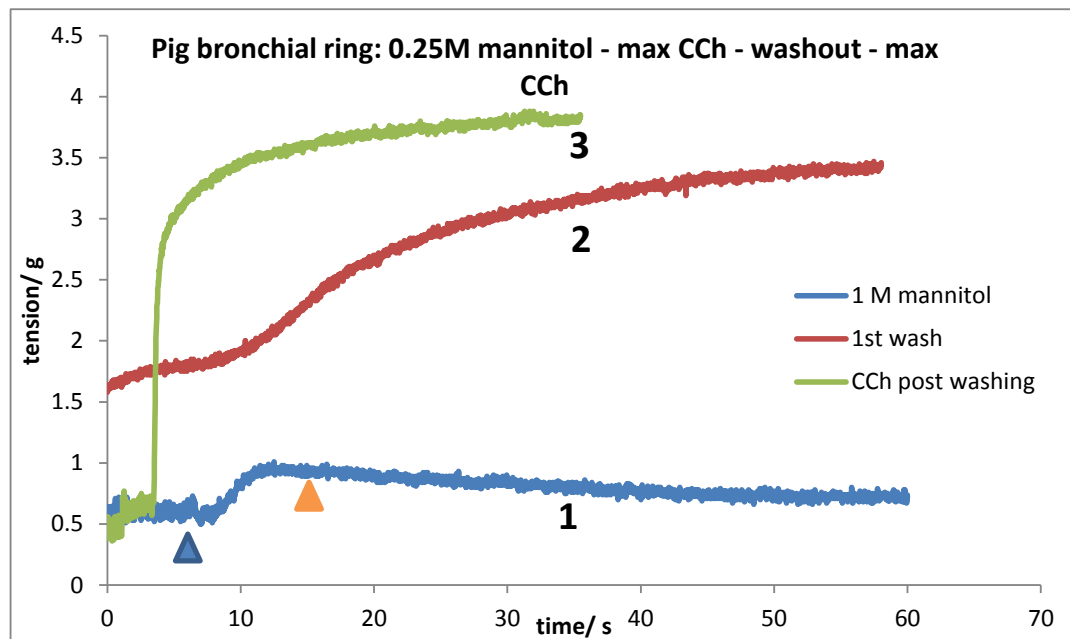


Figure 5.16. Pig bronchial ring. Trace 1: contraction with 1 M mannitol (first triangle) followed by addition of carbachol (second triangle), washing with fresh buffer (trace 2 - post first wash), return to baseline and re-challenge with carbachol (trace 3).

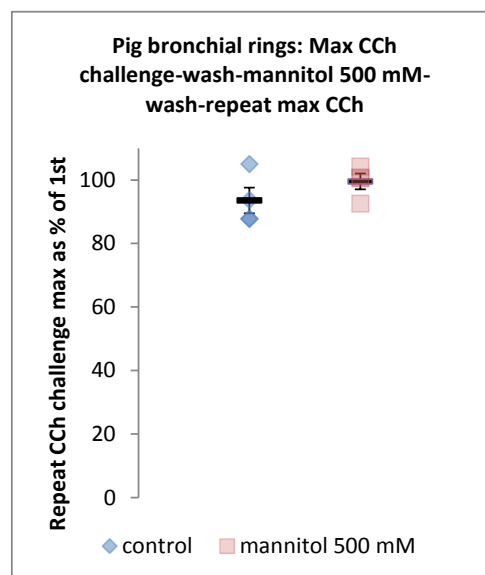


Figure 5.17. In this experiment 12 porcine bronchial rings were challenged with 300 μ M carbachol to establish their maximum degree of contraction. Following a prolonged period of washing the rings returned to their baseline level of contraction; 8 were then challenged with 500 mM mannitol and 4 with Krebs-Hensleit buffer as controls. Following wash out of the mannitol the rings were re-challenged with carbachol, and the magnitude of the resulting bronchoconstriction expressed as a percentage of the baseline carbachol challenge. Error bars indicate standard error.

A suppressive effect of high concentration mannitol on histamine-induced bronchoconstriction is also seen, although the dose-response relationship is less clear (Figure 5.18). Thus, although we have not been able to demonstrate that mannitol operates via a mast-cell mediated mechanism, it does induce a state of refractoriness bearing some similarity to the clinical phenomenon of the refractory period, albeit operating on directly-acting bronchoconstrictors. This is discussed more fully in 5.4.2 below; in Figure 5.19 and Figure 5.20 we demonstrate two experimental attempts to shed light on the mechanism of this mannitol-induced refractoriness. Figure 5.19 shows the effect of a 10 μ M concentration of the PGE₂ EP₂-receptor blocker AH6809 on mannitol-induced refractoriness; this concentration of AH6809 has, for example, been shown to inhibit the action of PGE₂ on human lung mast cells (Duffy, Cruse, Cockerill, Brightling, & Bradding, 2008). Figure 5.20 shows the effect of disrupting the bronchial ring epithelium with a cotton bud (an element of caution is needed here in that the epithelium is not visualised with the bronchial ring technique, unlike the PCLS method, and the presence or absence of epithelium was not directly seen; however, the cotton bud approach has been used successfully by others such as Flavahan et al.(1985)).

It can be seen that the EP₂-receptor appears to have no role in the mechanism of mannitol-induced refractoriness to carbachol, and similarly disruption of bronchial epithelium does not increase the carbachol response in the presence of mannitol.

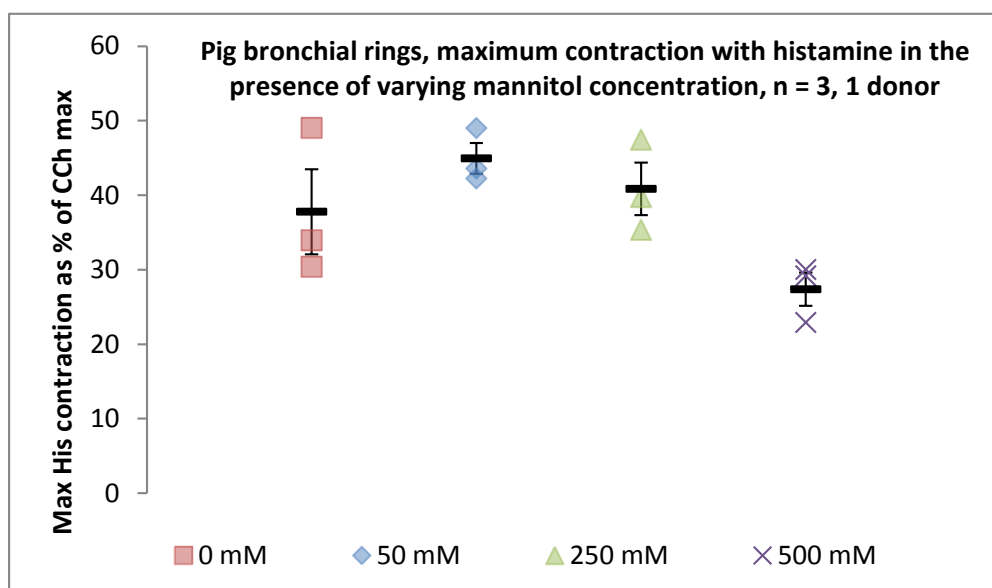


Figure 5.18. Dose-dependent inhibition of maximal histamine bronchoconstriction by mannitol in porcine bronchial rings. Contraction is normalised to the maximal carbachol response for each ring. Error bars indicate standard error.

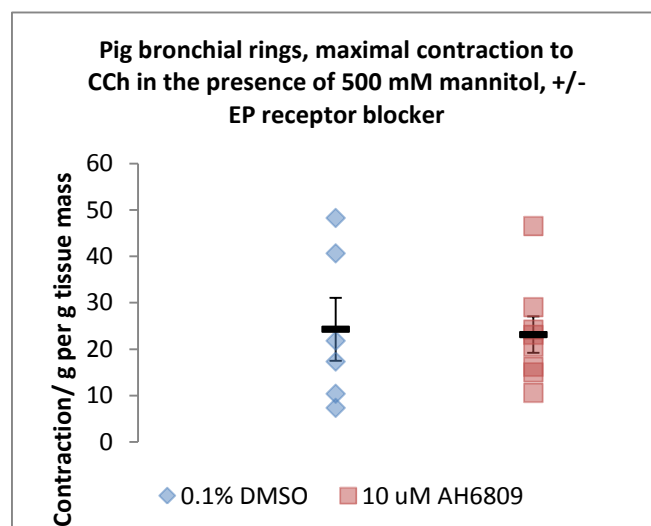


Figure 5.19. Effect of the EP₂-receptor blocker AH6809 on the magnitude of bronchoconstriction to carbachol in the presence of 500 mM mannitol. Contraction is normalised to ring tissue mass. Error bars indicate standard error.

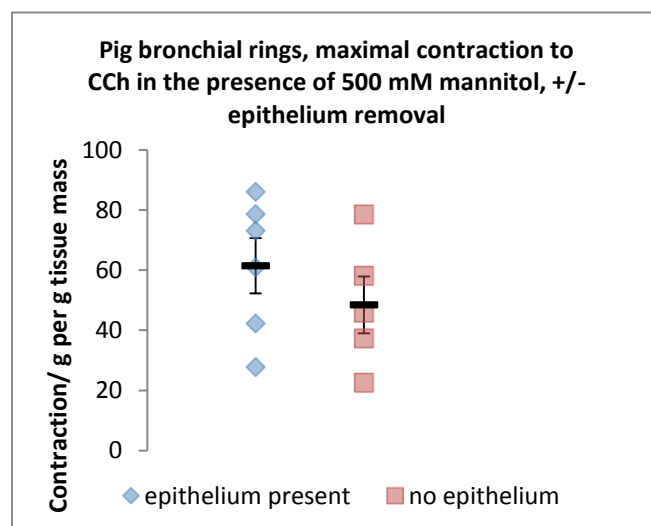


Figure 5.20. Effect of epithelium removal on the magnitude of bronchoconstriction to carbachol in the presence of 500 mM mannitol. Epithelium was disrupted by inserting the tip of a cotton-bud and rotating. Contraction is normalised to ring tissue mass. Error bars indicate standard error.

As it was not possible to establish the mechanism by which mannitol exerts its bronchoconstrictor or bronchoconstriction-suppressing effects, an extensive investigation of the effects of pro-resolving mediators in this model seemed unlikely to bear fruit. The effect of 10 nM MaR₁, which appears to have a small effect on histamine-mediated bronchoconstriction (Figure 5.7) was tested, as shown in Figure 5.21; no effect on mannitol-induced bronchoconstriction was seen.

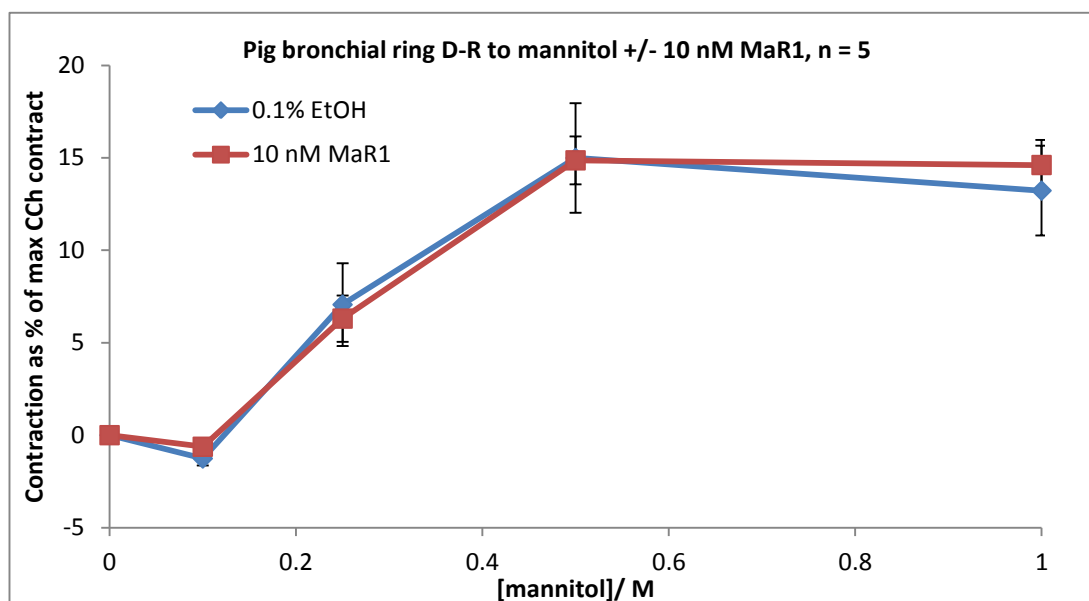


Figure 5.21. Mannitol dose-response curve in porcine bronchial rings, following 20 minute incubation with 10 nM MaR₁ or 0.1% ethanol. Contraction is normalised to the maximal carbachol response for each ring. Error bars indicate standard error.

In summary then, we have demonstrated that high concentration mannitol induces bronchoconstriction in pig bronchial rings *in vitro*. The mechanism is unclear, but does not operate *via* the mast cell mediators histamine, LTC₄ or PGD₂. Bronchoconstriction with mannitol renders an airway refractory to further contraction with the direct ASM agonists carbachol or histamine; again, the mechanism of this has not been elucidated, and this is discussed in 5.4.2 below. The next section of this chapter describes the results of experiments investigating the effect of pro-resolving mediators on human bronchial rings.

5.3.3 Human bronchial rings

Using human tissue for *in vitro* work is clearly the most desirable approach, and the attempts at developing animal models described above resulted from the difficulty in

obtaining human lung. However, small quantities of human lung were obtained as described in Section 5.2, and carbachol and histamine dose-response curves can be constructed using the same approach as for pig lung, as illustrated in Figure 5.22 and Figure 5.23. The carbachol EC₅₀ value determined using the 4-parameter logistical model described above was $(1.1 \pm 0.8) \times 10^{-6}$, consistent with the literature values of $0.4 - 1.1 \times 10^{-6}$ discussed above. The histamine EC₅₀ was $(7.5 \pm 6.1) \times 10^{-7}$, consistent with the value of 6.3×10^{-7} obtained by Finney et al. (1985). The standard errors are large as a result of the small number of airways. Due to the limited quantity of tissue it was decided that experiments should be concentrated on development of the mast cell-mediated model. Figure 5.24 shows a bronchial ring challenged with increasing doses of antibody against the mast cell receptor FcεR₁, known to induce mast cell degranulation (Moiseeva & Bradding, 2011). Once a threshold concentration of antibody is reached (1:10000), the airway contracts; no further contraction is seen on addition of further antibody. This approach was used to investigate the effect of the pro-resolving mediators LXA₄ and MaR₁ on mast cell-mediated bronchoconstriction; Figure 5.25, Figure 5.26 and Figure 5.27 show the results of experiments using 10 nM LXA₄, 10 nM MaR₁ and 100 nM MaR₁ respectively. Mediator concentrations are again based on literature data as discussed in 5.3.1. As relaxation following contraction with carbachol is much faster and more reliable with human bronchial rings than with porcine, rings were fully contracted with carbachol and then washed out until back to baseline before adding mediator and then antibody; results are expressed as a percentage of the maximum carbachol-induced contraction. Two experiments were conducted with RvD₁, but unfortunately insufficient tissue was obtained to acquire an adequate amount of data for analysis.

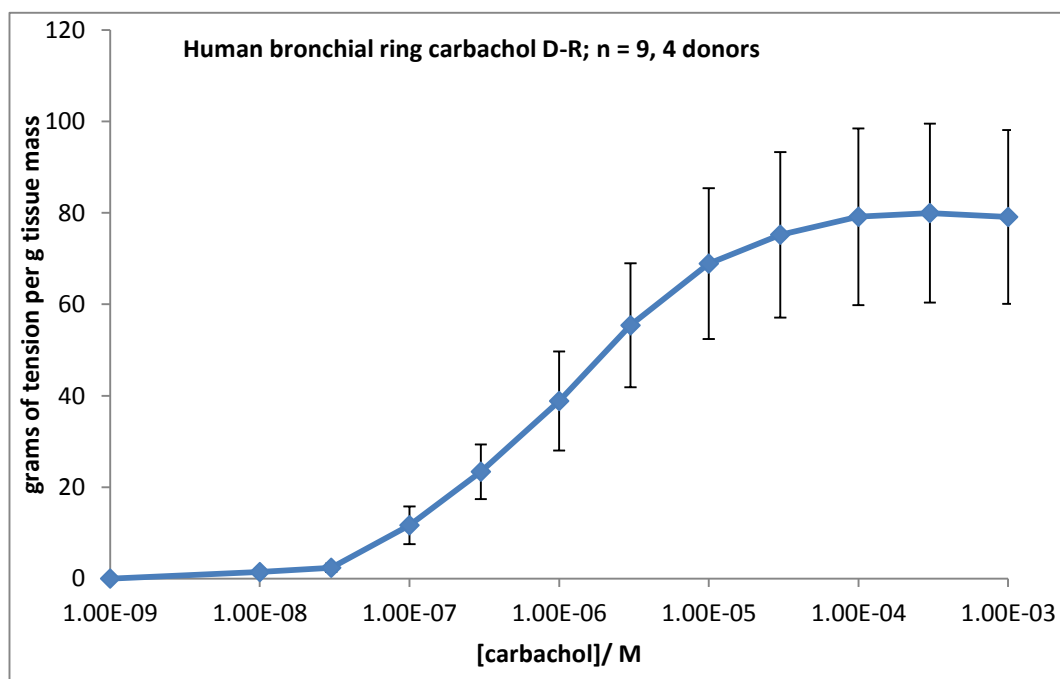


Figure 5.22. Mean carbachol dose response curve for 9 human bronchial rings obtained from 4 donors. Error bars indicate standard error. Contraction is normalised to ring tissue mass.

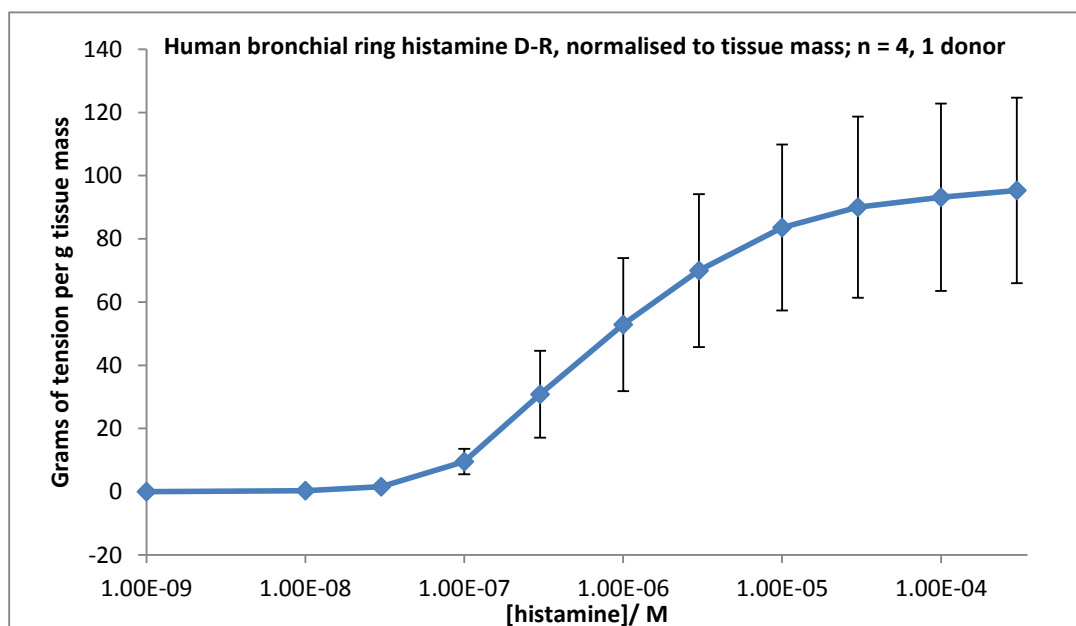


Figure 5.23. Mean histamine dose response curve for 4 human bronchial rings. Error bars indicate standard error. Contraction is normalised to ring tissue mass.

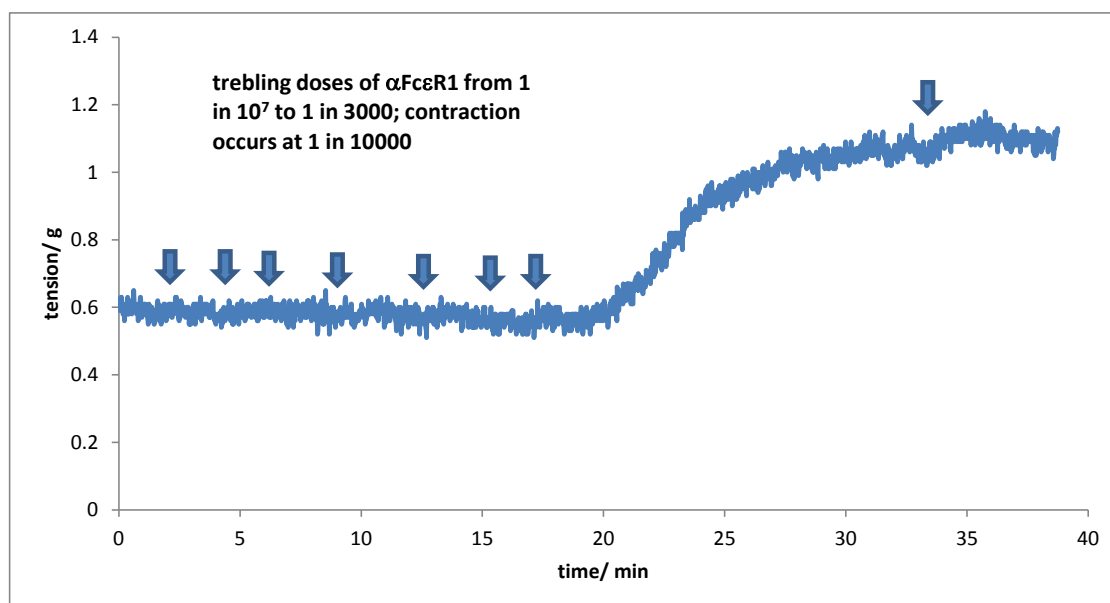


Figure 5.24. Contraction of a human bronchial ring with increasing concentrations of anti- $\text{Fc}\epsilon\text{R}_1$. Contraction occurs at a dilution of 1:10000.

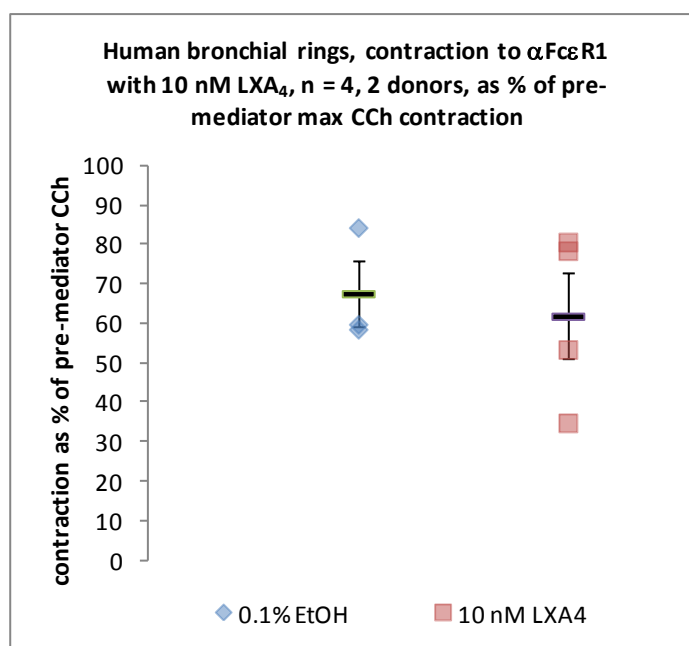


Figure 5.25. Contraction of human bronchial rings with $\alpha\text{Fc}\epsilon\text{R}_1$ following 20 minute incubation with 10 nM LXA_4 or 0.1% ethanol. Contraction is normalised to the maximal carbachol response for each ring. Error bars indicate standard error.

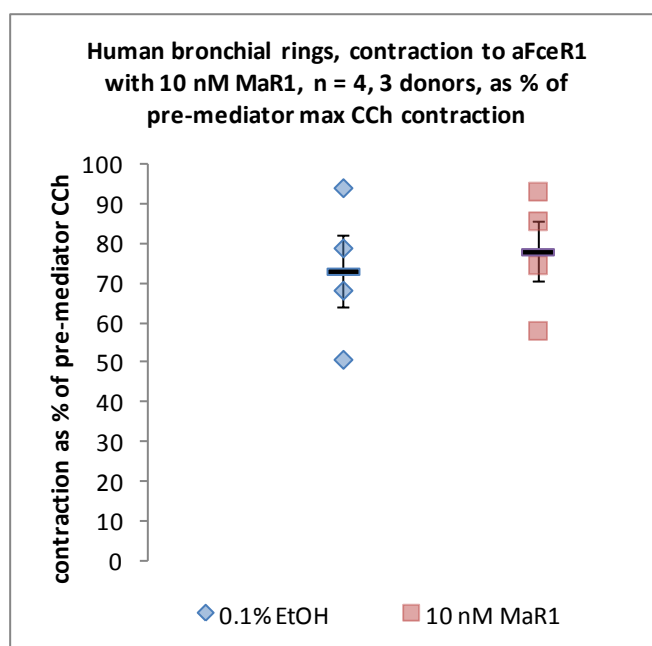


Figure 5.26. Contraction of human bronchial rings with $\alpha\text{Fc}\epsilon\text{R}_1$ following 20 minute incubation with 10 nM MaR₁ or 0.1% ethanol. Contraction is normalised to the maximal carbachol response for each ring. Error bars indicate standard error.

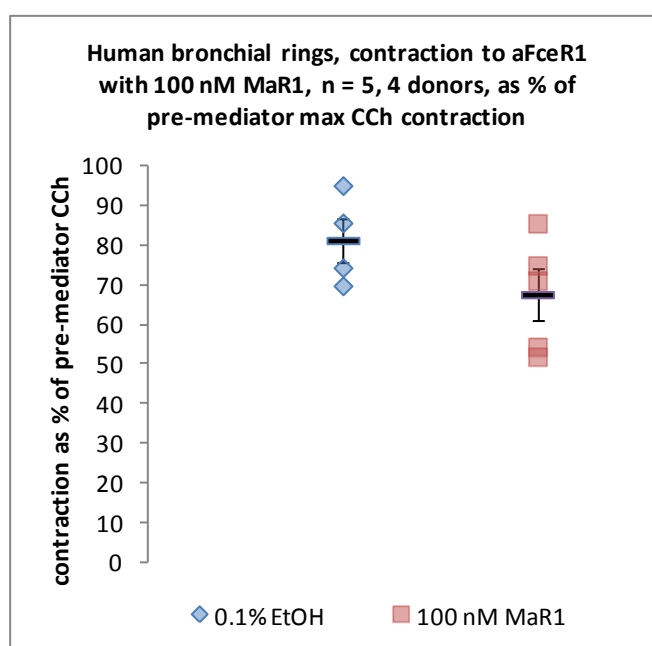


Figure 5.27. Contraction of human bronchial rings with $\alpha\text{Fc}\epsilon\text{R}_1$ following 20 minute incubation with 100 nM MaR₁ or 0.1% ethanol. Contraction is normalised to the maximal carbachol response for each ring. Error bars indicate standard error.

It is evident that neither 10 nM LXA₄ nor 10 nM MaR₁ have any effect on mast cell-mediated bronchoconstriction as induced by FcεR₁ activation. There appears to be a trend towards reduced bronchoconstriction in the presence of 100 nM MaR₁; however this is not statistically significant, with $p = 0.16$ for an unpaired t-test.

5.4 Discussion

5.4.1 Porcine bronchial rings: direct stimulation of ASM

The results of our investigation into the effect of pro-resolving mediators on histamine-induced bronchoconstriction have been largely negative. We have seen a small decrease in maximal histamine-induced contraction of porcine bronchial rings with MaR₁ at 10 nM but not at other concentrations, and no effect from other pro-resolving mediators. In some respects the lack of a substantial effect on direct smooth muscle stimulation is not surprising. To our knowledge there is to date no evidence of receptors for pro-resolving mediators such as the resolvin GPR32 or LXA₄ ALX receptor in airway smooth muscle, although the receptor for MaR₁ is not yet known (Parameswaran et al., 2007). In fact, published work on the effects of pro-resolving mediators has been focused on rodents and humans, at either the cell or whole body level, and there is no data on their effects in pigs, or the location and level of expression of their receptors. Furthermore, mediator concentration and incubation time for our experiments were chosen on the basis of published experiments on human cells; it may be that these conditions are not optimum for our porcine bronchial rings. However, in terms of our original hypothesis that pro-resolving mediators are a bronchoprotective factor responsible for the refractory period in asthma, a finding of little or no effect on direct smooth muscle challenge is not inconsistent. The existing literature on the refractory period does not

provide an entirely consistent picture, but overall the majority of studies favour refractoriness being an effect seen with indirect bronchial challenges such as mannitol, but not with direct stimulation of smooth muscle *via* histamine or muscarinic receptors (epithelium-dependent histamine tachyphylaxis of human airway *in vitro* is well documented however – Knight et al., 1992). It is noteworthy that in a clinical study inhaled LXA₄ did ameliorate the effect of LTC₄, a directly acting bronchoconstrictor (Christie et al., 1992); however it had no direct bronchodilator effect. Its action in this case is likely to be a result of competitive binding of LXA₄ at the CysLT₁ receptor (Gronert et al., 2001). Interestingly Dahlén et al. (1988) found that pre-incubation of guinea pig lung strips with LXA₄ induced desensitisation to LTC₄ (as did pre-incubation with LTC₄ itself) but not to histamine. Taking this evidence together it seems unlikely that LXA₄ acts as an EpDRF on ASM to cause refractoriness. There is no evidence for RvD₁ acting as a relaxing factor on ASM. MaR₁ remains a possibility, although the lack of effect seen at 1 nM and 100 nM concentrations does reduce confidence in the effect seen at 10 nM, despite its statistical significance. We have therefore not with any degree of certainty demonstrated a mechanism operating at ASM level which could induce refractoriness to mast cell-mediated bronchoconstriction. Any of the mediators studied could, of course, still act in the mast-cell mediated pathway upstream of the ASM, although this would not explain cross-refractoriness with neurally-mediated challenges. As a final note to this section, in all discussion of *in vitro* airway work it is important to bear in mind the great inter-species variation which exists in expression of mediators and receptors; it is not possible to make definitive statements about human airway behaviour based on animal models.

5.4.2 Porcine rings: mannitol-induced bronchoconstriction

Although there is good clinical data suggesting that mannitol causes bronchoconstriction via a mast cell mediated mechanism (J D Brannan et al., 2001; J.D. Brannan, Gulliksson, Anderson, Chew, & Kumlin, 2003) we have failed to demonstrate that this mechanism is responsible for contraction in our bronchial ring model: an H₁-blocker had no effect on the mannitol dose-response curve and LTC₄ and PGD₂ do not appear to be significant bronchoconstrictors in the pig. This is supported by a previous study: Setoguchi, Nishimura, Hirano, Takahashi, & Kanaide (2001) found that although LTC₄ increased sensitivity to other bronchoconstrictors, it did not itself produce a contraction in pig tracheal smooth muscle strips. It is not known what the major mast cell mediators are in pig – this varies from species to species, and with mast cell phenotype within species (Hsieh et al., 2005). It is therefore possible, though unlikely, that mannitol acts principally *via* another mast cell mediator which we have not investigated.

In view of the clinical data it is surprising that we have been unable to clearly demonstrate a mast cell-mediated mechanism for mannitol-induced contraction. However, some previous *in vitro* work with human lung found no effect of antihistamine on hyperosmolar contraction, although sodium chloride was used to induce contraction in this case (Jongejan, Jongste, Raatgeep, Bonta, & Kerrebijn, 1990). As discussed below, it may be that multiple mechanisms are at work, and that the mechanisms are different in human and pig. For example, osmotic stimulation of sensory nerves may also play a role in bronchoconstriction (S. D. Anderson & Holzer, 2000) as well as the direct cell-shrinking effect of a hyperosmolar environment, as discussed below.

The dose-dependent suppressive effect of mannitol on carbachol-induced bronchial ring contraction is an interesting finding; to some extent it mimics the effect of the refractory period seen clinically, although this is more clearly associated with indirectly-acting bronchoconstrictors. Another clear difference is that washout of mannitol immediately restores an airway's bronchoconstriction response. All the same, elucidating the mechanism of this phenomenon might offer important insights into the mechanism of airway hyper-responsiveness and the refractory period. A similar effect was noted previously in human tissue (Finney et al., 1987) using sodium chloride as a hyperosmolar agent, but in that study the mildly hyperosmolar conditions used induced relaxation of bronchial rings, not contraction. Jongejan et al, in contrast, found hyperosmolarity-induced contraction but *increased* sensitivity to methacholine using hyperosmolar sodium chloride (Jongejan et al., 1990). Our experiments have demonstrated that the mechanism of mannitol-induced refractoriness in the pig is unlikely to operate via PGE₂ or any mechanism involving the bronchial epithelium.

Overall, in our model mannitol appears to both induce bronchoconstriction and inhibit further contraction, at least in response to carbachol and histamine. Any mechanism underlying this behaviour is likely to be complex, and any discussion of it is speculative at present. Increasing extracellular osmolarity due to mannitol probably leads to shrinkage of epithelial and other cell types, triggering changes in a number of ion channel types in order to restore equilibrium - so-called regulatory volume increase or RVI (Hoffmann, Lambert, & Pedersen, 2009). RVI is complex and far from completely understood. Cell volume/osmosensors include integral membrane proteins such as integrins, ion channels such as TRPs and phospholipases, as well as cytoskeletal elements. Their signals are transduced by a variety of messengers including arachidonic acid metabolites, intracellular calcium, reactive oxygen species and proteins such as

MAPK; a range of ion channels such as Na^+/H^+ exchangers and $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ co-transporters effect the RVI response (Hoffmann et al., 2009).

A simple interpretation of the pattern of bronchoconstriction exhibited in Figure 5.10 would be that the initial contraction corresponds to osmotically-induced egress of water from ASM cells leading to cell shrinkage; there is then a partial relaxation which could represent the RVI response, followed by a refractory phase in which RVI processes cause the ASM cells to be resistant to further contraction (illustrated in Figure 5.28). This inhibition could occur at any level from receptors to the contractile apparatus of the cell, though against the latter is the fact that Finney et al found no effect of hyperosmolar conditions on electric field-induced contraction of bronchial smooth muscle (Finney et al., 1987). In our *in vitro* experiments the ASM is directly exposed to the surrounding fluid at the cut edges of the bronchial rings, in contrast to the situation *in vivo*; this may contribute to the apparent difference between clinical and laboratory data.

Overall, the behaviour of porcine airways in response to high concentration mannitol is likely to be determined by a complex web of pathways invoked by hyperosmolarity and consequent RVI, and untangling this complexity is beyond the scope of this study.

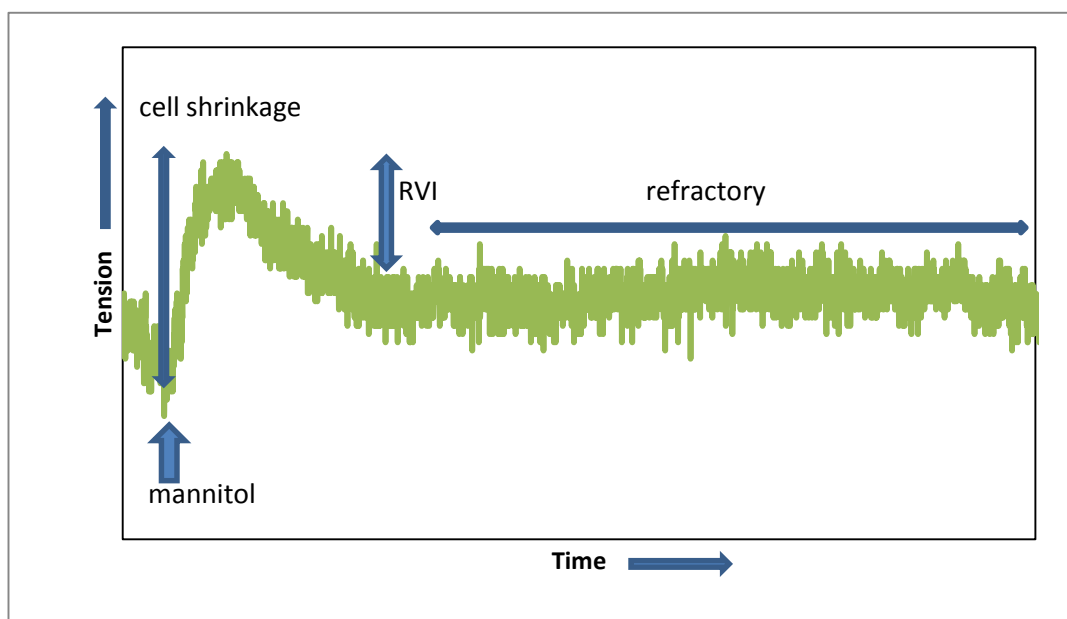


Figure 5.28. Proposed simplified interpretation of the response of a porcine bronchial ring to high concentration mannitol.

5.4.3 Human bronchial rings

The lack of an observed effect of pro-resolving mediators on mast cell-mediated contraction of human bronchial rings is at odds with our hypothesis, and somewhat surprising in view of some of the published findings discussed in Chapter 1 – resolving mediators inhibit human lung mast cell degranulation (N. Martin et al., 2012; Karra, Haworth, Levy, & Levi-Schaffer, 2013b); a LXA₄ analogue reduces airway inflammation and hyper-responsiveness in a murine model of allergic asthma (Bruce D Levy et al., 2002); An E-series resolvin has similar effects in a murine model, and increases LXA₄ levels (Aoki et al., 2010; Haworth, Cernadas, Yang, Serhan, & Levy, 2008). As with the porcine bronchial ring experiments, our negative findings may be a result of not having found the optimum mediator concentration or incubation time, although this is unlikely given that they are based on conditions used in *in vitro* studies in which pro-resolving mediators were shown to be active. Interestingly, *in vitro*

experiments with IgE-stimulated human airways have mostly shown no or only modest reduction of bronchoconstriction with H₁-antagonists, although synergistic effects with leukotriene receptor antagonists have been noted (Bartho & Benko, 2013). It may be that in our model histamine release from mast cells is indeed reduced by pro-resolving mediators, but that this effect does not extend to other mast cell products. Or alternatively, that although the histamine concentration in the airway is reduced, it is still sufficient to maximally stimulate ASM H₁ receptors. This could be investigated by measuring the concentration of histamine and other mast cell mediators in buffer from human bronchial rings following incubation with pro-resolving mediators and subsequent contraction with anti-FcεR₁ – is there a lower concentration of histamine or other mediators but still the same degree of contraction?

The studies described above in which pro-resolving mediators reduced airway hyper-responsiveness were conducted in ovalbumin-sensitised mice, rather than the non-asthmatic human tissue used in our experiments; as discussed above, there is huge species-to-species variation in airway function, and no certainty that results in a mouse asthma model will be applicable to non-asthmatic human tissue. A consistent finding in studies measuring LXA₄ (and in one case PD₁) levels in sputum and blood is that levels in mild asthma are increased compared to controls (for example Vachier et al., 2005; Bonnans et al., 2002; Levy et al., 2007). It may be that receptor expression is also lower in the normal airway, and hence resolving mediators have less effect.

Finally, one disadvantage of the bronchial ring technique in comparison to PCLS is that epithelial quality is not visually assessed. If the epithelium plays any role in the actions

of pro-resolving mediators in the airway, any degradation in epithelial quality during ring harvesting and incubation could reduce their effectiveness.

Chapter 6 Clinical study of the refractory period

6.1 Introduction

A number of clinical studies of the refractory period in asthma have been conducted, as outlined in Section 1.2. However, as well as the failure to elucidate a mechanism for the phenomenon a number of unanswered clinical questions remain. In particular, there remains uncertainty as to how best to demonstrate refractoriness; whether refractoriness is dependent on the magnitude of the initial response to the first challenge; whether refractoriness is associated with a measurable change in release of bronchoconstrictor and potentially bronchoprotective mediators in induced sputum and urine; and whether the subjects who develop refractoriness can be more clearly defined.

The intention of this study is to address these questions in a mixed population of subjects with asthma using inhaled mannitol as a bronchoconstrictor challenge. We have chosen this agent as it is commercially available, licensed for diagnostic testing in asthma and the response to the challenge has been shown to develop refractoriness (Larsson et al., 2011). Our hypothesis is that refractoriness is best demonstrated by repeated single dose challenges of inhaled mannitol (as refractoriness may begin to develop during administration of a cumulative dose), is independent of the magnitude of the initial response, is associated with increased production of protective mediators, and is seen particularly in patients with mild asthma. Our specific aims are:

1. To compare the refractory period induced by repeated challenge with a bolus dose previously shown to result in a 15% fall in FEV_1 (the PD15 dose) with that induced by repeated cumulative dose challenges (mannitol is given as a cumulative dose when using the commercially available bronchial challenge test kits). This is illustrated in Figure 6.1. We will quantify the refractory period as a refractory index (RI), determined as the change in the maximum FEV_1 reduction between the two challenges expressed as

a percentage of the maximum FEV₁ reduction from the first challenge; using the nomenclature of Figure 6.1, $RI = (a - b)/a \times 100$.

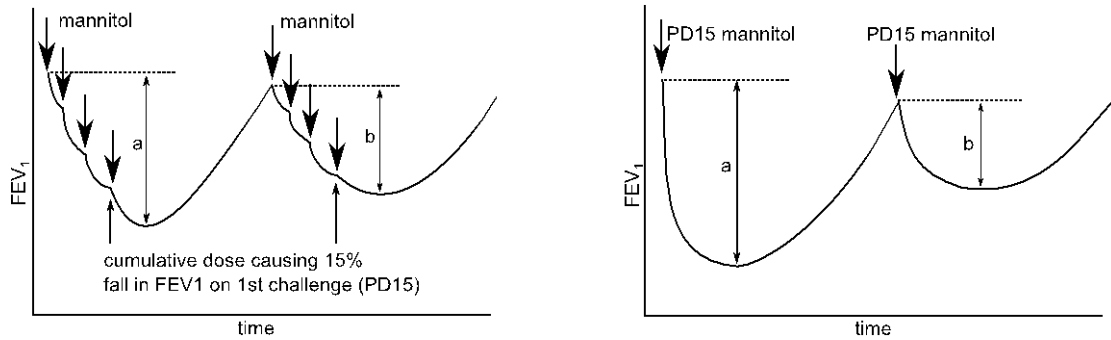


Figure 6.1. Cumulative dose versus bolus dose mannitol challenge.

2. To determine whether provocation of significant bronchoconstriction is necessary to induce a refractory period in mild asthma. This will be done by comparing the response during the first of two PD15 bolus dose mannitol challenges (*a* as shown in Figure 6.1) to the response following a PD15 dose challenge after prior PD5 challenge (dose causing 5% fall in FEV₁) *c* as shown in Figure 6.2.

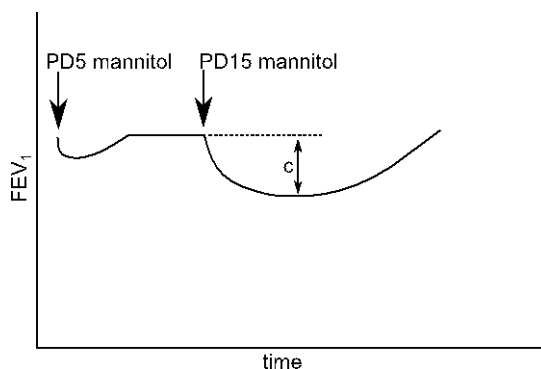


Figure 6.2. Bolus dose PD15 mannitol challenge following PD5 mannitol challenge.

3. To show that there are common demographic and phenotypic characteristics in the patient group with mild asthma who demonstrate refractoriness. It is our hypothesis that refractoriness will be negatively correlated with levels of airway inflammatory markers and positively correlated with steroid use.
4. To determine levels of inflammatory mediators (PGD₂ metabolites) and potential bronchoprotective factors (PGE₂, LXA₄, RvD₁) in our patient group to increase understanding of the mechanisms underlying the refractory period.

6.2 Methods summary

6.2.1 Materials and equipment

Mannitol challenge tests were administered using Osmohale kits obtained from Pharmaxis; methacholine and salbutamol were obtained from the hospital pharmacy. Spirometry was conducted using a Vitalograph Gold Standard spirometer.

6.2.2 Consent and recruitment

Full details are given in Chapter 2. Written consent was obtained from all volunteers before conducting any procedure for the study. Inclusion and exclusion criteria were:

Inclusion

- Age 18 or over
- Diagnosis of asthma

- Treatment with short-acting beta agonist +/- low dose steroids (400 µg per day beclomethasone or equivalent)
- Symptoms well controlled on treatment (never hospitalised with asthma, no more than one course of oral steroids in the past 12 months)
- Non-smoking (ex smoker >1 year, <10 pack year history)

Exclusion

- Major co-morbidity
- Pregnancy
- Previous hypersensitivity to methacholine or mannitol

6.2.3 Study visits

Volunteers made a screening visit followed, if recruited to the study, by four further visits. The visits are described in detail in Chapter 2; they are summarised in the flow chart in Figure 6.3 below. Informed consent was obtained at the screening visit.

Sputum samples from the baseline visit were analysed by microscopy for sputum eosinophilia. Urine and sputum supernatant from all visits was retained to test for markers of inflammation and possible bronchoprotective factors.

Study sputum samples were analysed using the LC-MS methods of Section 2.2 and the immunoassays described in 2.6. A dilution factor of 5:1 was used for the LXA₄ ELISA and 3:1 for the RvD₁ ELISA. Urine samples were analysed for PGD₂ and PGE₂ metabolites by immunoassay, using a dilution factor of 20:1.

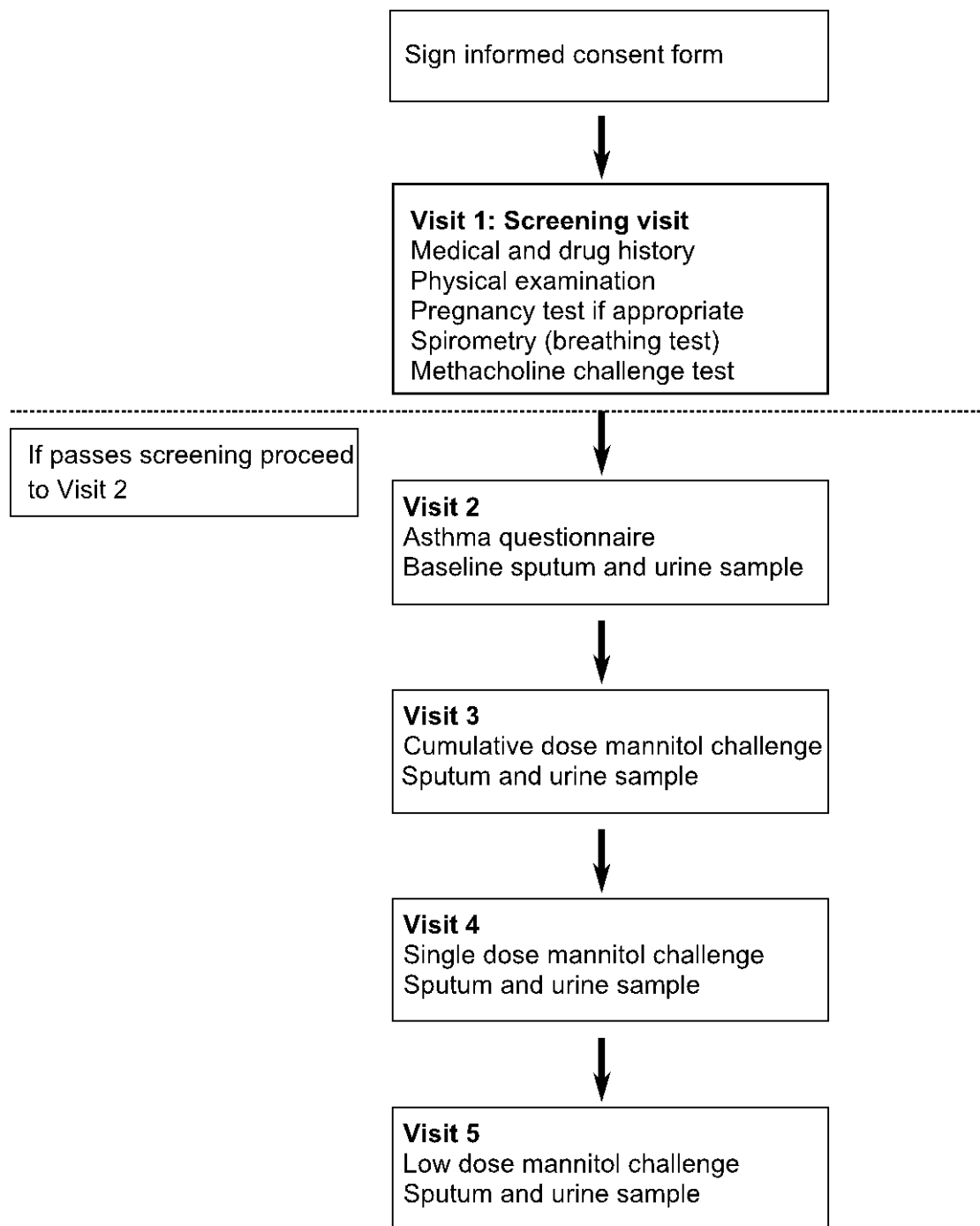


Figure 6.3. Summary flow chart of study visits.

6.3 Results

6.3.1 Recruitment

Recruitment proved difficult, primarily because members of the mild asthma cohort we wished to recruit are not usually hospital clinic attendees. Posters (see Appendix 1) were placed in public areas in the hospital and also at the local university, but these did not attract any volunteers. The principal source of study subjects was thus the small pool of mild asthma volunteers who had participated in previous studies in our hospital; a newspaper advert intended to attract volunteers to a range of projects in our centre also produced several suitable candidates. In total 15 volunteers were screened, of whom 10 were entered into the study; one of these was subsequently excluded from analysis due to a lack of airway responsiveness to mannitol. Table 6.1 details the reasons for screen failure; Table 6.2 summarises the characteristics of our study group.

Exclusion criterion	No of exclusions
Methacholine PC20 > 16 mg/ml	3
Recently re-started smoking	1
Recently started formoterol	1

Table 6.1. Reasons for study exclusion of screened candidates.

Male : female	4 : 5	
Ethnicity	6 White, 3 Asian	
Low dose inhaled steroids	6 (67%)	
Known atopic	6 (67%)	
	Mean (std. error)	Range observed
Age/ years	49 (6.2)	23 - 74
BMI	24.7 (3.8)	19.2 - 32.3
Smoking/ pack-years	0.56 (0.6)	0 - 5
Years since diagnosis	14 (4)	1 - 39
%predicted FEV₁	85% (5.5%)	62% - 111%
%sputum eosinophils	8.4% (4.7%)	0 – 40%
Methacholine PC20/ mg/ml	5.4 (0.81)*	1 - 16
Juniper ACQ score	1.02 (0.24)	0.1 – 2†

Table 6.2. Baseline characteristics of study participants. *Expressed as geometric mean (log SD). †Score out of 7.

6.3.2 Clinical data

Figure 6.4 shows the refractory indices for the cumulative and bolus mannitol PD15 doses. The mean RI \pm 95% confidence interval for the cumulative dose was (16.5 \pm 21)%; this is clearly not significantly different to 0. The mean bolus dose RI was (24.3 \pm 21.2)%.

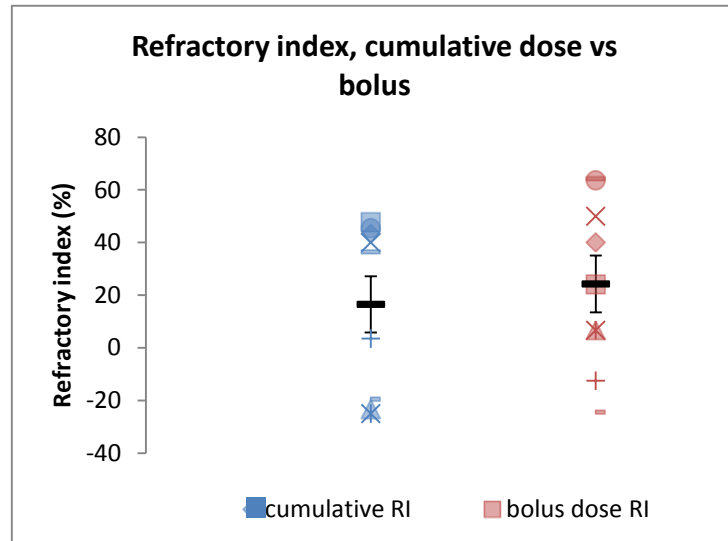


Figure 6.4. Refractory indices for cumulative and bolus dose mannitol inhalation challenges. Error bars indicate standard error. Each subject is represented by a different symbol.

Figure 6.5 shows a linear regression analysis of the relationship between cumulative and bolus dose refractory indices; subjects appear to fall into two groups, with those demonstrating refractoriness to the cumulative dose challenge also likely to do so to the bolus dose challenge.

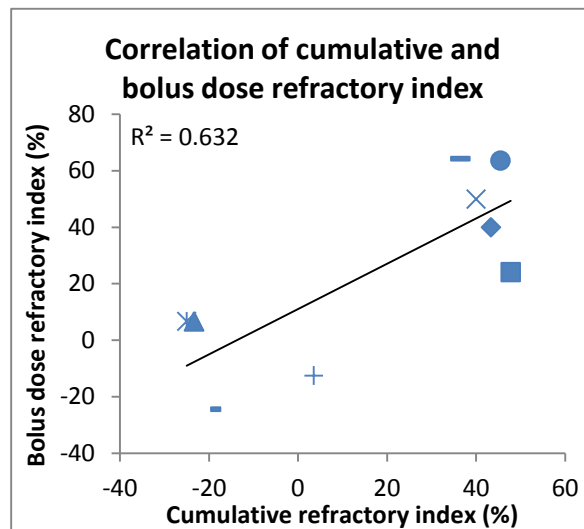


Figure 6.5. Linear regression analysis of the relationship between cumulative and bolus dose refractory indices. Each subject is represented by a different symbol.

To determine if a clinically significant degree of bronchoconstriction is necessary to induce refractoriness, Figure 6.6 compares the refractory index for consecutive PD15 bolus dose mannitol challenges with a refractory index calculated using FEV₁ values from the first dose of the consecutive PD15 bolus dose mannitol challenges from Visit 4 and the second dose of the PD5-PD15 dose challenges from Visit 5 (See Section 6.1). It was not possible to demonstrate that the PD5 dose of mannitol induces refractoriness; however, the variance in the PD5 refractory indices is too great for meaningful interpretation. As seen in Figure 6.7, the mean FEV₁ fall following the second Visit 5 challenge (PD15) is approximately the same as that following the first Visit 4 challenge.

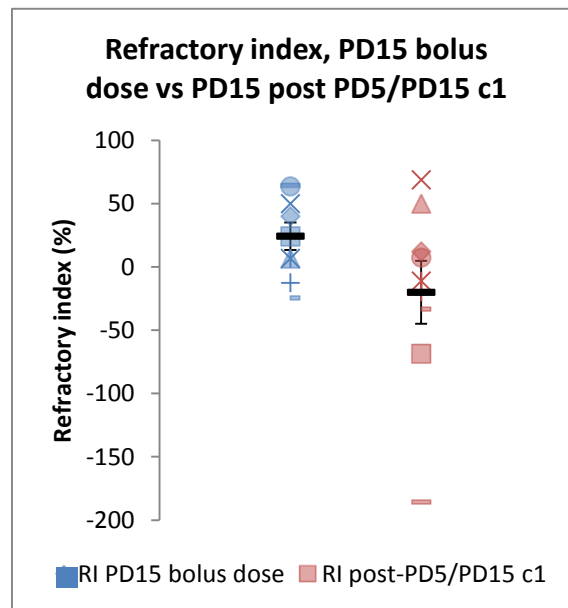


Figure 6.6. Refractory index for consecutive bolus PD15 dose mannitol challenges compared to refractory index calculated using FEV₁ values from the first of the consecutive bolus PD15 dose mannitol challenges and the second dose of the PD5-PD15 challenge. Error bars indicate standard error. Each subject is represented by a different symbol.

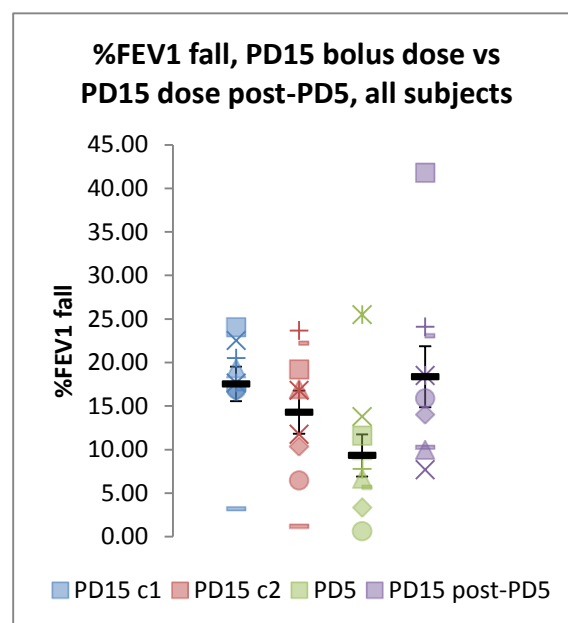


Figure 6.7. FEV₁ fall following consecutive bolus PD15 mannitol doses and a PD5 dose followed by a PD15 dose (Visits 4 and 5). Error bars indicate standard error. Each subject is represented by a different symbol.

There were no strong correlations between RI and asthma severity. Table 6.3 lists slope sign and R^2 values for linear regression analysis of bolus dose RI versus various markers of severity. The relationship between RI and %predicted FEV₁ is shown in Figure 6.8; however, for 9 subjects the R^2 value of 0.192 is not statistically significant at the 95% level (2-tailed t-test).

Severity Marker	Slope	R^2
%predicted FEV ₁	+	0.192
Juniper ACQ	-	0.002
Methacholine PC20	-	0.020
Sputum eosinophils	+	0.125

Table 6.3. Results of linear regression analysis of bolus dose RI (dependent variable) against asthma severity markers (independent variable).

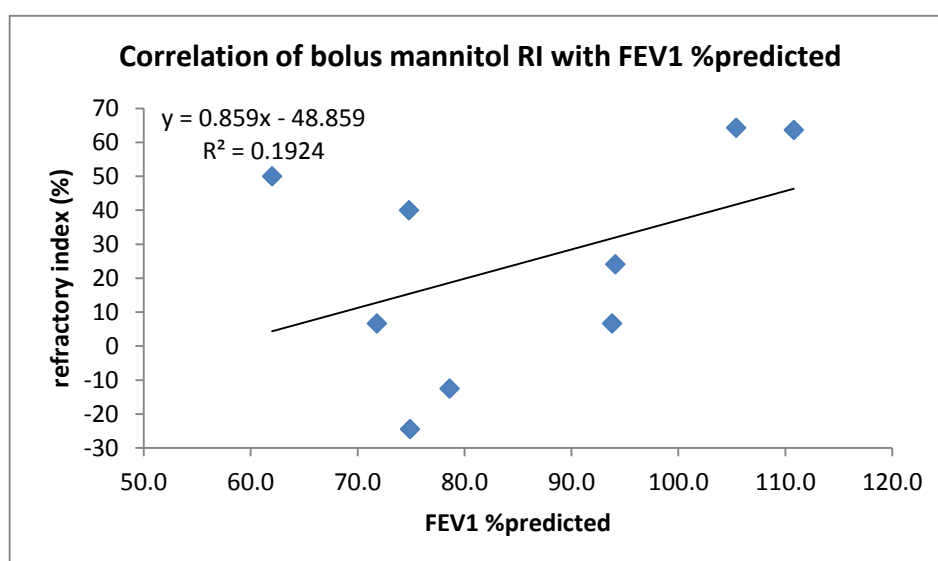


Figure 6.8. Linear regression analysis of bolus dose RI versus %predicted FEV₁.

6.3.3 Urine analysis

Urine samples were obtained post-challenge at each visit, in order to look for changes in the level of PGD₂ and PGE₂ metabolites. In each case concentrations were normalised to the urinary creatinine concentration; the calibration curve for this assay is shown in Appendix 1 (Figure A1.1). Figure A1.2 and Figure A1.3 show the calibration curves for the PGD₂ and PGE₂ metabolite ELISAs respectively. Figure 6.9 and Figure 6.10 show the variation in normalised urinary PGD₂ and PGE₂ metabolite concentrations with study visit. There is no correlation between bolus-dose refractory index and prostaglandin concentration (Visit 4) with linear regression analysis producing R² values of 0.002 and 0.003 for PGD₂ and PGE₂ respectively.

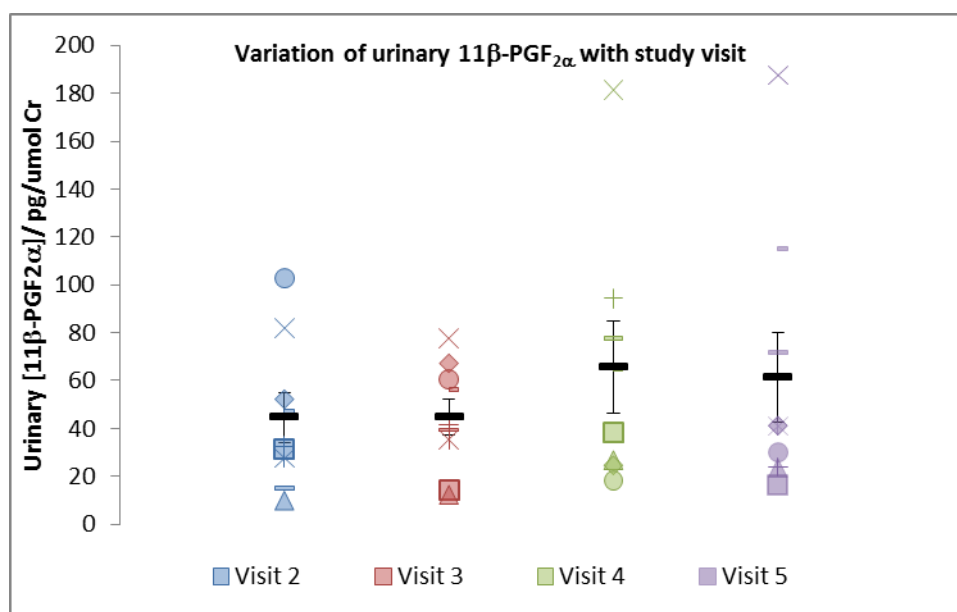


Figure 6.9. Variation of normalised urinary PGD₂ metabolite concentration with study visit. Error bars indicate standard error. Each subject is represented by a different symbol.

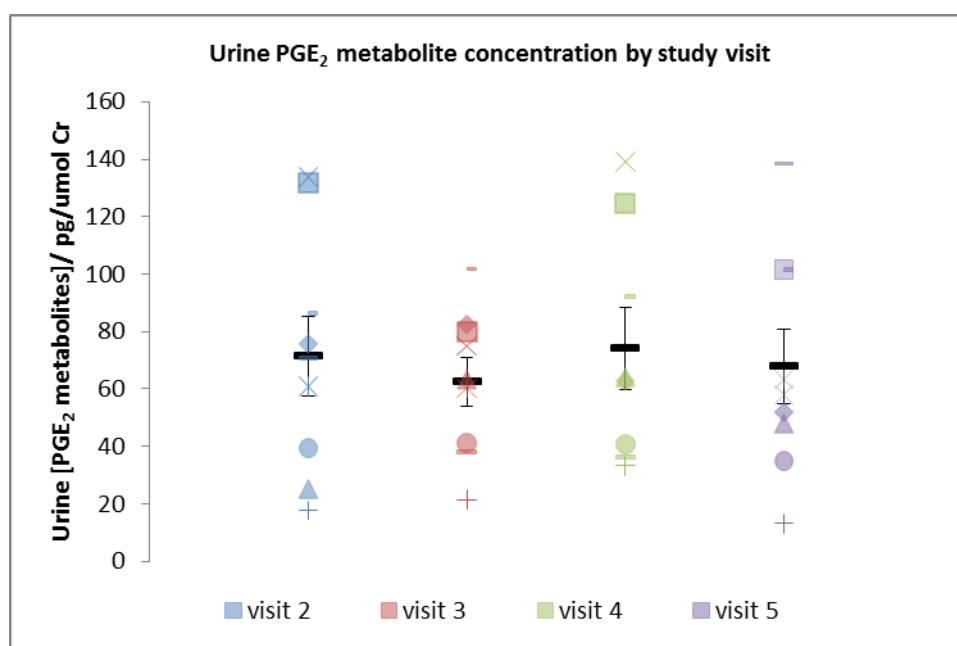


Figure 6.10. Variation of normalised urinary PGE₂ metabolite concentration with study visit. Error bars indicate standard error. Each subject is represented by a different symbol.

It can be seen that there is no significant difference in mean levels of either PGD₂ or PGE₂ between samples taken at baseline (Visit 2) and immediately following mannitol

challenge (Visits 3 – 5). In fact, as Figure 6.11 and Figure 6.12 show, urinary mediator levels for many subjects show a high level of intra-subject consistency between visits.

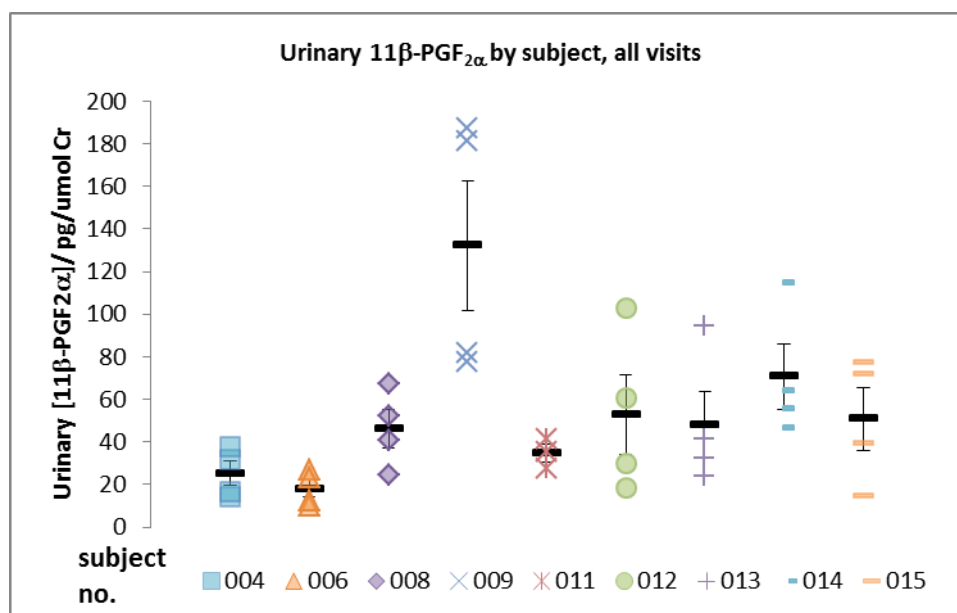


Figure 6.11. Urinary PGD₂ metabolite levels by subject across all study visits. Error bars indicate standard error.

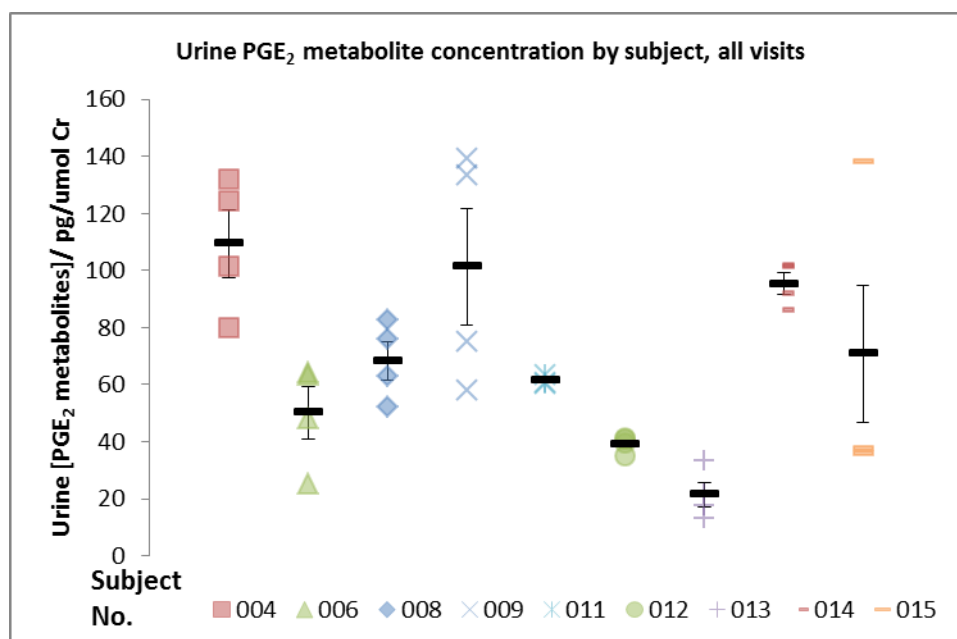


Figure 6.12. Urinary PGE₂ metabolite levels by subject across all study visits. Error bars indicate standard error.

6.3.4 Sputum analysis

As discussed in Chapter 3, it was necessary to conduct LC-MS analysis of the study sputum samples using the less sensitive TQ instrument, and as anticipated this did not detect any lipid mediators. It was thus necessary to use an immunoassay approach; LXA₄ and RvD₁ ELISAs were used as these are commercially available and affordable. Figures A1.4 and A1.5 in Appendix 1 show the calibration curves for these assays; variation of mediator levels with study visit is shown in Figure 6.13 and Figure 6.14. Subject 008 did not produce sputum at any visit, and several other subjects were unable to produce sufficient sputum at some visits to complete all analyses.

It should be noted that 11 samples were analysed for RvD₁ at two dilutions (1:3 and undiluted), as advised by the manufacturer. They state that a difference in measured concentration of >20% between the two dilutions indicates the likelihood of interfering substances in the sample; in our case the mean level of discrepancy was 69%, suggesting a degree of interference. This figure is based on only six sample pairs, as five of the samples were only detectable at the lower dilution.

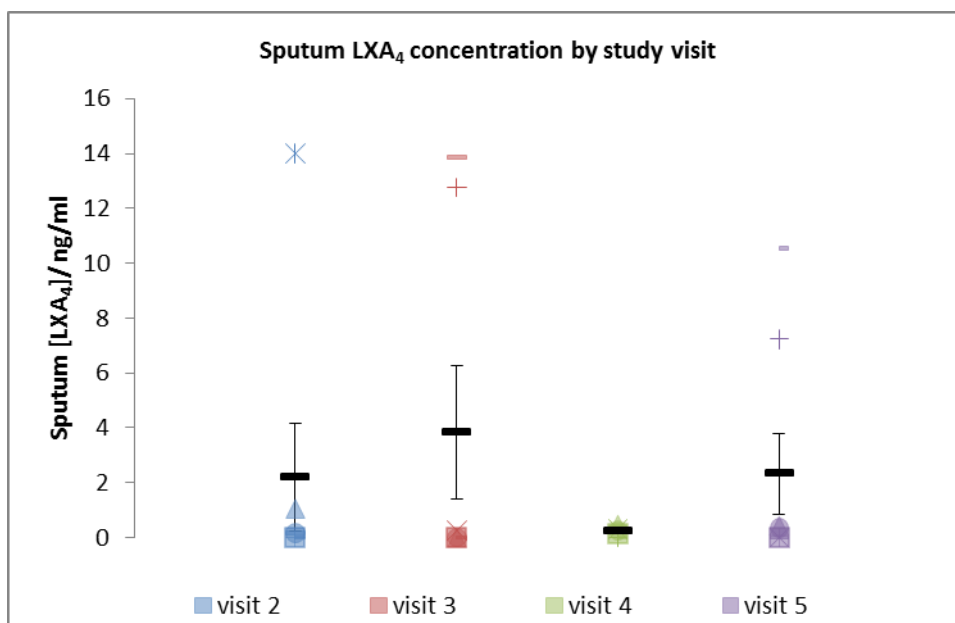


Figure 6.13. Variation of sputum LXA₄ concentration with study visit. Error bars indicate standard error. Each subject is represented by a different symbol. The four highest values are above the top of the calibration curve and should be treated with caution.

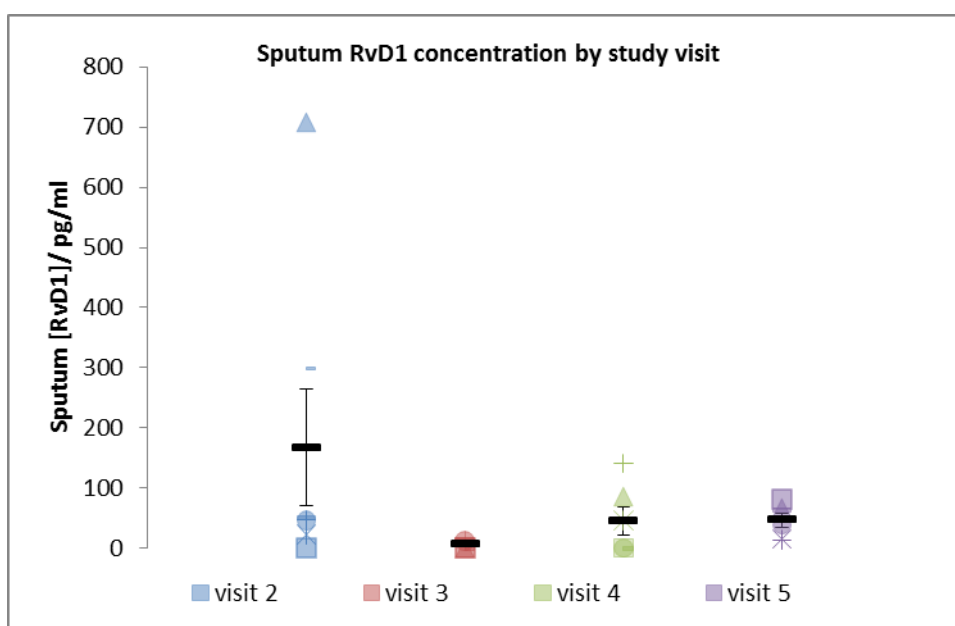


Figure 6.14. Variation of sputum RvD₁ concentration with study visit. Error bars indicate standard error. Each subject is represented by a different symbol.

There is no statistically significant difference in detected sputum LXA₄ or RvD₁ levels between baseline and post-mannitol challenge visits at a 95% confidence level. As shown in Figure 6.15 and Figure 6.16, there is less intra-subject consistency in detected levels than was seen for urinary prostaglandins.

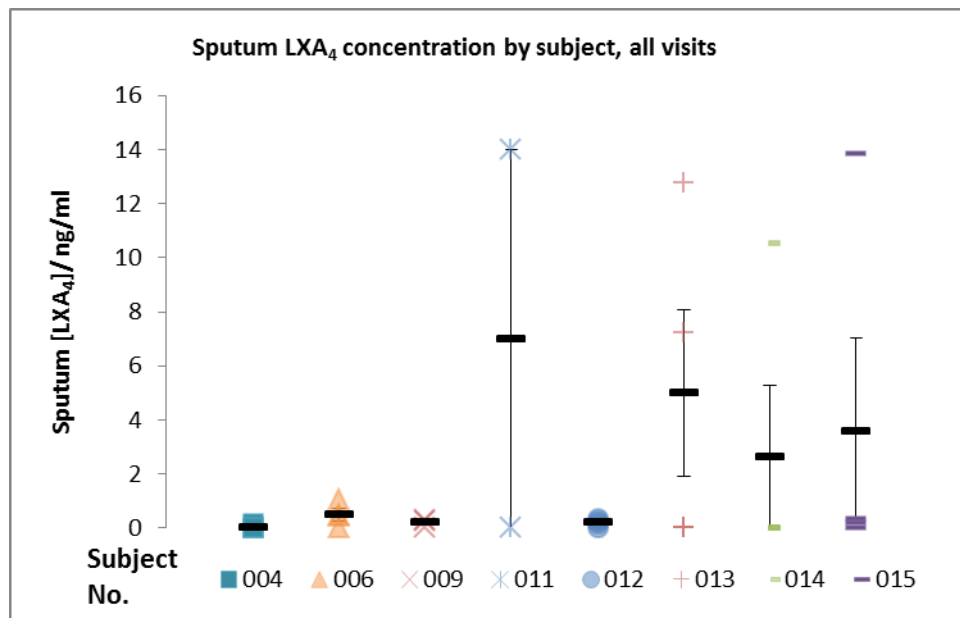


Figure 6.15. Sputum LXA₄ levels by subject across all study visits. Error bars indicate standard error.

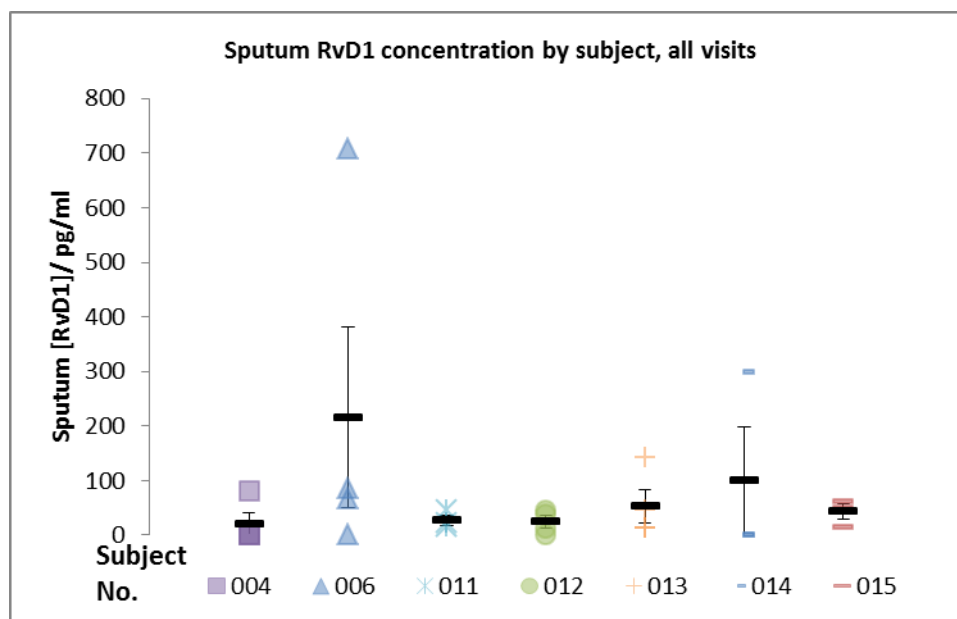


Figure 6.16. Sputum RvD₁ levels by subject across all study visits. Error bars indicate standard error.

6.3.5 Summary

In summary, the main findings of the clinical study are:

- Refractoriness to mannitol was not demonstrated overall with the cumulative dosing regimen, although there was a strong trend; with bolus dosing the mean refractory index was 24%. Those subjects showing refractoriness to bolus dosing tended to show refractoriness to cumulative dosing and vice versa.
- The degree of refractoriness did not correlate with markers of disease severity.
- We were unable to demonstrate any refractoriness following a PD5 dose of mannitol.

- Urinary PGD₂ and PGE₂ metabolite levels showed no significant change in response to mannitol challenge. Overall there was good intra-subject consistency in prostaglandin levels across study visits.
- Sputum LXA₄ and RvD₁ levels showed no consistent change in response to mannitol challenge.

These findings are discussed in 6.4 below.

6.4 Discussion

Although some useful data was obtained, this study is clearly handicapped by the small number of subjects recruited (9 suitable subjects, from an initial target of 20). The primary cause of difficulty with recruitment was the intention to recruit patients with mild asthma. This group of patients rarely attend hospital clinics and have less incentive to volunteer for studies than those with more severe disease, as the lack of response to our posters would attest. The distance between the university campus and our hospital may have further reduced the likelihood of responses to our advertising on campus. Financial constraints precluded more expensive means of recruitment to this study, but in future studies of the mild asthma cohort an approach to local General Practitioners to suggest potential study volunteers from their practice databases might be fruitful.

The small sample size is a likely explanation for our failure to clearly demonstrate refractoriness to mannitol with the cumulative dose method. There are two recently published studies of refractoriness to mannitol challenge. Larsson et al. (2011) used a different approach to ours, allowing 90 minutes between challenges 1 and 2. Six of Larsson's 16 patients were taking inhaled corticosteroids (37.5%) with 5 of these also

on a long-acting beta-agonist; baseline FEV₁ was 90.2%. The study found a mean RI to inhaled mannitol of 50% (range 0 - 88%) from the 16 subjects. Suh, Lee, Kim, & Koh (2011) carried out a study of mannitol refractoriness in 41 children. 43.9% were on inhaled steroids; mean baseline FEV₁ was 87.2%. This group used our method of waiting for a return to 95% of baseline FEV₁ before giving the second challenge, measuring FEV₁ every 10 minutes following challenge 1. They calculated several measures of refractoriness; using the method comparable to ours the RI was 49%. Again, they did not find any negative refractory indices, although a number of subjects had a PD15 dose >635 mg in one or both challenges.

Our small sample size and correspondingly wide confidence intervals may partly explain why our mean bolus dose RI of 24% (95% confidence interval \pm 21.2%) is lower than that seen in these two studies. Another reason for the disparity may be disease severity; baseline FEV₁ is comparable between the three studies, but 67% of our subjects required inhaled corticosteroids compared to 37.5% in Larsson's study and 43.9% in Suh's. In the case of Larsson's study, it may be that waiting 90 minutes between challenges regardless of FEV₁ allows more patients to reach a significantly refractory state than our approach. As seen in Figure 6.5, our patients appear to split into a refractory and non-refractory group, with 4 of the 9 subjects showing no or minimal refractoriness to either challenge method. It is worth noting that only around 50% of exercise-induced asthmatics demonstrate significant refractoriness (see discussion in 1.2.2).

Urine analysis found no change in the concentration of the PGD₂ metabolite 11 β -PGF_{2 α} following mannitol challenge. This is in contrast to two previous studies. Larsson et al, in the study discussed above, were able to obtain urine samples at 30 minute intervals

following both mannitol challenges. They found that the PGD₂ metabolite 11 β -PGF_{2a} and the cysteinyl-leukotriene LTE₄ were present at increased concentration following both challenges. The confidence intervals for the PGD₂ metabolite concentrations are wide, and the increase would probably not have reached statistical significance in a study the size of ours. Additionally, in our study we use a baseline metabolite concentration measured on a different day to the mannitol challenge, which will also introduce a greater degree of variability compared to Larsson's study. Brannan et al. (2003) measured 11 β -PGF_{2a} and LTE₄ in urine 30, 60 and 90 minutes following a single mannitol challenge in 12 asthma patients and 9 controls. They found the peak concentration of both mediators was increased post-challenge relative to baseline in both groups (although the control group did not demonstrate airway hyper-responsiveness). Commenting on the previous failure of some studies to show evidence of mast cell mediator release after exercise, the authors remark "*As a technical note, it should be recognised that the time course of the bronchoconstriction after exercise and mannitol is short and this makes it very different to the prolonged airway responses that occur after allergen challenge. This may explain why it has been difficult to find significantly increased levels of urinary LTE₄ after exercise in some studies*". However, our urine samples were taken immediately following maximal bronchoconstriction after the second mannitol challenge, so it seems unlikely that this is an explanation for the disparity with our study. Our data on urine mediators appear to be of good quality, so the low number of subjects, and the collection of baseline and post-challenge samples on different days, are the only obvious differences in our methodology which might offer an explanation. It is possible that our samples would show evidence of increased cysteinyl-leukotrienes post-mannitol despite the lack of PGD₂ response; urine samples

have been retained with consent for future studies, and this would be an interesting additional measurement.

Sputum analysis was clearly hampered by the unavailability of a high sensitivity LC-MS instrument, meaning that separate ELISA analyses had to be performed on each mediator of interest. This was exacerbated by the small sputum volumes produced by some of the relatively mild asthmatics in our study group, which did not allow analysis of sputum from every patient at every visit. The overall result is a rather small data-set which does not demonstrate any convincing relationship between pro-resolving mediator concentration and mannitol challenge. We have thus not found any evidence for a prominent role for LXA₄ or RvD₁ in the refractory period; as noted in Chapter 3, they appear to be either absent or present in very low concentrations in sputum. A proviso to this is that, as noted in 6.3 above, analysis for RvD₁ at two sample dilutions suggested that there may be substances present in the samples which interfere with the assay.

Our clinical study clearly does not shed much new light on the mechanism of the refractory period, and in fact does not confirm the previous findings of a mast-cell mediated mechanism for mannitol-induced bronchoconstriction. The lack of a PGE₂ response to mannitol is noteworthy. Larsson et al propose PGE₂ bronchoprotection as one potential mechanism for mannitol-induced refractoriness, as others have previously (see discussion in Chapter 1). Our data does not support this. Their alternative proposed mechanism is ASM receptor down-regulation, which our study does not address; this is discussed further in Chapter 7.

There are clearly a number of limitations to this study. By far the most important is the small sample size. As discussed above, if financial and time constraints allowed an

approach to recruitment via GPs might prove fruitful. Assessing bronchial hyper-responsiveness using the Forced Oscillometry Technique (FOT) rather than spirometry might maximise the robustness of a small data set, as FOT requires minimal subject co-operation and no airway manoeuvres, and so should not be affected by the coughing that deep inspiration during spirometry can sometimes induce following mannitol inhalation (Oostveen et al., 2003). Another issue identified above is the timing of baseline sample collection. As sputum induction with hypertonic saline can cause bronchoconstriction, salbutamol is given as part of the induction protocol; it is therefore not possible to collect baseline samples immediately pre-mannitol challenge, and the samples were obtained at a separate baseline visit. As noted above however, collection of pre-challenge urine samples is feasible with co-operative volunteers, and may have increased the likelihood of a statistically significant result from our prostaglandin data.

The other key issue is sample analysis. Although the urine prostaglandin ELISA data appears robust, the sputum lipoxin and resolvins data does not. Despite using DTT-free sputum, some of the detected LXA₄ levels are suspiciously high in comparison to the more robust LC-MS data seen in Chapter 3, and there is evidence of interfering substances in the RvD₁ assay. The ideal solution would of course be regular access to a high-sensitivity LC-MS instrument, for the reasons outlined in Chapter 3. If the ELISA approach must be taken however, a further period of method development would be advisable to devise a suitable sputum-processing method to eliminate potential interfering substances prior to analysis. For example, the manufacturers of the RvD₁ ELISA recommend a solid phase extraction-based technique for use with human plasma (Cayman Chemical, 2013b). The obvious disadvantage of any lipid-extraction method is that it introduces another source of experimental error. There is a large variability in

extraction efficiency using the solid phase extraction technique of Chapter 3, and with ELISA it would not be possible to correct for this using an internal standard.

Chapter 7 Discussion and future work

7.1 Summary of main hypotheses and findings

The primary hypothesis of this thesis is that refractoriness in asthma is due to release of bronchoprotective mediator(s) within the airway and that these may be one or more of the pro-resolving lipid mediators such as lipoxins or resolvins. A number of secondary hypotheses were developed for the clinical part of our study: that refractoriness is best demonstrated by repeated bolus dose challenges of inhaled mannitol (as refractoriness may begin to develop during administration of a cumulative dose); is independent of the magnitude of the initial response; is negatively correlated with levels of airway inflammatory markers; is associated with increased production of protective mediators; is positively correlated with steroid use; and is seen particularly in patients with mild asthma.

We have attempted to investigate these hypotheses via:

1. Development of a LC-MS assay for the detection of pro-resolving mediators in sputum.
2. Development of an *in vitro* airway model using sheep PCLS and porcine and human bronchial rings, in order to test the effect of pro-resolving mediators on histamine-induced airway contraction.
3. A clinical study of mannitol-induced refractoriness with collection of urine and sputum to assess changes in prostaglandin and pro-resolving mediator levels.

The main findings of our work are:

1. Approximate LC-MS limits of detection were 0.002, 0.01 and 0.04 pg/μl for PGE₂, LXA₄ and RvD₁ respectively, using a Waters Xevo TQ-S instrument. These LOD

compare favourably with ELISA, but the utility of the assay for our study was unfortunately limited by instrument availability.

2. LC-MS analysis of 10 sputum samples selected from our library of research samples showed evidence of low levels of LXA₄ in one sample, and no other pro-resolving mediators in any sample.
3. Serial LC-MS analysis of spiked sputum samples showed loss of 50% of the initial PGE₂ signal after 5 months; LXA₄ appeared stable, while RvD₁ showed loss of 20-30% of its initial signal.
4. A sheep precision-cut lung slice airway model was developed, and key elements for successful slice preparation were identified as: good quality, “still warm” fresh lung; good control of agarose temperature; a high quality tissue-slicing instrument; extensive washing of slices with growth medium in the first few hours post-preparation. Even with experience, good-quality slices were not always obtained and there was huge animal-to-animal variation in bronchial responsiveness.
5. A porcine bronchial ring airway model was developed. Incubation of bronchial rings with LXA₄ or RvD₁ had no effect on their response to histamine. Incubation with 10nM MaR₁ appeared to produce a small attenuation in the histamine response, but this was not repeatable with either 1 nM or 100 nM MaR₁ and is thus unlikely to be important.
6. High concentration mannitol induced bronchoconstriction in pig bronchial rings *in vitro*. The mechanism is unclear, but does not operate *via* the mast cell mediators histamine, LTC₄ or PGD₂. Bronchoconstriction with mannitol rendered an airway

refractory to further contraction with the direct ASM agonists carbachol and histamine.

7. In human bronchial rings, neither 10 nM LXA₄ nor 10 nM MaR₁ had any effect on mast cell-mediated bronchoconstriction as induced by FcεR₁ activation. There was a trend towards reduced bronchoconstriction in the presence of 100 nM MaR₁, but this was not statistically significant. The use of human bronchial rings was limited by a lack of available tissue.
8. A clinical study of the refractory period was conducted using inhaled mannitol as a bronchial challenge. Its primary limitation was small sample size. Refractoriness to mannitol was not demonstrated overall with a cumulative dosing regimen, although there was a strong trend; with bolus dosing the mean refractory index was 24%. Those subjects showing refractoriness to bolus dosing tended to show refractoriness to cumulative dosing and vice versa.
9. The degree of refractoriness to mannitol did not correlate with markers of disease severity. We did not demonstrate any refractoriness following a PD5 dose of mannitol.
10. Urinary PGD₂ and PGE₂ metabolite levels measured by ELISA showed no significant change in response to mannitol challenge. Overall there was good intra-subject consistency in prostaglandin levels across study visits.
11. Sputum LXA₄ and RvD₁ levels measured by ELISA showed no consistent change in response to mannitol challenge.

7.2 Discussion

7.2.1 Implications of this study

In Chapter 1 we outlined the various mechanisms which have been proposed to explain the refractory period. In Chapter 6, we described the recent study of mannitol-induced refractoriness by Larsson et al, and their proposed mechanisms – PGE₂ as a bronchoprotective factor or ASM receptor down-regulation – are essentially the same as those proposed in the 1980s and 90s; little progress has been made in understanding this phenomenon. Unfortunately this current study has not advanced our understanding very much; however, in this chapter we attempt to draw together what has been learned and what further work is needed.

Despite the attractiveness of the hypothesis, we have found no evidence that any of the pro-resolving mediators plays a role in the form of a bronchoprotective factor. As discussed above, their lack of effect on FcεR₁-mediated bronchoconstriction does not preclude an effect on mast cell histamine release, but taken along with their lack of effect on histamine-induced ASM contraction, their low sputum concentrations, and the absence of any clear correlation between mediator levels and bronchial challenge, it does make a significant role in the refractory period unlikely. There are caveats, as discussed above; there is uncertainty over the reliability of the immunoassays used in the clinical study, and species differences may be important in the pig experiments, but mediator incubation times in the *in vitro* work may be particularly important. Our 20 minute incubation time was based on the actions of pro-resolving mediators reported in the literature, but refractoriness tends to develop over a slightly longer time period, and if ASM receptor down-regulation is a component of the refractoriness mechanism it

may be that a longer incubation time is needed to see a significant effect. Further *in vitro* experiments would be needed to explore this.

Although not definitive, our clinical study offers no support to the hypothesis advanced by Larsson and others that PGE₂ mediates refractoriness by acting as a bronchoprotective factor – no significant increase in PGE₂ levels was seen following mannitol challenge. Given the limited size of the study it is possible that a small effect exists, but it is unlikely to be of a magnitude which would explain refractoriness.

Brief mention should be made of the refractoriness seen in the mannitol-induced bronchial ring model. Although this is an interesting effect, its resemblance to the clinical refractory period is superficial, with the underlying mechanisms likely to be multifactorial and complex, and further work in this area is unlikely to be useful in terms of elucidating a mechanism for the clinical phenomenon. In the final section, we summarise the current state of knowledge in this area and consider what clinical and laboratory studies are most likely to bear fruit in increasing our understanding of refractoriness.

7.2.2 Current hypotheses and future work

To a large extent, the work described in this thesis has not changed the focus of the discussion in Chapter 1 – the most satisfying unifying hypothesis to explain refractoriness to a range of different indirect airway challenges, and cross-refractoriness between them, remains the down regulation of distal bronchoconstrictor pathways as a result of release of a bronchoprotective substance during the initial challenge. This is not necessarily an alternative to the ASM receptor down-regulation concept – this may

be the downstream action of the bronchoprotective agent. However, receptor down-regulation alone, for example LTC₄ triggering reduced expression of its own receptor, does not readily explain cross-refractoriness. Other intracellular events leading to down-regulation of other ASM receptor types would be necessary. Given that refractoriness is seen against both mast cell and neurally-mediated challenges, the airway smooth muscle cell remains the most likely site of bronchoprotective action. Given the lack of convincing evidence of refractoriness to direct challenges such as methacholine, any bronchoprotective agent is likely to act relatively proximally in the internal pathways of ASM cells (for example by down-regulating receptors or reducing their sensitivity as discussed above) rather than inhibiting the contractile apparatus of the cell.

Alternative hypotheses to the single bronchoprotective agent acting on ASM can be proposed, though they are less satisfying. As discussed in Chapter 1, some authors such as Vanhoutte are critical of the concept of a single epithelium-derived bronchodilating agent on the basis that the range of bronchoprotective/relaxant effects it would have to mediate and the requirement for it to diffuse considerable distances across the sub-epithelial layers in larger airways are both implausible. They propose that “...*the epithelial cells release true messenger molecules (acetylcholine, GABA, and cytokines), which in turn alter the production of endogenous substances (NO and/or metabolites of arachidonic acid) by the subepithelial layers. These substances then diffuse to the airway smooth muscle cells, indirectly conveying epithelium dependency to their responses*” (Vanhoutte, 2013). A bronchoprotective cascade of this type, with a final single or multiple agents operating on ASM, is a possibility. Another alternative is that a single bronchoprotective agent exists but has multiple sites of action, such as mast cells, airway nerve-endings and ASM receptors for a range of bronchoconstrictors. For

example, the pro-resolving mediators discussed in this thesis have been found to have a broad range of actions across multiple tissue types. Finally, multiple mechanisms may be at work – but it is difficult to explain cross-refractoriness with this hypothesis.

Any programme of work intended to untangle the mechanism of refractoriness (or indeed of AHR) will inevitably be long and complex. Key to future studies would be the availability of high sensitivity LC-MS. As discussed in Chapter 3, this offers many advantages over ELISA, and its use in our study was limited only by instrument availability.

An exciting recent development in LC-MS is the new field of metabolomics. This technique attempts to profile the entire metabolome of a biological sample – all the small molecules including lipids, hormones, amino acids, small peptides, carbohydrates, exogenous metabolites and other molecules, excluding proteins and nucleic acids, which make up the metabolic pathways of an organism. More targeted approaches can focus on specific classes of molecules. The technique is employed in a data-driven, hypothesis generating approach by examining biological samples of differing exposure for differences in their metabolic profile. Examples of its application in clinical chemistry include identification of biomarkers in colorectal cancer, and studies of metabolic disorders related to respiratory chain dysfunction (Roux, Lison, Junot, & Heilier, 2010). An accurate mass, high resolution mass spectrometer is required to obtain good discrimination of the many hundreds of signals acquired, and allow an elemental analysis of analytes of interest. Following sample extraction and dilution, the LC-MS analysis is performed; the resulting metabolic fingerprint is then pre-processed using an automatic peak detection algorithm, followed by analysis using appropriate statistical tools. Preliminary identification of any signals of interest is achieved by a

combination of mass spectrum analysis, database consultation and other appropriate informatics tools.

The attractiveness of this approach for investigating refractoriness is clear. Although various putative bronchoprotective agents have been proposed to explain the refractory period, so far none of them have been confirmed in clinical studies. A new clinical study combined with a metabolomics approach would allow any associations between refractoriness and specific metabolites to be detected; these could then be investigated further in subsequent laboratory and clinical studies.

A further clinical study is clearly needed, both to firm-up the conclusions of our existing study and to generate and explore new hypotheses. Again, mild asthma patients would be required, ideally milder than the cohort in the current study, and involvement of general practice would be the most likely means of recruiting greater numbers in a reasonable time-frame. Greater subject numbers would be the most important improvement over the current study. Mannitol appears to be a convenient challenge with which to generate refractoriness; however, alternatives to FEV₁ could be considered for measurement of refractoriness index. Although widely used, FEV₁ has limitations as a measure of small airway obstruction; many patients with symptomatically uncontrolled asthma can have “normal” FEV₁ (Bourdin et al., 2006). The forced expiratory flow at 25-75% of FVC (FEF_{25-75%}) has in the past been widely considered to be more sensitive than FEV₁ to small airways disease, but recent studies have demonstrated that this is not the case (Quanjor, Weiner, Pretto, Brazzale, & Boros, 2014). As discussed in Chapter 6, FEV₁ measurement has the further disadvantage of requiring cooperation from the patient, further exacerbated in our study by the degree of upper airway irritation some volunteers experienced on mannitol inhalation.

Other measures of small airways obstruction are available. The gold standard is measurement of specific airway resistance (sRaw), a measure directly related to bronchial diameter, by whole body plethysmography. This has the advantage of requiring limited cooperation from the patient, and has therefore been used in the assessment of airway resistance in children, but has the major drawback that specialised and costly equipment is required (Olaguibel et al., 2005). Another method is the single breath nitrogen washout test, which can assess abnormalities at the small airway level (Bourdin et al., 2006). In this test the patient takes a single breath of pure oxygen and then exhales slowly to residual volume. The exhaled volume and the nitrogen concentration in that volume are measured to obtain the nitrogen-washout curve. During mid-expiration, when gas is being emptied from all parts of the lungs, the nitrogen washout curve is relatively straight (so-called phase III of the curve). Increases in the phase III slope are regarded as a marker of small airway alterations. The test is reasonably simple to perform but does require additional equipment over simple spirometry, and has not been widely used in the context of AHR assessment. In addition, the phase III slope is influenced by a variety of factors, and the quantitative effect of the different mechanisms is not clear (Barbini, Brighenti, & Gnudi, 2013). As discussed in Chapter 6, the most promising alternative technique for a future study is the forced oscillometry technique (FOT). This uses low-amplitude pressure oscillations superimposed on normal breathing to measure airway conductance (Grs, reflecting airway calibre) and reactance (Xrs, reflecting elastic and inertial properties of the airways) (Oostveen et al., 2003). As measurement is made during tidal breathing, minimal cooperation is required from the patient; it has been used to assess pulmonary function in patients who find spirometry difficult, such as children. FOT measurements

of Grs and Xrs have been successfully used for assessment of AHR, and have been shown to provide a sensitive and reproducible measure of response to mannitol challenge (McClean, Htun, King, Berend, & Salome, 2011). FOT is thus a strong candidate for replacing FEV₁ in the calculation of refractory indices to mannitol in a future study. It provides a more specific assessment of small airway diameter changes, and the much reduced requirement for patient cooperation is likely to improve reproducibility in a relatively small study cohort. The main drawback is the relative lack of data on which to derive appropriate cut-off values for bronchial challenge testing, particularly with mannitol.

A further enhancement over the current study would be the use of a same-day control for urinary mediator levels – this would allow increased confidence in our finding that PGE₂ is not correlated with refractoriness, as well as improved confidence intervals for any new mediators of interest. Sputum samples would again be taken at each visit, with LC-MS allowing more efficient use of small samples. Metabolomic analysis of both urine and sputum would be conducted to identify targets associated with refractoriness. Targeted LC-MS of molecules of interest would also be performed. A diagram for a preliminary study design is illustrated in Figure 7.1 below.

This study will clearly not be definitive, and has the potential to demonstrate only an association between specific mediators and the development of refractoriness. Further laboratory studies, and ultimately further clinical studies, will be necessary to show that a given candidate mediator actually induces refractoriness. A key element of this will be further development of an *in vitro* model of refractoriness.

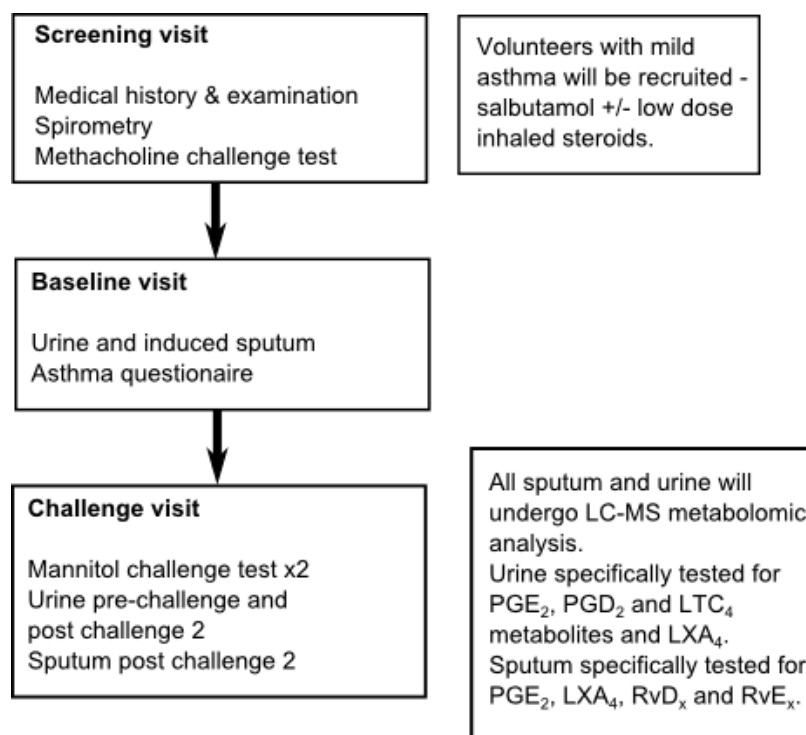


Figure 7.1. Proposed new clinical study to further investigate the mechanism of the refractory period.

A further programme of laboratory work will be needed to complement the clinical study. There are four objectives: 1. to test the hypothesis that a transferable bronchoprotective factor is responsible for refractoriness; 2. to test candidate bronchoprotective factors derived from metabolomics analysis in the clinical study; 3. to test whether refractoriness is mediated at the level of ASM or higher level, such as mast cell; 4. to test whether receptor down-regulation plays a role in the mechanism of refractoriness. Ideally, human bronchial rings will be used; it may be possible to obtain them from another centre where surgery is less lung-conserving. Failing this, in order to meet all objectives an animal model such as our pig model would need to be further developed by the synthesis of antibodies against mast cell FcεR₁; this would require a collaborative approach to access the necessary expertise. The following tentative programme of work is proposed:

1. An *in vitro* model of refractoriness. A clinically authentic refractory period cannot be simulated using direct ASM agonists, and a mast cell-mediated model may be vulnerable to mast cell mediator depletion in the organ bath environment. Although the mannitol model proved disappointing, other indirect challenges such as metabisulphite could be tried. An alternative possibility is suggested by the literature review of Chapter 1, which revealed uncertainty as to whether a direct ASM agonist can induce refractoriness to a subsequent indirect challenge. The use of a direct challenge or electric field stimulation followed by washout and then an indirect challenge would be investigated. Comparison would be made between the second challenge and a control set of rings receiving the indirect challenge only. The establishment of this latter type of model would be crucial for experiment 2.

2. Demonstrating that airway challenge induces release of a bronchoprotective agent. Bronchial rings are contracted in a minimal volume of medium using electric field stimulation. The supernatant medium is collected and used to incubate a second set of test rings, with control rings incubated in fresh medium. Both sets of rings are contracted using an indirect airway challenge. Reduced contractility of the test rings relative to controls would be suggestive of a transferable bronchoprotective factor. The experiment is obviously dependent on electric field stimulation inducing refractoriness to indirect challenges, as investigated in experiment 1; data from canine trachea provide evidence that this may be the case (Walters et al., 1984).

3. Testing potential bronchoprotective agents. Candidate molecules identified by metabolomic analysis in the initial clinical study will be tested for their effects on direct and mast cell-mediated airway contraction *in vitro*, in a manner similar to the current study. If the model of refractoriness outlined in experiment 1 is effective and

appropriate receptor agonists are available, then the effect on refractoriness of blocking the action of candidate molecules will be investigated.

4. Testing for receptor down-regulation. Expression of receptors for bronchoconstrictors such as histamine, LTC₄ and PGD₂ will be measured on airway smooth muscle in bronchial rings before and after challenge, to determine whether receptor down-regulation is likely to play a role in refractoriness.

The final part of the programme, and the most difficult to implement, would be a further clinical study to test *in vivo* the effect of any new bronchoprotective agent identified in the earlier phases of the research.

Appendix 1: ELISA Calibration Curves

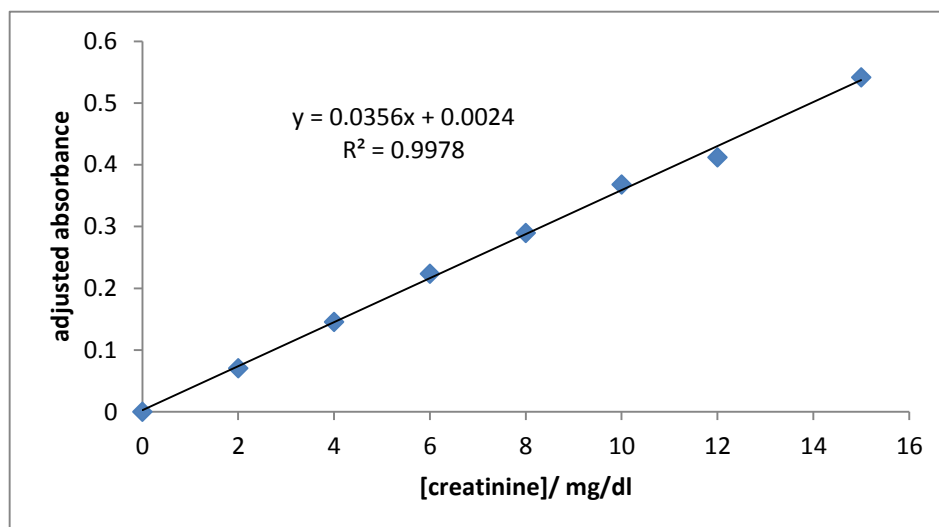


Figure A1.1. Calibration curve for urinary creatinine assay.

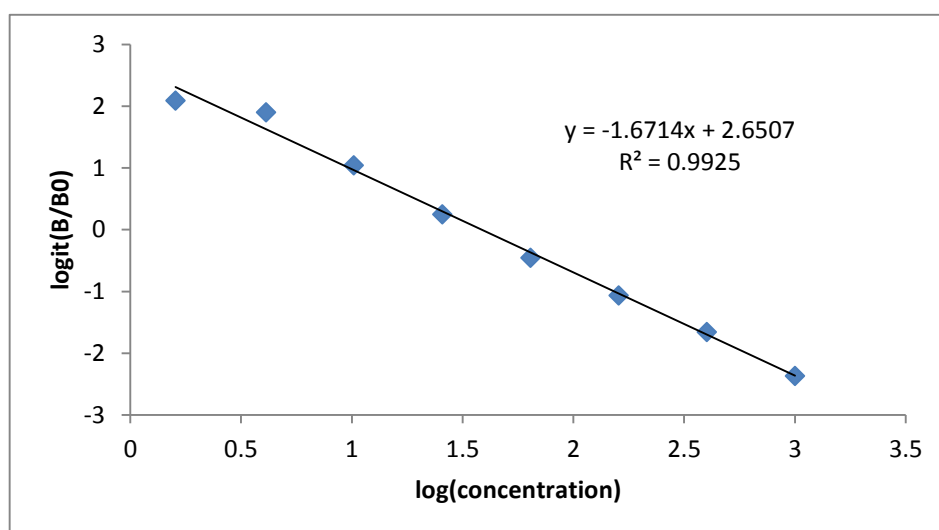


Figure A1.2. Calibration curve for PGD₂ metabolite assay.

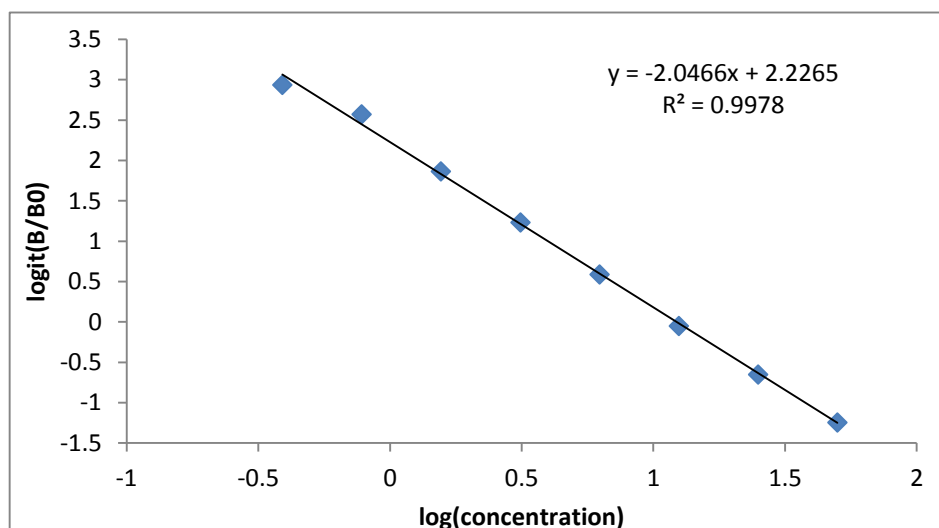


Figure A1.3. Calibration curve for PGE₂ metabolite assay.

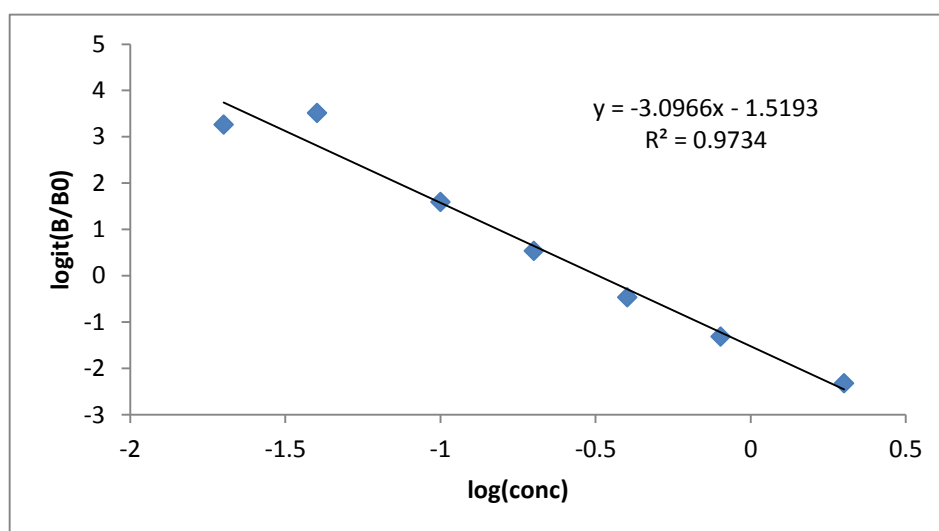


Figure A1.4. Calibration curve for LXA₄ ELISA.

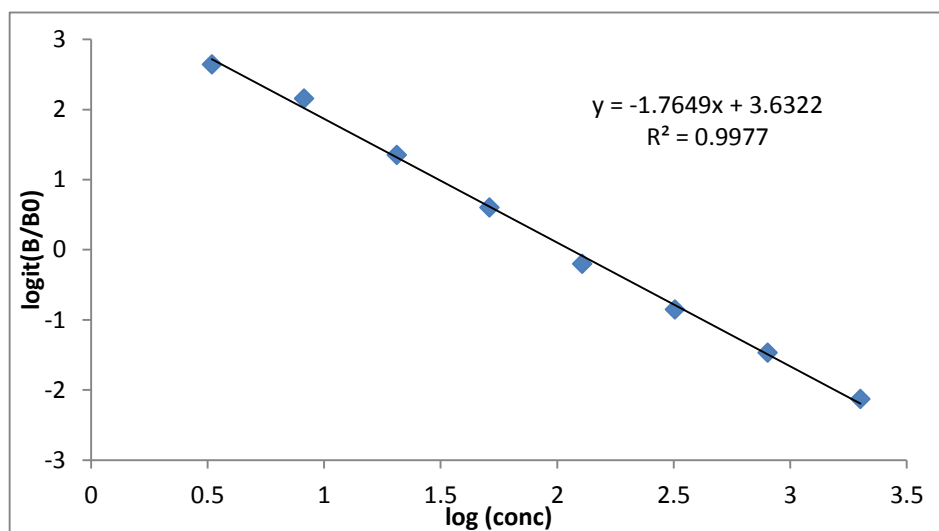


Figure A1.5. Calibration curve for RvD₁ ELISA.

Appendix 2: Clinical Study Documents

Study documents have been reproduced as figures in order to allow the documents to be accurately represented within the thesis format.

Study protocol

Assessment and Mechanism of the Refractory Period in Asthma

Chief Investigator: Prof Ian D Pavord, Dept of Respiratory Medicine, Glenfield Hospital, Leicester, LE3 9QP.

Co-investigator: Prof Peter Bradding, Dept of Respiratory Medicine, Glenfield Hospital, Leicester, LE3 9QP.

Sponsor: University Hospitals Leicester NHS Trust, Gwendolen House, Gwendolen Rd, Leicester, LE5 4QF.

Synopsis

Title	Assessment and Mechanism of the Refractory Period in Asthma.
Chief Investigator	Prof Ian D Pavord Department of Respiratory Medicine Glenfield Hospital Leicester, UK
Co-investigator	Prof Peter Bradding Department of Respiratory Medicine Glenfield Hospital Leicester, UK
Sub Investigator	Dr Andrew Ruddick Department of Respiratory Medicine Glenfield Hospital Leicester, UK
Sponsor	University Hospitals of Leicester NHS Trust
Objectives	<ul style="list-style-type: none"> How best to demonstrate the refractory period in asthma: <ul style="list-style-type: none"> Cumulative or bolus bronchoconstrictor challenge? Is clinically significant bronchoconstriction necessary? Are there common demographic and phenotypic characteristics in patients who demonstrate refractoriness? How do sputum and urine concentrations of inflammatory mediators change during the refractory period? Is an identifiable bronchoprotective factor involved?
Setting	Secondary Care

Number of participants	20 patients
Eligibility Criteria	<ul style="list-style-type: none"> • Aged over 18 • Non-smoking (ex smoker >1 year, <10 pack year history) • Diagnosis of Asthma • Treatment: SABA +/- low dose inhaled steroids (≤ 400 micrograms beclomethasone daily) only • Asthma symptoms well controlled (no previous asthma related hospital admissions, ≤ 1 steroid course in previous 1 year).
Duration of Study	Duration: 12 months Total duration for each patient: approximately 3 months

Introduction

Asthma is a major problem in terms of healthcare needs (1). In the UK 21% of children and 15% of adults have a diagnosis of asthma and asthma is the most common chronic disease in young males between the ages of 16-24 (2).

Asthma is characterised by an exaggerated bronchoconstrictor response to a variety of non-specific stimuli such as cold dry air, hyperosmotic challenge and exercise, and specific stimuli including methacholine and histamine. This leads to wheeze, breathlessness and cough (3). One interesting and poorly understood characteristic of the airway response to indirect stimuli in asthma is that the response diminishes on repeat challenge. This phenomenon is known as the refractory period. The refractory period lasts 1-3 hours; it occurs in up to 50% of subjects who experience exercise-induced bronchoconstriction and is particularly well recognised in subjects with mild asthma (4, 5). Studies have shown that subjects who are refractory to the effect of one indirect challenge, such as exercise, then also have a reduced response to a second stimulus such as metabisulphite (6). This is termed cross-refractoriness. However, the response to stimuli that cause bronchoconstriction by a direct effect on airway smooth muscle is retained. These findings suggest that the refractory period is due to a down regulation of a number of different bronchoconstrictor pathways proximal to airway smooth muscle contraction.

A number of mechanisms for the refractory period have been postulated but only the involvement of an endogenous bronchoprotective factor accounts for all the features outlined above (7). As the refractory period is abolished by the cyclo-oxygenase inhibitor indomethacin in either oral (8) or inhaled form (9) there have been suggestions that this bronchoprotective factor could be prostaglandin E2 (PGE₂) (7). In support of this, inhaled PGE₂ has been shown to inhibit the airway response to several bronchoconstrictor challenges (10). However, it remains possible that other factors are involved in the pathogenesis of the refractory period as the effect of cyclooxygenase inhibitors have been mixed (8, 11) and in vitro models of refractoriness have suggested involvement of a non-prostanoid, epithelium-derived factor (12). As a refractory period is particularly well recognised in mild asthma it may be that patients with milder disease are able to produce this bronchoprotective factor, but only in response to a significant initial stimulus whereas subjects with more significant disease have a more complete deficiency. If this is true, then the bronchoprotective factor may play a fundamental role in the pathogenesis of asthma.

We hypothesise that one group of compounds which may act as bronchoprotective agents are the resolving mediators of inflammation which include the resolvins, protectins, maresins and lipoxins (referred to collectively as resolving mediators for convenience). These are lipid mediators which are secreted in numerous tissues during resolution of inflammation (13). They are generated from arachidonic acid (Lipoxin A4 and B4), docosahexanoic acid (protectins D1-D4, resolvin D1-D4, maresin 1), and eicosapentanoic acid (resolvin E1) through biochemical synthesis involving the enzymes 5- and 15-lipoxygenase (5-LOX, 15-LOX) (13, 14). In addition, aspirin-triggered (AT) forms of these molecules exist (epi-lipoxin A4, AT-resolvins), where acetylated COX-2 generates the initial metabolite which is then modified further by 5-LOX. These can be formed endogenously in the absence of aspirin, possibly via a cytochrome P450-dependent pathway (15). The biosynthesis of these resolving mediators generally requires transcellular synthesis, with 15-lipoxygenase working in one cell type, and the product of this then being modified further by 5-LOX and further downstream enzymes in a further cell type (16). A key

feature of these molecules is that they promote resolution of inflammation, but are not innately immunosuppressive as they also activate anti-bacterial mechanisms (for example see ref 17). Examples of their pro-resolving activity include inhibition of eosinophil (18) and neutrophil (19) chemotaxis, stimulation of non-phlogistic phagocytosis of apoptotic neutrophils by macrophages (20), and inhibition of dendritic cell IL-12 production (21).

Lipoxin A4 is apparently down-regulated in asthma (22), and this impaired expression might therefore contribute to the chronic airway inflammatory response. Protectin D1 was also reduced in asthmatic exhaled breath in a very small study of patients with severe asthma (4 controls, 3 patients) (23). The source of these pro-resolving mediators in the airways is likely to involve both the airway epithelium and inflammatory airway cells. The airway epithelium is a rich source of 15-LOX (24), while airway eosinophils and mast cells express 5-LOX (25). In addition, eosinophils and cord blood-derived mast cells express 15-LOX (26), raising the possibility that uni-cellular synthesis might occur. Preliminary studies in vitro show that resolvins are potent and effective inhibitors of mast cell degranulation. Endogenous resolvins are known to be present in human plasma (27). Lipoxins have been given by inhalation in a study in humans with no ill effects, and were shown to reduce leukotriene C4-induced bronchoconstriction (28).

The refractory period remains an under researched phenomenon and there are many gaps in our knowledge. In particular, there remains uncertainty as to how best demonstrate refractoriness; whether refractoriness is dependent on the magnitude of the initial response to the first challenge; whether refractoriness is associated with a measurable change in release of bronchoconstrictor and potentially bronchoprotective mediators in induced sputum and urine; and whether the subjects who develop refractoriness can be more clearly defined. We plan to address these questions in a large and mixed population of subjects with asthma using inhaled mannitol as a bronchoconstrictor challenge. We have chosen this agent as it is commercially available, licensed for diagnostic testing in asthma and the response to the challenge

has been shown to be refractory. Our hypothesis is that refractoriness is best demonstrated by repeated single dose challenges of inhaled mannitol (as refractoriness may begin to develop during administration of a cumulative dose), is independent of the magnitude of the initial response, is associated with reduced production of constrictor mediators and increased production of protective mediators, and is seen particularly in patients with mild asthma. Our specific aims are:

1. To compare the refractory period induced by repeated challenge with a single dose previously shown to result in a 15% fall in FEV₁ (the PD15 dose) with that induced by repeated cumulative dose challenges. This is illustrated in Figure 1. We will quantify the refractory period as a refractory index (RI), determined as the change in the maximum FEV₁ reduction between the two challenges expressed as a percentage of the maximum FEV₁ reduction from the first challenge; using the nomenclature of Figure 1, $RI = (a - b)/a \times 100$.

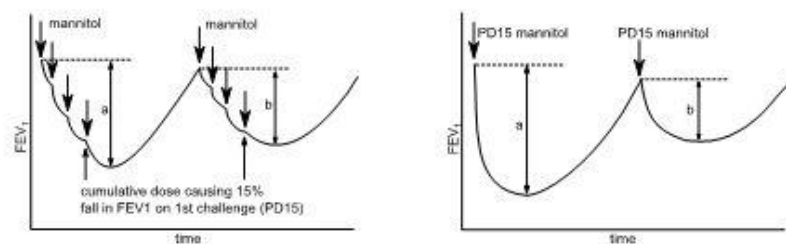


Figure 1. Cumulative dose versus single dose mannitol challenge.

2. To determine whether provocation of significant bronchoconstriction is necessary to induce a refractory period in mild asthma. This will be done by comparing the response during the second of two PD15 single dose mannitol challenges (*b* as shown in Figure 1) to the response following a PD15 dose challenge after prior PD5 challenge (dose causing 5% fall in FEV₁) *c* as shown in Figure 2.

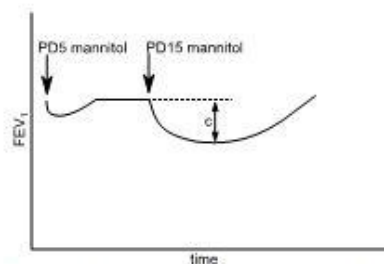


Figure 2. Single dose PD15 mannitol challenge following PD5 mannitol challenge.

3. To show that there are common demographic and phenotypic characteristics in the patient group with mild asthma who demonstrate refractoriness. It is our hypothesis that refractoriness will be negatively correlated with levels of airway inflammatory markers and sputum eosinophilia, and positively correlated with steroid use.

4. To determine levels of inflammatory mediators (namely sputum ~~cysteinyl-leukotrienes~~, histamine, PGD2 and urinary PGD2 metabolites) and potential bronchoprotective factors (e.g. PGE2, ~~resolvins~~, lipoxins) in our patient group to increase understanding of the mechanisms underlying the refractory period.

Study design

All subjects will receive oral and written information about the nature, purpose, possible risk and benefit of the study. Subjects will also be notified that they are free to withdraw from the study at any time. Potential subjects will be given the opportunity to ask questions and allowed time (>24 hours) to consider the information provided. The subject's signed and dated consent will be obtained before conducting any procedure for the study. The investigative centre will store the original, signed consent form. A copy of the signed consent form must be given to the subject.

Recruitment

Recruitment will be by invitation, based on the presence of mild asthma controlled by short-acting bronchodilators with or without low dose inhaled steroids. The subjects will be recruited mainly from newspaper advertising and posters placed in public places (see enclosed). We will also invite well characterised patients already known to the clinical study team.

We aim to recruit 20 patients to the study. There is a scarcity of data on which to base a sample size calculation. However, analysis of a previous study of 13 patients with asthma, in which RI was measured on two separate occasions, found a mean RI of 53%, with an intra-subject standard deviation of 13.3. In the current study, in each subject we will compare RI measured using cumulative and single dose mannitol tests. We intend to use a paired t-test for this purpose. A sample size calculation based on this method with the above standard deviation gives a value of 19 subjects necessary to demonstrate a mean absolute difference in RI of 10%, with $\alpha = 0.05$ and $\beta = 0.1$. (Sample size calculated using the equation $n = ((z_{\alpha} + z_{\beta}) s / \Delta)^2$, where n = sample size, z_{α} and z_{β} are z-scores corresponding to α and β errors of 0.05 and 0.1 respectively, s is the standard deviation of 13.3 and $\Delta = 10$, the target difference in mean RI between the two groups of measurements).

The total study duration will be 12 months, though individuals are expected to be in the study for 3 months or less. Inclusion and exclusion criteria are:

Inclusion

- Age 18 or over
- Diagnosis of asthma
- Treatment with short-acting beta agonist +/- low dose steroids (400 mcg per day beclometasone or equivalent)
- Symptoms well controlled on treatment (never hospitalised with asthma, no more than one course of oral steroids in the past 12 months)
- Non-smoking (ex smoker >1 year, <10 pack year history)

Exclusion

- Major co-morbidity
- Pregnancy
- Previous hypersensitivity to ~~methacholine~~ or ~~mannitol~~

Withdrawal

- If subjects develop any of the exclusion criteria during the trial
- Subject requests withdrawal
- Discretion of the investigator

Serious adverse events will be followed up. If a subject ceases to participate in the study then his/her enrolment number cannot be issued to another subject. If a subject withdraws consent to the use of donated biological samples then the samples will be disposed of or destroyed, if not already analysed and documented. In the event that analysis has already been performed the results will be retained but no further analysis will be performed and the sample will be disposed of.

Study visits

Subjects will make a screening visit followed, if recruited to the study, by four further visits. The visits are described below and summarised in the flow chart in Figure 3. Informed consent will be obtained at the screening visit.

The volunteer will receive the information and consent form at least 24 hours before the screening visit. If the investigator or their delegate is satisfied that the volunteer has read and understood these and if the volunteer gives their written consent, they will then be assigned a unique volunteer number and their details entered into an enrolment log.

The screening visit will include medical history and brief physical examination, baseline spirometry, a pregnancy test and a challenge with ~~methacholine~~ to establish

that the subject demonstrates airway hyper-responsiveness. If recruited to the study, volunteers will be advised of the need to withhold their short-acting beta agonist for 12 hours prior to the next visit (Visit 2). It will be emphasised that they should not withhold medication if they feel that it is essential for symptom control. All other medication should be continued as usual.

At Visit 2, initial assessment will include measurement of height and weight and a detailed questionnaire on level of physical activity, respiratory symptoms, asthma treatment and asthma control using the validated Juniper Asthma Control Questionnaire (ACQ) (29). Induced sputum and urine will be obtained to determine a baseline for cell and molecular markers of airway inflammation and potential bronchoprotective factors.

On the third visit they will receive a cumulative dose of inhaled mannitol until a 15% reduction in FEV₁ is achieved (the PD15 dose). This cumulative dose will then be repeated once FEV₁ is >95% of the original baseline, and further FEV₁ measurements made to determine if the subject is now refractory. Induced sputum and urine will be obtained following this to determine cell and molecular markers of airway inflammation and potential bronchoprotective factors. If a subject does not demonstrate a 15% FEV₁ reduction at the maximum cumulative mannitol dose of 635 mg then they will not proceed to the second challenge.

On the fourth visit the subject will receive the PD15 dose of mannitol as a single bolus. FEV₁ will be measured 2, 5, 7, 10, 15, 20 and 30 minutes after. This bolus dose will then be repeated after 30 minutes or when FEV₁ is >95% of the original baseline and FEV₁ measured at the same times. Induced sputum and urine will again be obtained. Those subjects at visit 3 who did not develop a 15% fall in FEV₁ may have developed refractoriness during the challenge procedure, before a 15% fall was achieved. These subjects will receive a single dose of 635 mg mannitol at visit 4 to assess whether a single dose is able to induce a greater fall in FEV₁ than cumulative challenge.

On the fifth visit the subject will receive a single dose of mannitol previously shown in visit 3 to cause a 5% reduction in FEV₁. This dose does not correlate to significant bronchoconstriction. A single PD15 dose of mannitol will be given after 30 minutes to determine if the subject has become refractory. Induced sputum and urine will again be obtained.

Sputum samples from the baseline visit will be analysed for sputum eosinophilia, a marker of atopic airway inflammation, in the local research laboratory at the Glenfield Hospital, Leicester. Sputum supernatant will be retained to test for markers of inflammation and possible bronchoprotective factors at a later stage.

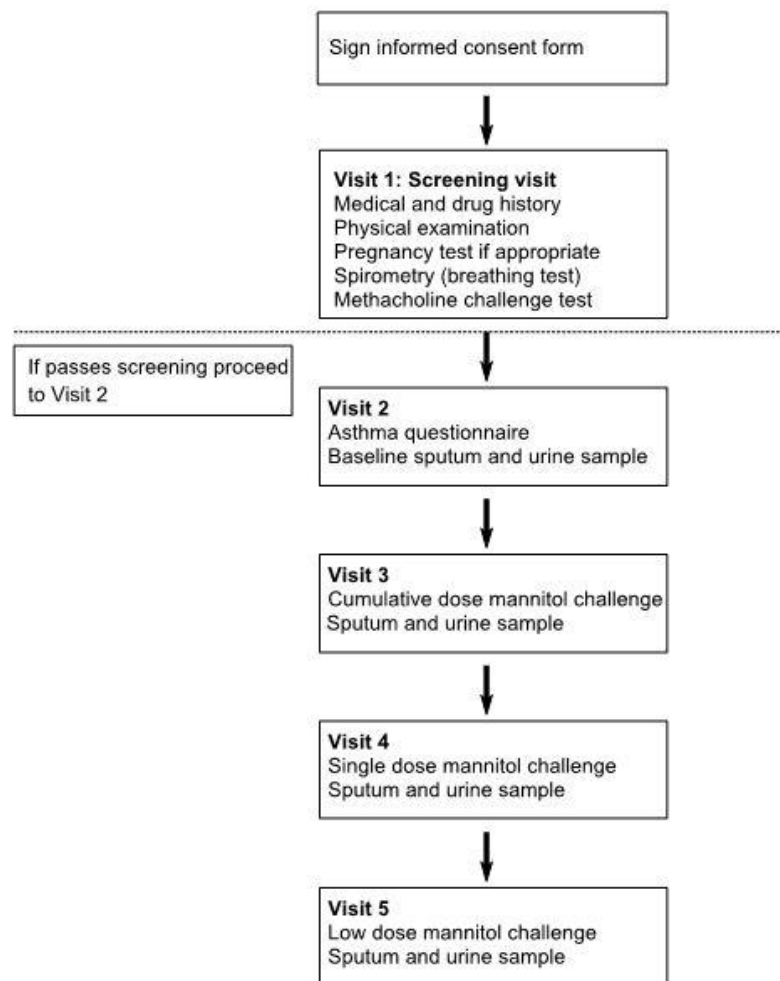


Figure 3. Summary flow chart of study visits.

Details of protocol tests

Spirometry

Forced expiratory volume in one second and forced vital capacity will be recorded in all subjects, with the best value of attempts being taken. Results will also be expressed as percentage of predicted. FEV₁ will be measured at 2, 5, 7, 10, 15, 20 and 30 minutes after each challenge test.

Methacholine Challenge Test

Methacholine challenge test will be performed using a tidal breathing method. Methacholine will be delivered using a Wrights nebuliser in doubling concentrations from 0.03 mg/ml – 16 mg/ml or until at least a 20% decrease in FEV₁ compared with baseline is reached. Full details are given in reference 30.

Mannitol challenge test

Bronchial provocation to mannitol will be performed using a dry powder preparation contained in capsules and administered from an Osmohaler. For cumulative dose testing mannitol will be administered up to a cumulative dose of 635mg or until a 15% decrease in FEV₁ compared with baseline is reached. Full details are given in reference 31. For bolus dosing, the dose previously shown to give a 15% FEV₁ decrease will be used.

Induced sputum

Sputum induction will follow a standardised technique compliant with the European Respiratory Society guidelines, using pre-treatment with inhaled bronchodilator to offset the potential constrictive effect of nebulised hypertonic saline. It will use an ultrasonic nebuliser with 3-5% hypertonic saline and be performed under strict infection control procedures. Full details are given in reference 32.

Urine collection

The subject will be asked to provide a specimen of urine into a suitable container.

Recording of adverse events

Only serious adverse events related to study procedures will be recorded in the volunteers' notes. An assessment of causality of the adverse event should be performed.

Data storage & protection

Study data for the patients will be stored electronically and/or on paper and information on this will be filed in the study master file.

The informed consent document will incorporate wording that complies with relevant data protection and privacy legislation. Pursuant to this wording, volunteers will authorise the collection, use and disclosure of their study data by the investigator and by those persons who need it for the purposes of the study.

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Patient Information Leaflet

Patient Information Sheet & Consent Form

Title: Assessment and Mechanism of the Refractory Period in Asthma.

Chief Investigator: Prof Ian D Pavord
Study Co-ordinator: Dr Andrew Ruddick

This research is being conducted as part of a Doctor of Medicine degree.

Dr Ruddick can be contacted by telephone on 0115 2583034 or via email on andrew.ruddick@uhl-tr.nhs.uk.

Invitation

You are being invited to take part in a research study which is attempting to better understand what happens in the airways of people with asthma. Before you decide to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you want to take part.

What is the purpose of the study?

In people with asthma their airways narrow in response to a variety of things (known as stimuli) which include cold air, exercise and allergy-triggering substances such as pollen. This leads to the symptoms of wheeze, breathlessness and cough. One poorly understood aspect of this airway-narrowing response to these stimuli is that the response is smaller if the stimulus is given again after a short period of time, i.e. after a second stimulus the airways do not narrow, or narrow less than previously. This period of reduced airway response is known as the refractory period. The refractory period lasts 1-3 hours, and occurs in up to 50% of people who experience exercise-related asthma. Our theory is that a substance that protects the airway from narrowing is released in the airway during the first stimulus which then prevents triggering of airway muscle contraction following a second stimulus.

The refractory period is poorly understood and there are many gaps in our knowledge. In particular, there is uncertainty about how best to measure the amount of refractoriness; whether refractoriness depends on the size of the response to the first stimulus; whether refractoriness is associated with a change in the release of airway narrowing and airway protecting substances in sputum and urine; and whether the group of individuals who develop refractoriness share other features in common. We hope to address these questions in people with mild asthma using inhaled mannitol, a substance which can cause airway narrowing in people with asthma.

What will happen to me if I take part?

If you consent to taking part in the study you will need to make five visits of up to 2 hours each to the hospital where the tests will be carried out. The timing of the visits is flexible to suit the schedule of the volunteer, but they would typically be completed over a period of 2 weeks - 2 months. Each visit should ideally be made at the same time of day.

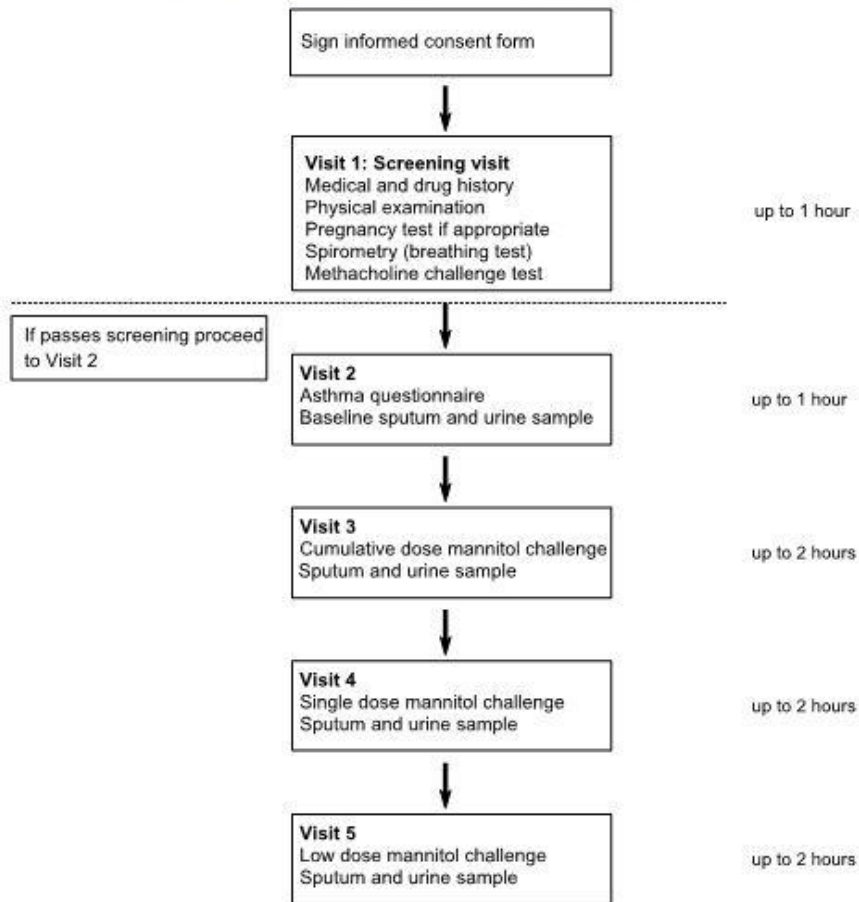
Each visit and test will assess a different aspect of your lung function and its response to inhaling mannitol, and collect samples to assess the underlying inflammation in your airway. The tests performed on each visit are summarised in the flow chart on the next page, with an indication of the likely length of each visit. An explanation of the different tests is given below the flow chart.

Included in the visits are a pregnancy test for women of child-bearing age and methacholine and mannitol challenge tests. The pregnancy test is included because pregnancy sometimes affects an individual's control of their asthma and might confuse the results of the study. The methacholine and mannitol challenge tests involving inhaling an airway irritant and are described in more detail in the section *About the tests* below, but both are used routinely for the diagnosis of asthma and can cause temporary face flushing and mild headache and symptoms (wheezing, chest tightness or cough) similar to those of a mild asthma attack. The methacholine challenge test in Visit 1 is used as a screening test to see if you are suitable for the study, i.e. to see if your airways narrow in response to an irritant. There are several different mannitol challenge tests used in Visits 3 - 5 to investigate the refractory period described above in the *"what is the purpose of this study?"* section.

You will need to avoid using your short-acting bronchodilator (reliever) inhaler for 12 hours prior to each visit. This is because the effects of the inhaler could affect the results of the study. **You should not omit your inhaler if at any time you feel that you really need it to control your symptoms-** your visit could be re-scheduled if necessary.

Flow Chart of the Study

Please see the text below the chart for a full explanation of the various tests.



About the tests:

Physical Examination

The study doctor will feel your pulse and listen to the front and back of your chest with a stethoscope.

Spirometry (breathing tests)

Lung function tests that will measure how well your lungs are working by asking you to blow at different times into a machine. This will be done at the screening visit and repeated throughout the methacholine and mannitol challenge tests. The test is not painful but can sometimes cause some temporary light-headedness and coughing.

Urine tests

You will be asked to provide a urine sample during most of your visits. This will be used for analysis of markers of airway inflammation. In Visit 1 a urine sample may be used for pregnancy testing in some female participants.

Sputum induction and collection

A test that enables you to produce sputum for us to analyse and see how many inflammation-causing cells it contains, and carry out tests for substances which are associated with either inducing or reducing airway inflammation. You will be asked to breathe in a mist of salty water and cough up any secretions at any time during the procedure. This may cause some chest tightness, wheezing and/or cough. These are all readily reversed by inhaling a bronchodilator (salbutamol).

Methacholine challenge test

A test used in Visit 1 to determine how sensitive your airways are to becoming narrowed. This will be used as a screening test to see if you are suitable for the study. You will be asked to inhale a mist containing increasing concentrations of an airway irritant called methacholine. This substance causes the airways to become narrowed in people with asthma and the concentration of methacholine that is needed to cause a certain amount of airway narrowing is used as a measure of how twitchy your air passages are. The amount of airway narrowing will be assessed by regular spirometry (breathing tests) during the procedure. This test is routinely used to diagnose asthma. Inhaling methacholine can cause temporary face flushing and mild headache and can cause symptoms (wheezing, chest tightness or cough) similar to those of a mild asthma attack.

Cumulative mannitol challenge

Mannitol is another airway irritant used to assess the sensitivity of your airways; it works through a different pathway to methacholine. You will receive a gradually increasing dose of inhaled mannitol from a handheld inhaler until your FEV1 (a measure of airway narrowing made during spirometry) is reduced by 15%. This cumulative dose will then be repeated once your FEV1 is back to 95% of your baseline, and further FEV1 measurements will be made to see if you are now refractory to the challenge. Mannitol inhalation can cause similar side-effects to methacholine.

Single dose mannitol challenge

During this test in Visit 4 you will be given the amount of mannitol which caused a 15% FEV1 drop on your previous visit, this time as one single dose. FEV1 will be monitored and the mannitol dose repeated when FEV1 is >95% of your baseline. Further FEV1 measurements will be made to see if you are refractory to this repeat challenge. Again, side-effects similar to those listed for methacholine can occur.

Low dose mannitol challenge

During this test in Visit 5 you will receive a single dose of mannitol previously shown to cause just a 5% reduction in your FEV1. The dose of mannitol which causes a 15% FEV1 drop will then be given after 30 minutes to see if the low dose initial challenge made you refractory. Side-effects such as those described for methacholine can occur, but will be very mild at the low dose.

Why have I been chosen?

You have been chosen because you have mild asthma controlled with short-acting bronchodilators +/- low dose inhaled steroids, and are otherwise in good health.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. You may withdraw your permission to the use of your data and samples at any time. If you withdraw your permission **before** your tissue samples and data are used, we will not use the data and the samples will be destroyed. If you withdraw your consent **after** your tissue sample has been sent for analysis we will ensure that your samples are destroyed, but we will need to use the data collected up to your withdrawal. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What do I have to do?

As a participant in this study, you would need to attend for all of your scheduled visits. You will need to avoid using your short-acting bronchodilator (reliever) inhaler for 12 hours prior to each visit. **You should not omit your inhaler if at any time you feel that you really need it to control your symptoms**- your visit could be re-scheduled if necessary. Steroid inhalers should be continued as usual, as should all your other regular medication. There are no restrictions on your lifestyle.

Women of child bearing age who could potentially be pregnant will be asked to have a pregnancy test before taking part to exclude the possibility of pregnancy. Pregnancy sometimes affects an individual's control of their asthma and might confuse the results of the study. We would ask participants to inform us immediately if they find out they have become pregnant; we might not be able to include their data in the final analysis.

What will happen to any samples I give?

Sputum and urine samples will be tested throughout the study at the Glenfield Hospital, Leicester. Any samples you give will be labelled with a unique number that will allow us to work out that the samples came from you but will ensure that no one outside the research group can identify you. With your consent, at the end of the study any unused samples will have their cells removed and be stored as "supernatant" (this just means the liquid portion of a sample without any cells). These samples may then, if appropriate, be used in other studies of lung disease conducted within the Institute for Lung Health at Glenfield Hospital, but would not be sold or

donated to any other organisation. The samples will in any case be destroyed within 5 years of completion of the study.

Will any genetic tests be done?

No

What are the alternatives for diagnosis or treatment?

If it appears that the treatment you are on for your asthma is no longer appropriate we would write to your GP suggesting an alternative.

What are the possible disadvantages and risks of taking part?

The effects of the different tests you will undergo are discussed above.

We have taken reasonable steps to protect your research information. This is done to reduce the potential for harm to you from an unintended disclosure of information resulting from research on your body substance.

What are the possible benefits of taking part?

There are no direct benefits to you during this study; however, your participation will contribute to research into asthma and the information that we get from this study may help us to improve treatment of asthma in the future.

What will happen when the research study stops?

During and after the study you will continue on your regular medication.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the disease that is being studied. If this happens, your research doctor will inform you of this and discuss whether you wish to continue in the study. If you decide to withdraw, your research doctor will make arrangements for your care to continue. If you decide to continue in the study, you may be asked to sign an updated consent form.

Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue.

What if something goes wrong?

In the unlikely event of you being harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns

about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms would be available to you. The telephone number for the Trust's Patient Advice and Liaison Service (PALS) is 08081788337.

Will my taking part in this study be kept confidential?

Yes, all information that is collected about you during the course of the research will be kept strictly confidential. If you agree to participate in this study, we will seek your permission to notify your GP and other doctors who are routinely involved in your medical care.

During the study, your sample and medical information will be labelled ("coded") with your participant number, not your name. Only your study doctor and some members of the clinical study team directly involved with the study will be able to link your subject number to your name. Your medical information and any results will be stored on paper and on a computer electronic database. These records will be kept separate from your medical records. Your name will not be in any publications or reports about this research. You have the right to ask your study doctor about the data being collected on you for the study and about the purpose of this data. You have the right to ask your study doctor to allow you to see your personal information and to have any needed corrections of it, made.

What will happen to the results of the research study?

We expect to present the results of the study at National and International meetings and the results will be formally published in a medical journal. You will not be personally identified in any report/publication. You will be given any information about publications and presentations when it becomes available.

Who is organising and funding the research?

The Institute for Lung Health – a department of the University of Leicester - is funding the study. The study has been organised by Professor Ian Pavord, a consultant physician at Glenfield Hospital. No member of the clinical study team will receive any payments above their salary for conducting this study.

Who has reviewed the study?

All research that involves NHS patients or staff, information from NHS medical records or uses NHS premises or facilities must be approved by an NHS Research Ethics Committee before it goes ahead.

The East Midlands Research Ethics Committee and the Adult Respiratory Medicine Programme Board of the University Hospitals of Leicester NHS Trust have reviewed this study. Approval does not absolutely guarantee that you will not come to any harm if you take part. However, approval means that the Committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and

balanced against possible benefits and that you have been given sufficient information on which to make an informed decision to take part or not.

Expenses and Payments

Travel expenses and car park charges can be reimbursed.

Contact for Further Information

If you have any questions at all about this study please do not hesitate to contact the following member of the clinical study team:

Dr Andrew Ruddick – Study co-ordinator

Tel: 0116 2583034 e-mail: andrew.ruddick@uhl-tr.nhs.uk

Research Nurses

Tel: 0116 258 3119

Thank you again for reading this information leaflet.

If you offer your consent for participation in this study, you will be given a copy of this information sheet and a signed consent form for this study to keep.

Centre: Glenfield Hospital

Glenfield Hospital
Groby Road
Leicester
LE3 9QP

Patient Identification Number for this trial:

Tel: 0300 303 1573
Fax: 0116 258 3950
Minicom: 0116 287 9852

CONSENT FORM

Title of Project: Assessment and Mechanism of the Refractory Period in Asthma.

Chief Investigator: Prof Ian D Pavord

Please initial box

1. I confirm that I have read and understand the information sheet dated 21-Nov-11. Version 5 for the above study and have had the opportunity to ask questions. ☐
 2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
 3. I understand that relevant sections of my medical notes and/or data may be looked at by responsible individuals from the study team, the sponsor, NHS Trust, Research Ethics Committee or from regulatory authorities where it is relevant to my taking part in the research. I give permission for these individuals to access my records. ☐
 4. I agree to my GP being informed of my participation in the study. ☐
 5. I understand that I am consenting for samples of my sputum and urine to be used in research along with coded study data by Glenfield Hospital. ☐
 6. I agree that cell-free sputum and urine samples may be retained for up to 5 years and if appropriate used in other studies of lung disease within the Institute for Lung Health at Glenfield Hospital, but not donated or sold to any other organisation. ☐ ☐
- Yes No
7. I agree to take part in the above study. ☐

Name of Patient Date Signature


Name of Person taking consent
(if different from researcher) Date Signature

Researcher Date Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

The Refractory Period in Asthma. Patient Information Sheet and Consent Form. Version 5, 21/11/2011

Invitation letter

University Hospitals of Leicester 
NHS Trust

Glenfield Hospital
Groby Road
Leicester
LE3 9QP

Tel: 0300 303 1573
Fax: 0116 258 3950
Minicom: 0116 287 9852

Date:

To:

Dear

Study title: Assessment and Mechanism of the Refractory Period in Asthma.

A research study is being carried out at the Glenfield Hospital by Prof Ian Pavord.

The study has been designed to further our understanding of the abnormalities which occur in the airways of people with asthma.

If you would like to take part in this study, details of which are given on the information leaflet enclosed, please complete the reply slip enclosed with this letter and return it in the pre-paid envelope. A member of the clinical study team will then contact you to arrange a convenient time to answer any questions and if appropriate invite you to a screening visit.

I would like to thank you for taking time to read this letter and hope to hear from you soon. If you have any queries, please feel free to contact me on the telephone number or email address below.

Yours sincerely

Dr Andrew Ruddick (study co-ordinator)
0116 2583034
andrew.ruddick@uhl-tr.nhs.uk

or

Research Nurses
0116 258 3119

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Yours sincerely

Dr Andrew Ruddick (study co-ordinator)
0116 2583034
andrew.ruddick@uhl-tr.nhs.uk


OR

Research Nurses
0116 258 3119

Recruitment Poster

University Hospitals of Leicester NHS Trust 

Wanted: Volunteers for Asthma Research



- Have you been diagnosed with asthma?
- Are your symptoms well controlled with a reliever inhaler or low dose steroid inhaler?
- Are you over 18 years of age?

If you can answer yes to the above questions you may be able to help with a research study here at Glenfield Hospital.

Some people with asthma are able to protect themselves against airway narrowing for a time following an initial asthma attack. They probably do this by releasing a natural protective substance within their airway. This study aims to assess the best way of measuring this protective effect and to look for the substance responsible.

If you would like to find out more, or volunteer for involvement then contact:

Dr Andrew Ruddick, Glenfield Hospital, Leicester
andrew.ruddick@uhl-tr.nhs.uk
Telephone: 0116 258 3034

Research Nurses
Telephone: 0116 258 3119

The Refractory Period in Asthma.Version 3, 25th July 2011.Advertisement Poster

GP Letter

<p>I</p> <p><i>GP name</i></p> <p><i>GP address</i></p> <p><i>Date</i></p> <p>Dear <i>(name of GP)</i></p> <p>Re: <i>(name and DOB of patient)</i></p> <p>Assessment and Mechanism of the Refractory Period in Asthma.</p> <p>I am writing to inform you that <i>(patient name)</i> is taking part in a research study. The research is concerned with understanding mechanisms underlying the refractory period in asthma. This is a phenomenon, particularly noted in exercise-related asthma, whereby an initial bronchoconstrictor challenge renders an asthmatic individual refractory to a subsequent challenge for a short period. The study does not involve any change to the patient's usual medication other than the need to refrain from using their short-acting beta agonist for a short period prior to each visit. A copy of the patient information sheet is enclosed for your information.</p> <p>Please contact us on 0116 2583034 if you would like any further information about this study.</p> <p>Yours sincerely,</p> <p>Prof Ian Pavord Consultant in Respiratory Medicine</p>	<p>University Hospitals of Leicester NHS Trust</p> <p>Glenfield Hospital Groby Road Leicester LE3 9QP</p> <p>Tel: 0300 303 1573 Fax: 0116 258 3950 Minicom: 0116 237 9652</p>
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The Refractory Period in Asthma. Version 2, 25th July 2011. GP Letter

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