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STUDIES OF EXERCISE-INDUCED BRONCHOCONSTRICTION TO DEFINE PROTECTIVE MECHANISMS IN ASTHMA

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Exercise-induced bronchoconstriction (EIB) occurs in the majority of asthmatics following vigorous exercise. EIB is caused by a loss of water from the airways creating a hyperosmolar environment in the tissue that in turn triggers the release of bronchoconstrictive mediators. These bronchoconstrictive mediators, including histamine, cysteinyl leukotrienes (CysLTs) and prostaglandins, act at their respective receptors on the airway smooth muscle to induce bronchoconstriction. Because the mechanism of EIB involves drying of the airways, provocations mimicking this drying, such as eucapnic voluntary hyperpnea (EVH) and mannitol inhalation can be used to study EIB. With repeated challenge, a smaller response is observed following the second challenge and this decreased responsiveness is called refractoriness. Defining the mechanism of refractoriness may lead to new treatments for asthma.

In this thesis a range of airway challenges were performed to study the urinary excretion of mediators released in the lung following EIB and the effect of different interventions. Furthermore, urinary mediator excretion during refractoriness was also studied.

For the first time we demonstrated urinary excretion of CysLT and Prostaglandin D_2 metabolites after EVH. Mediator release was no different in subjects who did not experience bronchoconstriction following EVH compared to those who did react with bronchoconstriction. This indicates that a necessity of EIB is for the airways to be sensitive to the mediators released. Pre-treatment with the mast cell stabilising drug sodium cromoglycate (SCG) inhibited the airway response to EVH, and the inhibition was accompanied by a decreased release of mediators into the urine. The same effects were observed following pre-treatment with a single high dose of inhaled corticosteroid (ICS). Pre-treatment with fish oil, rich in omega-3 fatty acids, had no effect on the basal excretion of urinary mediators, or airway responsiveness to mannitol challenge.

We also report the novel finding of refractoriness following repeated mannitol challenge. Mast cell mediators were excreted into the urine to the same extent after both the first challenge and the repeated challenge 90 min later. Also, those that were most refractory displayed the highest mediator release. This contradicts depletion of mediator release at the time of the second challenge as being the mechanism of refractoriness. These findings were then replicated by repeated EVH challenge. We also demonstrate for the first time an extended spectrum of urinary mediators excreted following EVH. Increased levels of the bronchoprotective mediators PGE_2 and PGI_2 were seen, which supports the release of protective prostaglandins as being a mechanism of refractoriness.

In summary, this thesis provides evidence that the mechanism of refractoriness does not involve mediator depletion. Rather, it indicates that there is a decreased sensitivity at the level of the airway smooth muscle to the mediators released. The induction of this decreased sensitivity may include the release of PGE_2 and PGI_2, which are likely to mediate protective responses.

LIST OF SCIENTIFIC PAPERS

Published as Johan Larsson until early 2013, and thereafter as Johan Bood

I. **Larsson J**, Perry CP, Anderson SD, Brannan JD, Dahlen S-E, Dahlen B. The occurrence of refractoriness and mast cell mediator release following mannitol-induced bronchoconstriction. Journal of Applied Physiology. 2011 Apr;110(4):1029-35.

II. Kippelen P, **Larsson J**, Anderson SD, Brannan JD, Delin I, Dahlén B, Dahlén S-E. Effects of Beclomethasone on Hyperpnoea-Induced Bronchoconstriction. Medicine and Science in Sports and Exercise. 2010;42(2):273-80.

III. Kippelen P, **Larsson J**, Anderson SD, Brannan JD, Dahlen B, Dahlen S-E. Effect of sodium cromoglycate on mast cell mediators during hyperpnea in athletes. Medicine and Science in Sports and Exercise. 2010;42(10):1853-60.

IV. **Bood J**, Sundblad BM, Delin I, Sjödin M, Larsson K, Anderson SD, Wheelock CE, Dahlén S-E, Dahlén B. Urinary excretion of lipid mediators in response to repeated eucapnic voluntary hyperpnea in asthmatics. *Submitted*

V. Brannan JD, **Bood J**, Alkhabaz A, Balgoma D, Otis J, Delin I, Dahlén B, Wheelock CE, Nair P, Dahlén S-E, O'Byrne PM. The effect of omega-3 fatty acids on bronchial hyperresponsiveness, sputum eosinophilia, and mast cell mediators in asthma. Chest. 2015;147(2):397-405

Work not included in the thesis:
- Balgoma D, **Larsson J**, Rokach J, Lawson JA, Daham K, Dahlén B, Dahlén S-E, Wheelock CE. Quantification of lipid mediator metabolites in human urine from asthma patients by electrospray ionization mass spectrometry: controlling matrix effects. Analytical Chemistry. 2013;85(16):7866-74
- **Larsson J**, Anderson SD, Dahlén S-E, Dahlén D. Refractoriness to exercise challenge: A review of the mechanisms old and new. Immunology and Allergy Clinics of North America. 2013;33(3):329-45
- Hallstrand TS, Kippelen P, **Larsson J**, Bougault V, van Leeuwen J, Driessen JM, Brannan JD. Where to go from here for EIB; the unanswered questions. Immunology and Allergy Clinics of North America. 2013 Aug;33(3):423-42.
- Säfholm J, Manson ML, **Bood J**, Delin I, Orre A-C, Bergman P, Al-Ameri M, Dahlén S-E, Adner M. Prostaglandin E_2 inhibits mast cell-dependent bronchoconstriction in human small airways via the EP_2 receptor. Journal of Allergy and Clinical Immunology in press and on-line

CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ATS	American Thoracic Society
COX	Cyclooxygenase
CysLT	Cysteinyl leukotriene
$CysLT_1$	Cysteinyl leukotriene receptor 1
EIA	Enzyme immunoassay
EIB	Exercise-induced Bronchoconstriction
EP_{1-4}	Prostaglandin E Receptor 1-4
ERS	European Respiratory Society
EVH	Eucapnic voluntary hyperpnea
FDA	Foods and Drug Administration
FEV_1	Forced expiratory volume in 1 second
FLAP	5-lipoxygenase activating protein
FVC	Forced vital capacity
GPCR	G-protein coupled receptor
IgE	Immunoglobulin E
IL	Interleukin
LT	Leukotrienes
5-LOX	5-lipoxygenase
NSAID	Non-steroidal anti-inflammatory drugs
ω-3	Omega-3 fatty acids
PG	Prostaglandin
PLA_2	Phospholipase A_2
UPLC-MS/MS	Ultra-performance liquid chromatography tandem mass spectrometry
TX	Thromboxane

1 INTRODUCTION

Asthma is a common disease occurring in about 10% of the population worldwide. It is a disease of the airways characterised by a variable degree of airway obstruction, airway inflammation and airway hyperresponsiveness. The most common symptoms of asthma are wheezing, cough, shortness of breath and chest tightness. Even though the disease was first described centuries ago, the pathogenesis is still not fully understood, and lack of treatment options remain a problem for many patients with asthma.

An attack of asthma can be triggered by a wide range of different stimuli ranging from common cold or other respiratory tract infections to environmental factors such as allergens or exposure to cold dry air. The association between exercise and asthma is known since long ago and was first described by Areatus in the first century. Areatus stated "if from running, gymnastics exercise or any other work the breathing becomes difficult, it is called asthma" (Adams 1856). More recently, studies have shown that the majority of untreated asthmatics will experience bronchoconstriction after exercise (Anderson et al. 1975; Godfrey et al. 1991; Cabral et al. 1999). The prevalence of this effect in patients with asthma, receiving treatment with inhaled corticosteroids, is about 50% (Waalkens et al. 1993; Anderson and Daviskas 2000; Weiler et al. 2005). Bronchoconstriction upon exercise confirms a diagnosis of asthma and an inhibition of the bronchoconstrictor response by a drug is a recognised indication for treatment (FDA Guidance for Industry, http://www.fda.gov./cder/guidance)

Interestingly, when the exercise challenge is repeated within 2-4 h, about 50% of asthmatics will demonstrate a bronchoconstrictor response that is less than half of that seen after the initial challenge (Anderson et al. 1979; Schoeffel et al. 1980). This decreased responsiveness to challenge is called refractoriness and its duration is usually called the refractory period.

The mechanism underlying refractoriness has been studied since it was first discovered, however it remains largely unclear. Identification of this physiological protective mechanism is important since it may lead to the discovery of new targets for treatment, and ultimately aid the development of new drugs to treat asthma.

The aim of my doctoral studies was to further elucidate the mechanisms of refractoriness and better understand mast cell mediator release in exercise-induced bronchoconstriction (EIB). We found evidence to suggest that refractoriness is not caused by depletion of bronchoconstrictive mediators, which has previously been a dominating theory. We also demonstrated for the first time that release of the bronchoprotective mediators PGE_2 and PGI_2 could play a role in the development of refractoriness.

2 AIMS OF THE THESIS

The overall objective of the project was to define the mechanisms that explain why an episode of exercise-induced bronchoconstriction (EIB) is followed by refractoriness, i.e. a reduced response to subsequent challenges. The identification of endogenous protective factors may establish new targets for diagnosis, treatment and prevention of asthma.

The project included a series of different exercise challenge studies in subjects with asthma. The effects of the challenges and specific interventions on airway function and release of bioactive mediators was used to test the following hypotheses:

1. Refractoriness occurs after mannitol challenge.
2. EIB following eucapnic voluntary hyperpnea (EVH) is associated with release of mast cell mediators into the urine.
3. Treatments preventing EIB will also affect the mediator release
   a. One high dose of inhaled corticosteroids blunts the EIB following EVH by preventing mast cell mediator release
   b. Inhalation of the mast cell stabilising drug sodium cromoglycate prevent EIB following EVH by inhibiting mast cell mediator release
   c. Dietary supplementation with omega-3 fatty acids reduces airway responsiveness to mannitol challenge by changing the pattern of the mediators released
4. The refractoriness/tolerance phenomenon after EIB occurs at the level of the airway bronchial muscle. The corollary of this hypothesis being that release of bronchoconstrictive mediators remains unaltered during the refractoriness/tolerance.
5. Endogenous molecules produced in response to the initial bronchoconstrictive episode, determines the degree of refractoriness/tolerance.
   a. Greater release of bronchoconstrictive mediators, such as leukotrienes and prostaglandin D_2, during the first response, favours increased refractoriness/tolerance during subsequent challenges.
   b. Prostaglandin E_2 is one key protective factor in the refractoriness/tolerance response.

3 BACKGROUND

3.1 ASTHMA

Asthma has been described by Professor Ann Woolcock as "Airways that constrict too much, too often and too easily, resulting in impaired lung physiology and quality of life". It is a common disease affecting about 8-12% of the Swedish population. Asthma can present at any time throughout life, even if it is most common during childhood (To et al. 2010). The life-time risk of developing asthma is the same as the risk of developing cancer or diabetes, however since it often presents early in life, compared to the other diseases, it may cause a substantial lifelong burden for the individual (To et al. 2010). The cause of asthma is largely unknown; though genetic susceptibility seems to account for one part (Moffatt et al. 2010), while infections (Bartlett et al. 2009; Caramori et al. 2012) and environmental factors and exposure to allergens may account for another part (Lemanske and Busse 2010). Also, a lack of certain microorganisms in the commensal flora has been suggested to affect the immune system and trigger the development of deleterious effects that may eventually present as asthma (Heederik and von Mutius 2012). However, we still have an incomplete understanding of why some develop asthma, whereas others do not. Asthma is thus a multifactorial and complex disease, where dysfunction of the airways is however the common denominator.

3.1.1 Asthma phenotypes and why they matter

Accordingly, asthma is a very heterogeneous disease comprised of patients with a wide range of clinical characteristics. Throughout the years several efforts have been made to subgroup subjects with asthma, however, there is still no widely accepted definition for what should be considered a phenotype and what should not. Classically, asthma was divided into different subgroups depending on clinical characteristics; e.g. allergic asthma, exercise-induced asthma, fixed airflow limitation, exacerbation prone, obesity related, and eosinophilic asthma (Holgate 2011; Wenzel 2013). With the emerging knowledge on genes and molecules, the subgrouping has moved from phenotypes to endotypes, instead dividing the disease into groups according to molecular mechanisms. Examples of these endotypes could be: Th2 associated early onset allergic, late onset, IL-5 associated eosinophilic, mast cell associated exercise-induced, late onset obese, mild non-CS responsive (Holgate 2011; Wenzel 2013; Holgate 2014; Meyers et al. 2014; Fahy 2015). Each suggested endotype is related to specific molecular mechanisms; the early onset allergic is for example related to the involvement of IL-4/-13 whereas late-onset eosinophilic is more closely related to mediators such as IL-5, leukotrienes and eotaxin2.

This search for phenotypes and endotypes is more than just an academic exercise, the goal of this subgrouping being to predict which treatments are useful for which group of patients (Holgate 2013; Holgate 2014; Meyers et al. 2014). Perhaps the clearest example of the usefulness of this subgrouping is the initial failure of anti-IL-5 treatment when given to unstratified groups of subjects with asthma. However, when identifying a subgroup of

subjects with severe asthma having a high sputum eosinophil count, the medication was proven to be very effective (Nair et al. 2009; Castro et al. 2011; Pavord et al. 2012; Bel et al. 2014a; Bel et al. 2014b; Ortega et al. 2014). As the treatments become more and more specific, targeting specific receptors or signalling molecules, we also need to be better at defining which patients will benefit from the treatment. This is a major focus of asthma research today.

3.2   AIRWAY CHALLENGES

Asthma is characterised by variable airway obstruction, airway inflammation and airway hyperresponsiveness. Each of these features can be examined, e.g. the degree of airway obstruction can be tested by measuring lung function by spirometry (Miller et al. 2005), and the airway inflammation can be assessed by measures such as exhaled nitric oxide (Alving et al. 1993). A slightly unique aspect of asthma is that we can provoke attacks of asthma in a safe and controlled way in the lab by bronchial challenges (Joos et al. 2003). The bronchial provocation is usually classified as "direct" or "indirect".

Figure 1 Schematic description of the mechanism of action of direct and indirect challenges

The direct challenges including histamine, methacholine, and leukotriene D_4 act at their respective receptors on the bronchial smooth muscle, thereby causing bronchoconstriction

(Joos et al. 2003). The indirect challenges, includes exercise, allergen, adenosine, hypertonic saline and aspirin (in aspirin-intolerant patients). They cause airflow obstruction by inducing the release of bronchoconstrictive mediators from inflammatory cells such as the mast cell. The direct challenges are very sensitive, however they are not specific for asthma (Hopp et al. 1984; Cockcroft et al. 1992). The indirect challenges, on the other hand, are less sensitive but very specific, meaning that a positive reaction confirms a diagnosis of asthma and the degree of responsiveness is closely associated with the degree of airway inflammation (Joos et al. 2003).

3.3 MAST CELLS AND LIPID-DERIVED MEDIATORS

Mast cells are tissue resident inflammatory cells, often located around blood vessels and close to body surfaces such as the skin and the lungs. They play an important role in tissue homeostasis as well as in host defence against pathogens (Metcalfe et al. 1997). Mast cells have been shown to be one of the key disease-triggering cells in asthma, both in acute reactions as well as in the chronic changes observed in the airways (Brightling et al. 2002; Bradding et al. 2006; Wenzel 2012).

Mast cells can be activated by a wide range of stimuli. The classical route is via cross-linking of immunoglobulin (Ig)E that binds to the Fc receptor on the mast cell, causing the mast cell to degranulate. The mast cell releases three classes of mediators: pre-formed (e.g. histamine, tryptase), *de novo* synthesised lipid mediators (e.g. prostanoids and leukotrienes) and both preformed and newly synthesised chemokines and cytokines (e.g. TNF-α, and interleukins) (Galli et al. 2008; Galli and Tsai 2012). Mast cell can also be activated by other stimuli such as hyperosmolarity (Gulliksson et al. 2006), and by drugs, physical stimuli etc. (Rothschild 1970; Metcalfe et al. 1997).

3.3.1 Mast cell-derived lipid mediators

As stated above, activation of mast cells does not only induce the release of preformed mediators but also initiates *de novo* synthesis and release of lipid-derived substances. Among these lipids are the eicosanoids, so called because they contain 20 carbon atoms. The eicosanoids are derived from arachidonic acid (AA) which is found in phospholipids in the cell membrane. The eicosanoids include prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), lipoxins (LX) and a range of hydroxyl and hydroperoxy fatty acids (HETE and HPETE). The first group of eicosanoids to be characterised were the prostaglandins, a discovery for which the Nobel Prise in Physiology or Medicine in 1982 was granted to Bergström, Samuelsson and Vane (Bergström and Samuelsson 1962; Bergström et al. 1964a; Bergström et al. 1964b; Piper and Vane 1971; Vane 1971).

Essential fatty acids are the main elements of the cell membrane in all cells and are called essential since they cannot be synthesised in the human body. In humans there are two types of essential fatty acids; the ω6-series derived from cis-linoleic acid (LA, 18:2 - meaning it has 2 double bonds) and the ω3-series derived from α-linoleic acid (ALA, 18:3). LA is converted to γ-linoleic acid (GLA) by the enzyme Δ^6-desaturase. GLA is then elongated to form

dihomo-GLA (DGLA, 20:3ω6 – 3 double bonds with the first located at the 6th carbon atom). DGLA is the precursor of the 1-series PGs but it can also be converted to AA by Δ^5-desaturase; AA is the precursor of the 2-series PGs and the 4-series of cysteinyl leukotrienes (CysLTs). ALA is converted to eicosapentaenoic acid (EPA, 20:5ω3) by Δ^{5-6}-desaturase; EPA is the precursor of the 3-series of PGs and 5-series of CysLTs.

Upon activation of the mast cell, the polyunsaturated fatty acid AA (20:4ω6 is liberated from the cell membrane by phospholipase A_2 (PLA_2) via a calcium-dependent mechanism (Smith 1989).
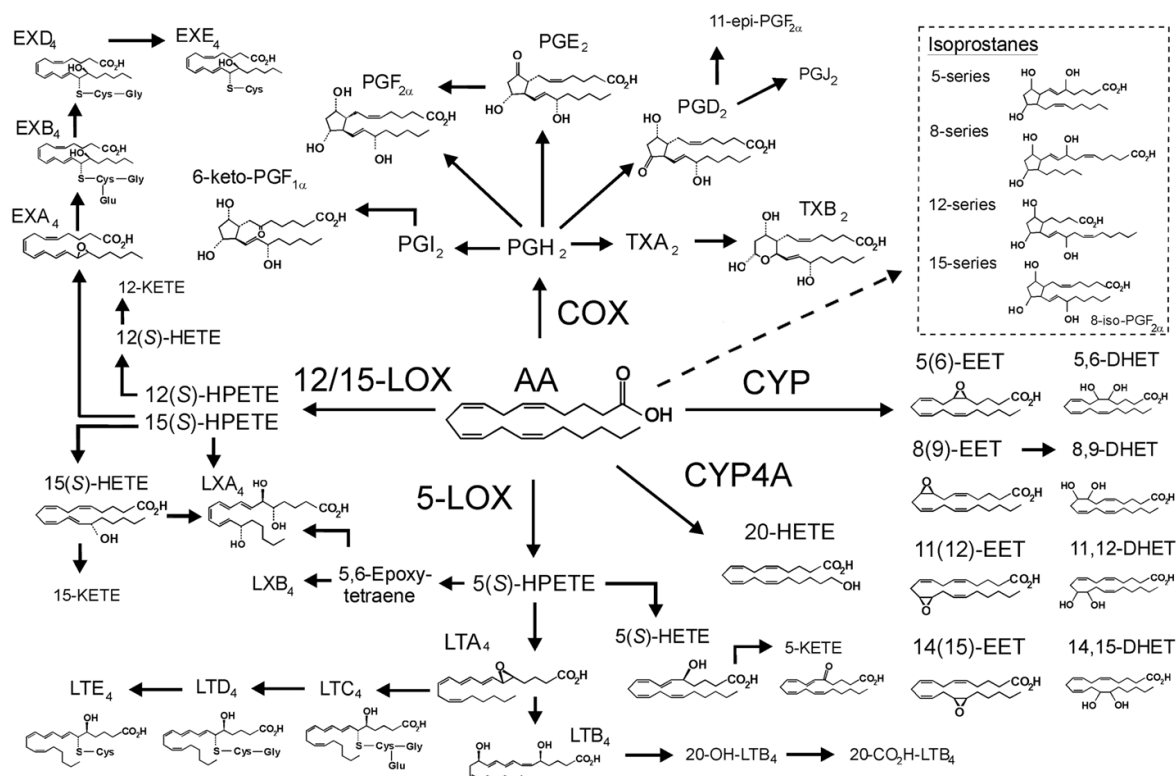
Figure 2 Overview of the arachidonic acid (AA) metabolism. The arrows indicates enzyme catalysed pathways, dashed arrows indicates non-enzymatic pathways.
COX – cyclooxygenase; 5-LOX – 5-lipoxygenase; 12/15-LOX – 12/15-lipoxygenase; CYP P450 – cytochrome P450 monyoxygenase

3.3.1.1 The cyclooxygenase pathway

Prostaglandins are formed from arachidonic acid (AA) in a two-step reaction; first AA is converted to PGG_2 by cyclooxygenase (COX). PGG_2 is highly unstable and is further metabolised to PGH_2 by a peroxidase reaction.

PGH_2 is then metabolised to the individual prostanoids by specific synthases. Which of the prostanoids a cell produces is dependent on the synthases expressed by each individual cell (Smith et al. 2000).
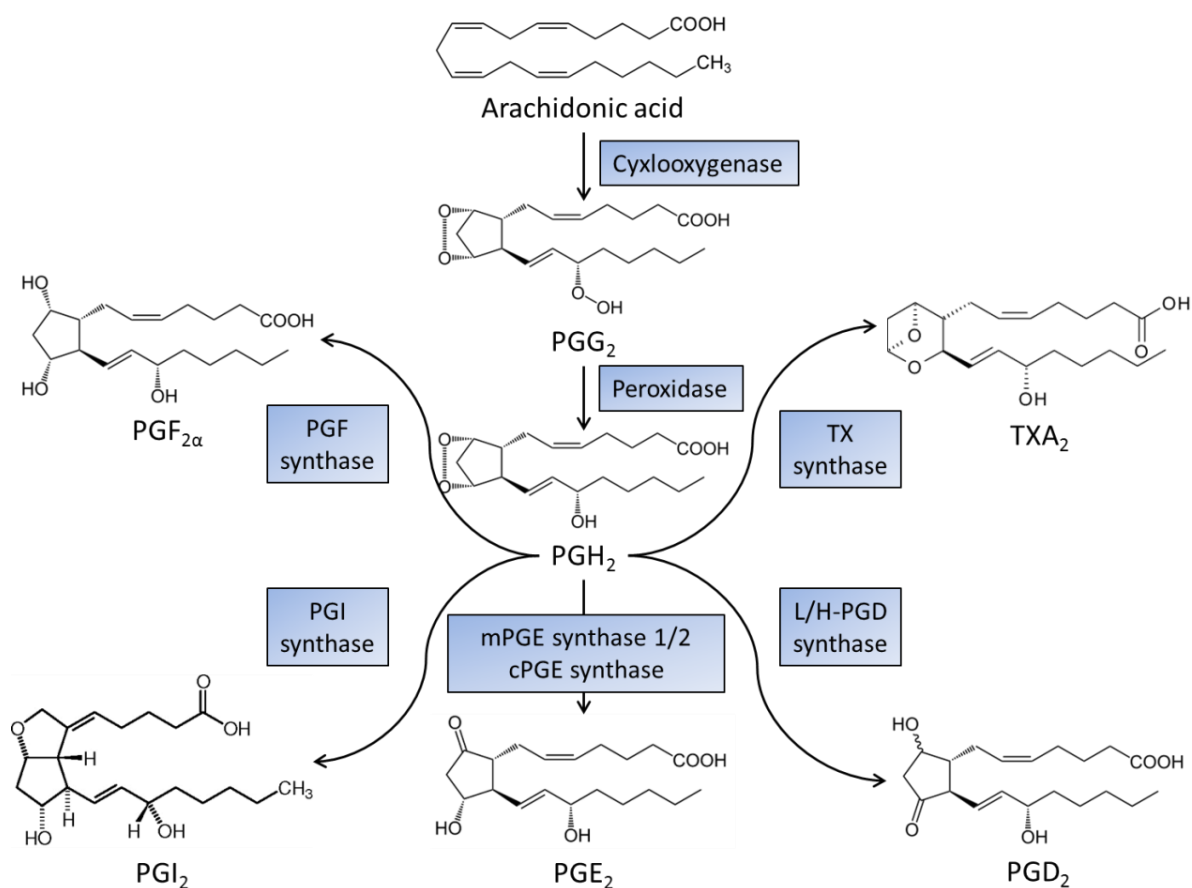
Figure 3 Overview of prostaglandin (PG) metabolism

The prostanoids are further metabolised, primarily via a pathway initiated by the oxidation of the 15(S)-hydroxyl group by 15-hydroxyprostaglandin dehydrogenase (15-PGDH). This is then followed by reduction of the 13 position double bond by 13-15-ketoprostaglandin reductase (13-PGR). A separate pathway exists for the oxidation of TXB_2 at C-11 by 11-hydroxythromboxane B_2 dehydrogenase (11-TXB_2DH) (Westlund et al. 1986).

The final degradation products are extensively oxidised before being excreted into the urine. The oxidation includes both β- and ω-oxidation, and leads to shortening of the carbon chains.

3.3.1.2 The lipoxygenase pathway

Following cellular activation, 5-lipoxygenase (5-LOX) is activated by increases in intracellular calcium. This activation causes the enzyme to translocate to the nuclear membrane where it interacts with 5-LOX activating protein (FLAP) (Miller et al. 1990). Following interaction between 5-LOX and FLAP it converts arachidonic acid (AA) to the unstable 5-hydroxyperoxyeicosatetraenoic acid (5-HPETE). 5-HPETE can then non-enzymatically be reduced to 5-HETE, or converted to LTA_4 by 5-LOX (Borgeat and Samuelsson 1979c; Borgeat and Samuelsson 1979b; Borgeat and Samuelsson 1979a; Borgeat and Samuelsson 1979d; Smith 1989).
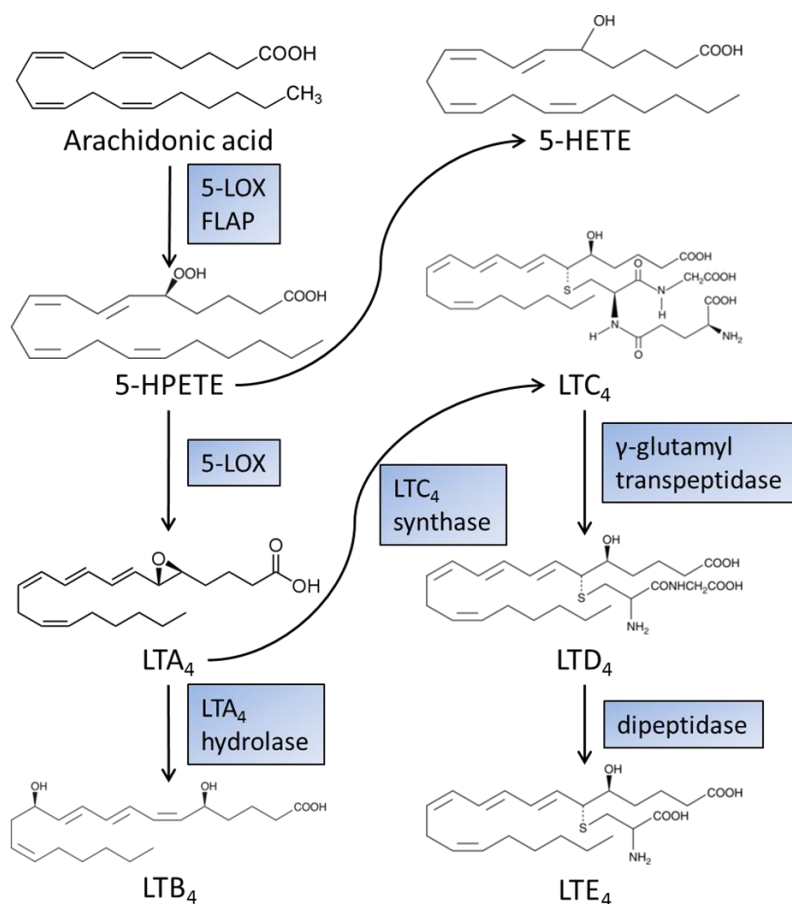
Figure 4 Overview of leukotriene (LT) metabolism
5-LOX – 5-lipoxygenase; FLAP – 5-LOX activating protein

In cells containing LTA_4 hydrolase (e.g. neutrophils), LTA_4 can be converted to LTB_4 and in cells containing LTC_4 synthase (e.g. mast cells and eosinophils), be converted to the first of the family of cysteinyl leukotrienes; LTC_4 (Welsch et al. 1994; Haeggstrom et al. 2007). In human mast cells, an alternative pathway from LTA_4 to LTC_4 via GSH-s-transferase type 2 (MGST2) has been shown to exist (Sjostrom et al. 2002).

Following exit from the cell, LTC_4 is rapidly converted to LTD_4 and then to LTE_4 by peptidases. The majority of the LTE_4 is eliminated via the faecal route whereas the other 15-20% is rapidly filtered in the kidneys and excreted into the urine within 24h (Maltby et al. 1990).

3.3.2 Urinary excretion of lipid mediators

The primary eicosanoids are potent biologically active mediators, but they are troublesome to measure since they are very rapidly metabolised and cleared from the circulation (Samuelsson et al. 1975). Also, following withdrawal of blood it has been shown that e.g. TXB_2 can be generated *ex vivo* (Patrono et al. 1986). Taken together, this often makes the measurement of these primary compounds very difficult, and the interpretation of such data complicated. Instead, a lot of focus has been put on determination of their respective metabolites in body fluids. As an alternative to blood, analysis of excretion products into the urine has emerged (Hamberg and Samuelsson 1971).

Accordingly, PGD_2 is metabolised by the above mentioned reactions to form 13,14-dihydro-15-keto-PGD_2. This metabolite is then further degraded by oxidation and the final metabolites are filtered in the kidneys and released into the urine. The urinary release of PGD_2 metabolites has been characterised in man by infusing radiolabeled PGD_2 into the circulation and then collecting urine to measure what is excreted (Liston and Roberts 1985). Primary PGD_2 was not detectable in urine. Instead, 25 different metabolites were discovered, of which 23 showed an F-ring configuration instead of the original D-ring. The earliest appearing metabolite was 11β-$PGF_{2\alpha}$ (also commonly known as $9\alpha,11\beta$-PGF_2). The metabolite 11β-$PGF_{2\alpha}$ is known to have some biological activity such as, bronchoconstriction (Beasley et al. 1987; Seibert et al. 1987a; Seibert et al. 1987b). 11β-$PGF_{2\alpha}$ is then further metabolised to 2,3-dinor-$PGF_{2\alpha}$. The most abundant metabolite of PGD_2 in the urine is tetranor-PGDM.

Similar metabolic studies to those performed for PGD_2, have been carried out to examine the urinary excretion of PGE_2, PGI_2 and TXB_2 (Zipser and Martin 1982). Following infusion, about 60% of the radioactivity was recovered in urine within 24 h, with most of it recovered within 2 h.

For PGE_2 this revealed that only a small proportion of the primary mediator is found in the urine (<0.1% when infused in brachial vein and 3.7% when infused in renal artery). The main metabolite is tetranor-PGEM.

PGI_2 was recovered as the metabolite 6-keto-$PGF_{1\alpha}$, and the more abundant 2,3-dinor-6-keto-$PGF_{1\alpha}$.

TXB_2 was only recovered in small amounts, whereas 11-dihydro-TXB_2 and 2,3-dinor-TXB_2 are known to be the major urinary metabolites.
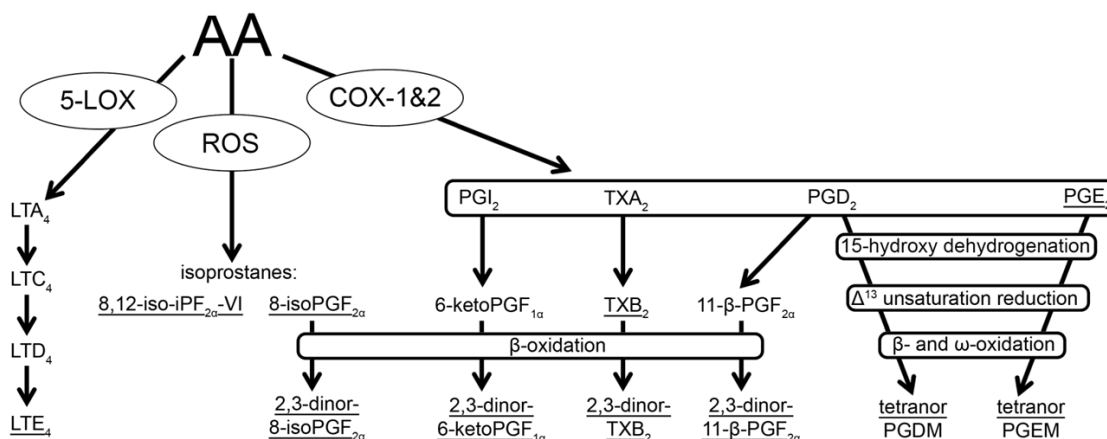
Figure 5 Simplified overview of eicosanoid metabolism. Underlined metabolites can be measured in urine

The use of urinary metabolites to study mechanisms in asthma has become widely accepted, especially using 11β-$PGF_{2\alpha}$ and LTE_4 (Kumlin et al. 1992; Dahlen and Kumlin 1998; O'Sullivan et al. 1998a; O'Sullivan et al. 1998b; O'Sullivan et al. 1999; Brannan et al. 2003; Dahlen and Kumlin 2004). The rationale for using urine samples to study the mechanisms involved in the reactions that follow an airway challenge, is that upon provocation of the lung only, an increase in the urinary excretion of e.g. PGD_2 is observed. There is then little doubt

that the increase in the urine originates from an increased release in the lung.

While the CysLTs can originate from other cells, e.g. eosinophils, PGD_2 is known to be predominately released by mast cells, and only to a small extent by macrophages (Hsueh 1979). PGD_2 and its metabolites are therefore often used as markers of mast cell activation *in vivo* (Dahlen and Kumlin 1998; O'Sullivan et al. 1999; Dahlen and Kumlin 2004).

3.4   EXERCISE-INDUCED BRONCHOCONSTRICTION (EIB)

After Areatus, another early description of EIB was made by Sir John Floyer who in 1717 stated "all violent exercise makes the asthmatic to breathe short – and if the exercise be continued it occasions a fit". Over the years, EIB has prevented many from performing physical activities, as well as made many people avoid any exercise that might provoke asthma. This is very unfortunate since the lower the level of fitness; the higher the respiratory strain during exercise (Åstrand and Rodahl 1970). Also, with higher strain, the severity of EIB is increased, whereas improved fitness is known to decrease the severity of EIB (Henriksen and Nielsen 1983; Carlsen et al. 2000).

3.4.1   How does exercise provoke an attack of asthma?

With the evidence that increased ventilation is an important factor in EIB, Deal and colleagues performed a number of studies focusing on the respiratory heat exchange and found a correlation between the airway response and the degree of respiratory heat loss (Deal et al. 1979a; Deal et al. 1979b; Deal et al. 1979c). This made the authors suggest that it is the cooling of the airways that is responsible for the bronchoconstriction following exercise (McFadden et al. 1986). This thermal theory proposes that the increased ventilation during exercise causes airway cooling and vasoconstriction, which is followed by rapid rewarming and a reactive hyperaemia of the bronchial vasculature at the cessation of exercise. These vascular events cause the airways to narrow, and the theory does not encompass bronchial smooth muscle or mediator release. The thermal theory initially gained much support, however, the documentation of EIB as a result of breathing hot dry air has provided evidence that an abnormal cooling of the airways is not a prerequisite for EIB (Anderson et al. 1985).

The findings of Anderson and colleagues led to the osmotic theory, which proposes that the water loss due to increased ventilation during exercise transiently increases the osmolarity of the airway surface (Anderson 1984; Anderson et al. 1985; Anderson et al. 1989; Anderson and Daviskas 2000). This event creates a favourable environment for the release of mediators such as prostaglandins, leukotrienes and histamine from inflammatory cells and neuropeptides from sensory nerves in the airways.

It is now recognised that the primary stimulus of EIB is indeed the loss of water from the airway surface, caused as a result of the humidification of large volumes of air over a short period of time during exercise. The volume of the airway lining fluid is less than 1ml throughout the lung and this small volume makes it vulnerable to water loss (Anderson et al. 1989). In support of this theory, is the finding that EIB can be inhibited or completely prevented, by the inhalation of air at a temperature of $37^{o}C$ and a relative humidity of 100% (body conditions), during exercise (Anderson et al. 1979; Hahn et al. 1984a). While the

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vascular effects may play an important role in subfreezing conditions, this effect will become less important as the temperature of the inspired air increases. While vascular events will amplify the airway narrowing effects of bronchial smooth muscle contraction, it is the release of mast cell mediators, and the effects they mediate at their respective receptors, that appear to be the most important factor contributing to EIB.

3.4.2 Pathogenesis of EIB

Classically, the term exercise-induced asthma (EIA) was used to describe a fall in lung function following exercise in subjects with a previous diagnosis of asthma. EIB on the other hand, has been used to describe the exercise-induced fall in lung function both in asthmatic subjects as well as in subjects without any previous asthma diagnosis. These two groups of subjects with EIB may differ slightly when it comes to pathogenesis and treatment, but the term EIB is more commonly being used for both groups now. Subjects with asthma and EIB most often have an eosinophilic inflammation, and respond well to treatment with corticosteroids (Backer et al. 2013). Elite athletes with EIB and no previous history of asthma however, seem to have an inflammation that is more often dominated by macrophages and neutrophils (Sue-Chu et al. 1999a; Sue-Chu et al. 1999b).

   EIB is known to be determined by the loss of water from the airway lining fluid, causing the release of mast cell mediators which in turn induce bronchoconstriction (Anderson and Daviskas 2000). The loss of water from the airway lining fluid may also lead to epithelial disruption as indicated by the release of CC16 into urine following EVH, mannitol and exercise challenge (Bolger et al. 2011b; Bolger et al. 2011c; Kippelen et al. 2013; Simpson et al. 2013). This epithelial damage has been shown to lead to mast cell infiltration in dogs upon hyperventilation (Davis et al. 2003). In marathon runners, an influx of neutrophils has been seen in conjunction with the epithelial damage (Chimenti et al. 2010). There is also evidence to suggest the epithelial damage leads to reduced PGE_2 release (Hallstrand et al. 2005a; Hallstrand et al. 2005b). In the long term, epithelial damage may also lead to permanent changes in the airways. Furthermore, there are indications of an involvement of sensory nerves in EIB, although the evidence is not clear (Kippelen and Anderson 2013).

3.4.3 Mast cell mediators in EIB

Evidence supporting the release of mast cell mediators following hyperosmolar stimuli comes from studies using mannitol, both *in vivo* and *in vitro* (Eggleston et al. 1987; Brannan et al. 2003; Brannan et al. 2006; Gulliksson et al. 2006). The mast cell stabilising agent sodium cromoglycate (SCG) also has a protective effect against both exercise and mannitol induced bronchoconstriction, supporting an important role for mast cell mediators in the effects of both challenges (Tullett et al. 1985; Brannan et al. 2006; Anderson et al. 2010). Further support comes from studies of the cysteinyl leukotriene receptor antagonists, which significantly attenuate exercise-induced bronchoconstriction (Manning et al. 1990; Dahlen et al. 2002) and enhance recovery from mannitol, suggesting that leukotrienes are important in both initiating and sustaining the bronchoconstriction (Kemp et al. 1998; Leff et al. 1998).

3.4.4 Diagnosis of EIB

Self-reported symptoms are a poor predictor of the bronchial response to exercise, and also the subjective effect of treatment is of little value when diagnosing EIB (Rundell et al. 2001; Simpson et al. 2015). This may be problematic in the clinical setting, since a diagnosis of EIB in primary care will most often be based on symptoms and the subjective effect of treatment alone. Ideally, a diagnosis of EIB requires bronchoprovocations to complement the medical history (Anderson and Kippelen 2013).

Since EIB depends on the ventilation achieved (Carlsen et al. 2000), it is important that the challenge is intense, and not too short. Another important factor to consider is the number of airway generations involved in the conditioning of the inspired air; challenges that involve recruitment of the small airways are more effective in identifying EIB (Daviskas et al. 1991). This may relate to the high density of mast cells in the small airways (Carroll et al. 2002; Carroll et al. 2011). Furthermore, there are several factors that can affect the response to challenge, including medications and air conditions; all of which need to be taken into consideration to optimise the chance of a positive response (Anderson and Kippelen 2013).

A diagnosis of EIB should be based on a fall in lung function following airway challenge, and many different challenges can be used, such as exercise, mannitol and EVH. The different challenge procedures have their pros and cons, but the American Thoracic Society (ATS) has published practice guidelines on how to diagnose EIB (Parsons et al. 2013).

3.4.5 Treatment of EIB

The treatment of EIB is generally based on the same principals as the treatment of asthma (Boulet and O'Byrne 2015). This often means using a β_2-agonist for bronchodilation and inhaled corticosteroids (ICS) for anti-inflammatory therapy. Guidelines for the treatment of asthma suggest that patients with daily symptoms should be treated with ICS, and also that all patients should be able to perform unlimited activity or the treatment needs to be intensified (Bousquet 2000). For a subject with EIB, however, there are no clear guidelines. It is not obvious whether a subject experiencing daily EIB when exercising, but who is symptom free at rest, should be considered to have uncontrolled asthma (Backer et al. 2013).

Treatment with short-acting β_2-agonists (SABAs) or long-acting β_2-agonists (LABAs) is fundamental. SABAs are often used as monotherapy and have been shown to reduce the fall in FEV_1 by 70% if taken at least 5 min prior to exercise (Anderson et al. 2006). Also LABAs have been shown to be effective in preventing EIB (Richter et al. 2002). However, there is evidence that regular use of SABAs or LABAs induces tolerance and a decreased protective effect against EIB (Ramage et al. 1994; Simons et al. 1997; Nelson et al. 1998).

ICS have been shown to inhibit EIB in asthmatics when given as a regular treatment (Weiler et al. 2005; Subbarao et al. 2006). It has also been suggested that a single high dose of ICS can be effective in inhibiting EIB (Thio et al. 2001). In guidelines for asthma treatment, low-dose ICS treatment is described as add-on to SABAs, but this may be insufficient in EIB (Backer et al. 2013). There is evidence of a dose-dependent effect of ICS (Subbarao et al. 2006), which suggests that it may be better to increase the dose of ICS rather than adding a LABA, which is often suggested in guidelines. The dose of ICS should be

titrated according to the response to exercise, and the treatment should give complete protection against EIB (Duong et al. 2008).

The combination of ICS and LABA is commonly used in the treatment of asthma, and it has been shown that treatment with this combination as needed, is as effective as daily treatment with ICS (Lazarinis et al. 2014).

Antileukotrienes, such as the $CysLT_1$ receptor antagonists montelukast and zafirlukast, have been shown to be effective in inhibiting EIB (Manning et al. 1990; Dessanges et al. 1999; Dahlen et al. 2002; Anderson 2004). Montelukast can be used as monotherapy or in combination with the other asthma medications. However, the effect is heterogeneous and not of benefit to all subjects.

Sodium cromoglycate and other mast cell stabilising agents have been shown to be effective in preventing EIB (Tullett et al. 1985; Woolley et al. 1990; Brannan et al. 2006; Anderson et al. 2010). These mast cell stabilisers are less potent than the other treatments, and also their duration of action is short, which is probably why they are seldom used in the treatment of EIB nowadays (Backer et al. 2013). However, they are effective and essentially free of side-effects, and may become useful again.

In some recent studies, treatment with fish oil that is rich in omega-3 fatty acids has been reported to inhibit EIB (Mickleborough et al. 2003; Mickleborough et al. 2006; Tecklenburg-Lund et al. 2010). These studies are in contrast to previous studies, showing no beneficial effects of omega-3 supplement in asthmatic subjects (Arm et al. 1988; Arm et al. 1989b). There are currently no official recommendations, suggesting omega-3 for the treatment of EIB or asthma in general.

Apart from pharmacological interventions, there are also non-pharmacological ways to prevent EIB. A short warm-up has been proven effective (Stickland et al. 2012). Also, the use of face masks to condition the inspired air is widespread, especially among winter athletes (Millqvist et al. 1995).

3.5 REFRACTORINESS

McNeill and colleagues were the first to describe refractoriness, namely a decreased airway response following repeated exercise challenge (McNeill et al. 1966). It was however, not until more than a decade later than Edmunds and colleagues set out to describe refractoriness in greater detail (Edmunds et al. 1978). They performed repeated challenges with different intervals, and also characterised the effect of work load on the degree of refractoriness. They found that the duration of the refractory period varied from 2 to 4 h. The studies also showed that the more intense the initial challenge, the more refractory the subjects became to further challenge and the shorter the interval between challenges, the higher the degree of refractoriness.

Refractoriness occurs not only following exercise challenge, but also following other indirect challenges such as EVH (Bar-Yishay et al. 1983; Argyros et al. 1995), hypertonic saline (Hawksworth et al. 1992), adenosine (Daxun et al. 1989), and ultrasonically nebulised distilled water (Mattoli et al. 1987b). All of these indirect challenges are associated with the release of mast cell mediators, and the reactions are inhibited by pre-treatment with the mast

cell stabilising drug sodium cromoglycate (SCG). The indirect challenges are also thought to induce refractoriness via similar pathways (Joos et al. 2003).

Further support for similar pathways in the development of refractoriness comes from studies of cross-refractoriness between indirect challenges where cross-refractoriness has been found between exercise and EVH (Ben-Dov et al. 1983), between hypertonic saline and exercise (Belcher et al. 1987), and between exercise and antigen-induced bronchoconstriction (Weiler-Ravell and Godfrey 1981).

EVH has proven to be a good surrogate for exercise challenge, in that it produces a very similar fall in lung function and it leads to the development of a similar degree of refractoriness (Rosenthal et al. 1990). Prior to this thesis, it was however not known whether refractoriness existed following mannitol challenge.

3.5.1 Suggested mechanisms

Since the discovery of refractoriness, there have been speculations as to why it occurs, yet little is known about the mechanisms involved. The most common suggestions for the mechanism are presented below, for more details see (Larsson et al. 2013).

3.5.1.1 High levels of catecholamines

One of the first suggestions to be put forward was that catecholamines released during the first challenge provide protection during the second (Larsson et al. 1982). However, in studies measuring the levels of epinephrine and norepinephrine, no support for this theory could be found (Dosani et al. 1987). In this study, the levels of epinephrine and norepinephrine increased both in healthy and asthmatics following exercise challenge, but returned to baseline levels before the second challenge was initiated. Of the different airway challenges, hypertonic saline is not believed to induce the release of cathecholamines; however, this has not been measured locally in the lung. The fact that refractoriness also occurs following hypertonic saline, questions the role of catecholamines in the development of refractoriness. It should be noted, however, that inhalation of β-receptor antagonists causes bronchoconstriction in asthmatics, suggesting that catecholamines play a role in regulating airway tone (Okayama et al. 1987). Taken together, it remains to be determined whether catecholamines can provide an explanation for the mechanism of refractoriness.

3.5.1.2 Depletion of mast cell mediators

As mast cell mediator release became accepted as an integral part of EIB, it was suggested that there were not enough mediators released to induce bronchoconstriction following the second challenge. This theory was further strengthened by the finding of a relationship between the workload during the first challenge and the degree of protection afforded (Edmunds et al. 1978). However, the studies that have actually measured levels of the mediators suggested to be depleted during the second challenge, have failed to find evidence for this (Belcher et al. 1988). This question was therefore further addressed in this thesis.

3.5.1.3 Decreased general responsiveness of the airway smooth muscle

During the time that mediator depletion was a popular theory, the suggestion also emerged that the airway smooth muscle itself was less responsive during the second challenge. This theory proposes that the airway smooth muscle does not respond normally to the mediators released. This theory has been tested in several different studies where the authors have used different direct stimuli following various indirect challenges. Following exercise, the sensitivity to histamine and methacholine has been reported to be unchanged in refractory subjects (Hahn et al. 1984b; Magnussen et al. 1986; Boulet et al. 1987). The same is true for methacholine following mannitol challenge (Suh et al. 2011). However, if a histamine challenge is performed first, a decreased response to a subsequent exercise challenge is observed although interestingly, the opposite was not found (Hamielec et al. 1988).When an exercise challenge is performed first, a decreased responsiveness to histamine has been seen only after two repeated exercise challenges (Carpentiere et al. 1988). This is in line with the finding that it takes multiple histamine challenges before tachyphylaxis is apparent (Schoeffel et al. 1980; Boulet et al. 1987).

Together these studies do not provide clear evidence that the mechanism of refractoriness involves a decreased responsiveness of the airway smooth muscle.

3.5.1.4 Specific tachyphylaxis to the released mast cell mediators

Support for the theory that refractoriness is caused by tachyphylaxis to the mediators involved in the bronchoconstrictive response, such as histamine, PGs and LTs comes from studies of repeated histamine challenge (Schoeffel et al. 1980; Boulet et al. 1987). Also, it has been shown that $PGF_{2\alpha}$ induces bronchoconstriction upon inhalation, but when continuing to inhale higher doses of $PGF_{2\alpha}$, there was a return to baseline lung function (Fish et al. 1984). Similar effects have been noted previously with repeated $PGF_{2\alpha}$ inhalation in asthmatic subjects, but not in healthy controls (Mathe and Hedqvist 1975). It is unclear what the mechanism underlying this decreased response is; whether it is the result of a down-regulation of the receptor for each mediator, or perhaps the release of a protective substance preventing the bronchoconstrictive mediator from exerting its action. In the study of Fish et al, the protective response was not specific to $PGF_{2\alpha}$, but rather a decreased response to histamine was also seen after $PGF_{2\alpha}$ challenge. This led the authors to suggest the release of a protective substance, with reference to *in vitro* studies, showing the release of the bronchoprotective prostaglandins PGE_2 and PGI_2 in animal models (Piper and Vane 1971).

Histamine is only one of the mast cell mediators that has been shown to play a role in EIB, and studies of the effect of histamine antagonism have shown that it exerts only a minor role in the bronchoconstrictive response following exercise (Dahlen et al. 2002), and EVH (Wiebicke et al. 1988). However, this does not rule out the possibility that histamine may exert its effects via receptors other than the H_1 receptor. *In vitro* studies have indicated the importance of the histamine H_2 receptor in the development of tachyphylaxis to repeated histamine challenge (Knight et al. 1992). There are also indications that repeated histamine challenges induce release of protective prostaglandins as the smooth muscle response was

increased in bronchial preparations from patients pre-treated with NSAIDs (Knight et al. 1995).

3.5.1.5   Release of protective prostaglandins

To evaluate the suggestion that refractoriness is caused by the release of a protective prostaglandin, several studies of the effect of NSAIDs on the refractory period were performed. It was found that pre-treatment with indomethacin blocks the development of refractoriness following exercise, EVH, and hypotonic challenge with ultrasonically nebulised water (O'Byrne and Jones 1986; Mattoli et al. 1987a; Margolskee et al. 1988; Wilson et al. 1994). In these studies, the indomethacin pre-treatment had no effect on the response to the initial challenge. The fact that indomethacin abolished refractoriness, strongly supports a role for prostaglandins in the protective response. This was then further investigated in a comprehensive study by manning et al (Manning et al. 1993). They found that there was refractoriness both to repeated LTD_4 and exercise challenge, and that there was cross-tolerance between the challenges. The refractoriness and the cross-tolerance were blocked by pre-treatment with flurbiprofen, suggesting prostaglandins to be involved in the protective response. The same authors also tested the effect of histamine H_2 receptor antagonism on repeated exercise challenge and found no effect on either the first, or the second challenge.(Manning et al. 1992)

It is not known whether LTD_4 inhalation can induce tolerance to a subsequent exercise challenge. However, CysLTs have previously been shown to induce the release of PGs in the guinea pig *in vivo,* as well as *in vitro*.(Piper and Samhoun 1982; Dahlen 1983; Dahlen et al. 1983; Paruchuri et al. 2008) This suggests that the release of prostaglandins occurs upon LTD_4 inhalation. Also, evidence for tachyphylaxis has been observed with repeated CysLT exposure in isolated guinea pig airways.(Dahlen et al. 1983)

The protective prostaglandin has been suggested to be PGE_2, which has been shown to reduce the duration and severity of bronchoconstriction following exercise.(Melillo et al. 1994) It has also been shown that PGE_2 does not alter the responsiveness to methacholine suggesting that it is not working as a functional antagonist.(Pavord et al. 1991; Melillo et al. 1994). The PGE_2 is thought to be released by the airway smooth muscle and the epithelium (Delamere et al. 1994; Knight et al. 1995) and its release has also been shown to be stimulated by hyperosmolarity (Hjoberg et al. 2000). However, exactly how PGE_2 might exert its action was, and has largely remained, unclear. More recently, interesting data has emerged indicating that PGE_2 may have mast cell stabilising effects preventing the release of mediators via the EP_2 receptor both in mice (Torres-Atencio et al. 2014), an in man (Säfholm et al, J Allergy Clin Immunol *in press*).

3.5.2 Humid air and refractoriness

Studies where subjects inhaled hot humid air during the exercise challenge provided strong evidence for the hyperosmolar theory of EIB (Strauss et al. 1978; Hahn et al. 1984a). It became clear that breathing hot humid air during the challenge prevented bronchoconstriction. Several groups then went on to investigate the effect of humid air on the

development of refractoriness. Two studies found that exercise challenge performed at room temperature induced a marked bronchoconstriction , when performed 20 min after an initial exercise challenge breathing hot humid air, where the initial challenge did not cause any bronchoconstriction (Anderson et al. 1979; Hahn et al. 1985). Conversely, refractoriness to exercise has been seen when the initial challenge was performed while breathing hot humid air (Ben-Dov et al. 1982; Wilson et al. 1990). Wilson and colleagues later showed that this protective response is sensitive to NSAIDs (Wilson et al. 1994). The reason why an initial non-bronchoconstricting challenge can cause refractoriness to a further challenge remains unclear, even though prostaglandins seems to somehow be involved, considering the effect of NSAID pre-treatment. However, little is known about how humidity may affect mediator release. Previous results suggest that humid air itself can induce a relaxing factor, the effects of which last sufficiently long to protect against subsequent exercise challenge (Johnston et al. 1992). To further understand the mechanisms involved in the protective effect of hot humid air, a study of its effect on EIB-induced mediator excretion was initiated as part of this project.

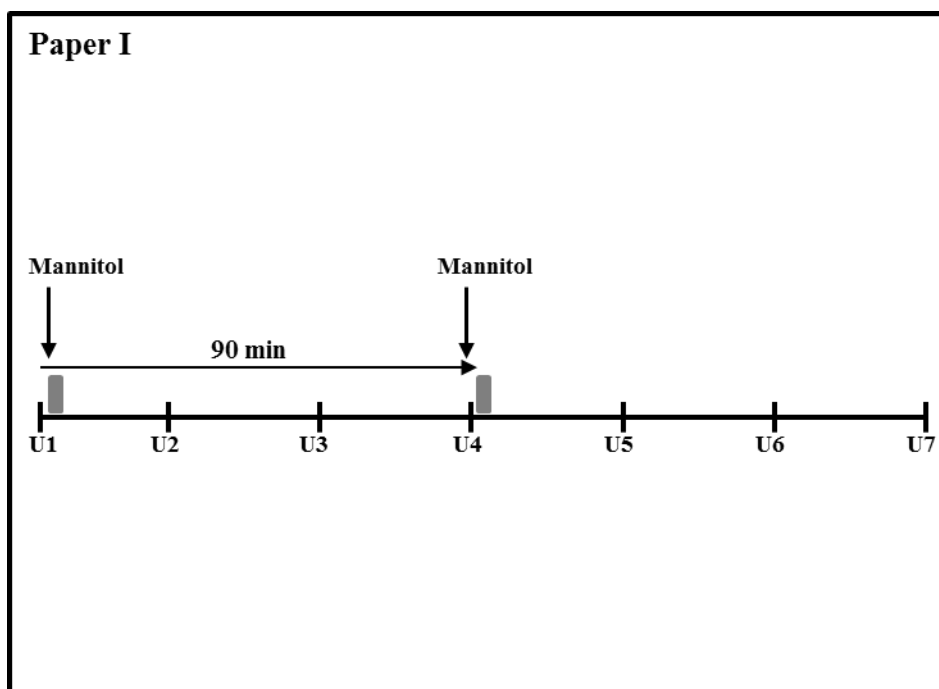# 4 MATERIAL AND METHODS

4.1 SUBJECTS AND STUDY DESIGN

Figure 6 Study design Paper I. U-urine sample.

The aim of **Paper I** was to test the hypothesis that refractoriness is due to decreased release of mast cell mediators following the second challenge. Sixteen asthmatic subjects were recruited to Sandra Anderson's lab in Sydney to perform repeated mannitol challenge. Subjects were included if they had a history of asthma, a positive response to mannitol ($>15\%$ fall in FEV_1 following inhalation of ≤ 635 mg of mannitol) and a spontaneous recovery of lung function within 90 min after the challenge. The protocol was approved by the institutional Ethics Committee (X05-0068), and the study was carried out under the Therapeutic Goods Administration of Australia Clinical Trial Notification scheme (CTN No. 2005/362). All participants gave their written informed consent before inclusion.

On the screening day, a skin prick test was performed. A mannitol challenge was performed to confirm a positive response. FEV_1 was measured at baseline, 5 min following challenge, then at 10 minute intervals for 90 min. If a spontaneous return to within 95% of baseline lung function was seen within 90 min the subject was included and called back for the study day.

On the study day the subjects performed two mannitol inhalation challenges with a 90 min interval. The dose that provoked a 15% fall during the first challenge was used during the second challenge. Urine samples were collected at baseline and then every 30 min until 180 min following the first challenge. The sample collected at 90 min before the start of the second challenge was used as the second baseline.
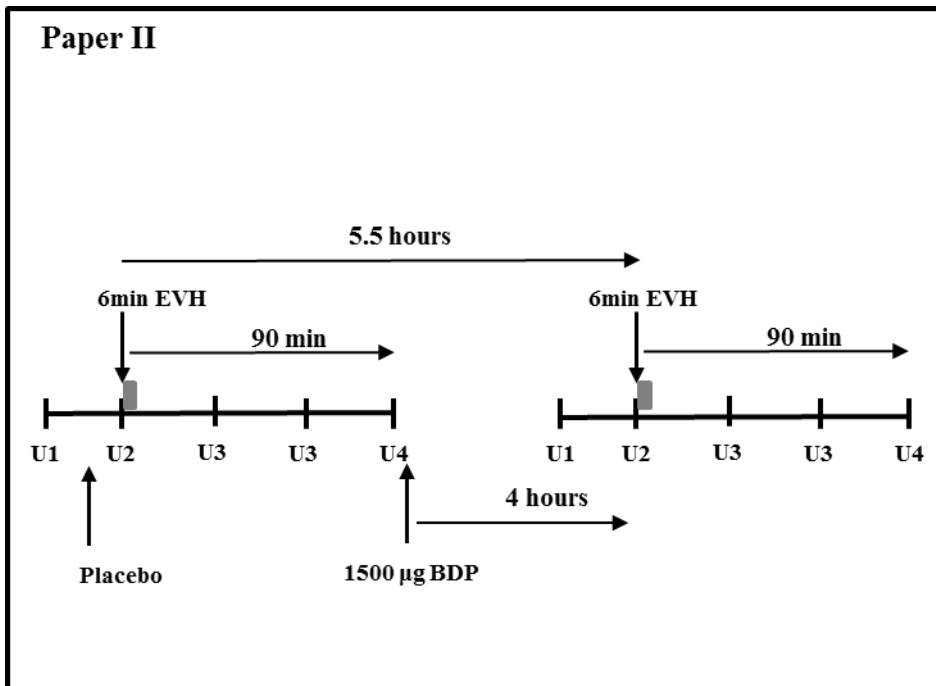
Figure 7 Study design Paper II. U-urine sample.

The aim of **Paper II** was to assess whether a single high dose of beclomethasone diproprionate (BDP) could blunt mast cell activation and bronchoconstriction following eucapnic voluntary hyperpnea (EVH). Seven endurance-trained athletes with exercise-induced bronchoconstriction (EIB) and 8 asthmatics were recruited to Sandra Anderson's lab in Sydney to participate in the study. Written informed consent was obtained. The protocol was approved by the Central Sydney Area Health Service Ethics Committee (X03-0164). Skin prick tests were also performed.

To confirm a diagnosis of EIB, the athletes were called into the lab to perform an EVH challenge. If a fall in FEV_1 of $\geq 10\%$ from baseline occurred, the subjects were eligible for inclusion.

On the study day each subject received a placebo aerosol in the morning, 10 min before the EVH challenge. In the afternoon 4 h after the EVH challenge the subjects received 1500 µg BDP (QVAR; 3M Pharmaceuticals, Loughborough, UK). The administration was single-blinded. A second EVH test was performed 5.5 h after the first. Lung function was measured at baseline and 1, 5 and 10 min after challenge, then every 10 min until 90 min after the challenge. Urine samples were collected 60 min before each challenge, just before the challenge and then every 30 min until 90 min after the challenge.
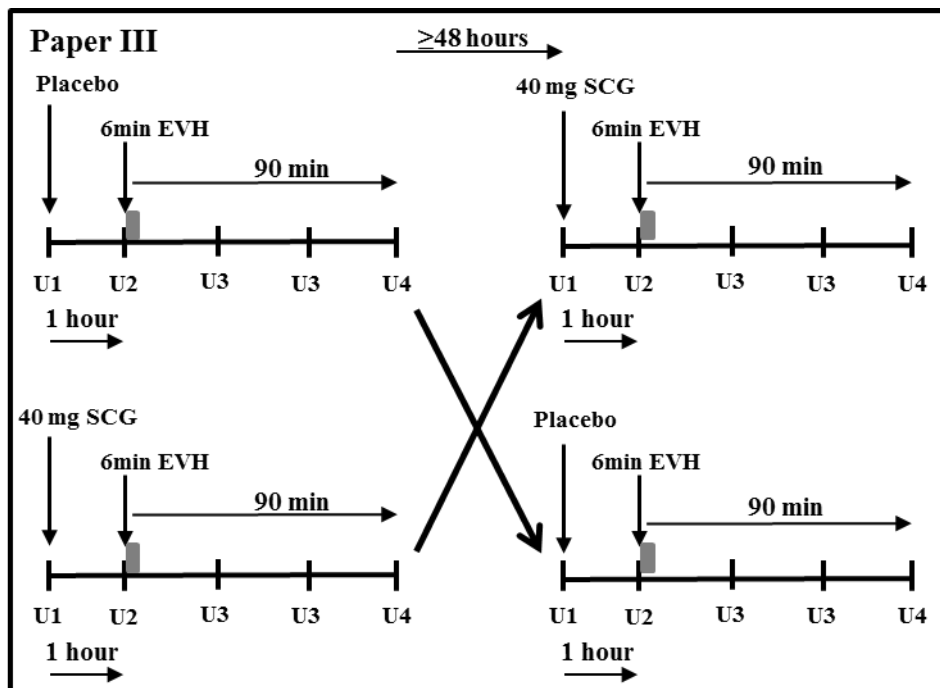
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Figure 8 Study design paper III. U-urine sample.

The aim of **Paper III** was to clarify the role of mast cell mediators in the airway response to exercise in athletes, and also to investigate the effect of the mast cell stabilising drug sodium cromoglycate (SCG). Eleven endurance-trained athletes with exercise-induced bronchoconstriction (EIB) and 11 without EIB were recruited to Sandra Anderson's lab in Sydney to participate in the study. Written informed consent was obtained. The protocol was approved by the Central Sydney Area Health Service Ethics Committee (X03-0164).

On the screening day, baseline lung function was measured and a skin prick test carried out. An EVH challenge was then performed. The subjects were divided into groups according to the fall in FEV_1 following EVH; if a fall >10% from baseline lung function occurred the subject was classified as EIB+, and if a fall <10% occurred they were classified as EIB-.

On the study days, the subjects received either placebo or 40 mg of SCG (8 inhalations of Intal Forte; Sanofi Aventis; France) from a pressurised metered-dose inhaler. The administration of drugs was randomised and single-blinded. The drug was administered 15 min before the start of the EVH challenge. Urine samples were collected when subjects arrived at the lab, before the start of the EVH challenge and then every 30 min up to 90 min. Lung function was recorded at baseline, following administration of the drug and then at 1, 5 and 10 min after the EVH challenge, and then every 10 min up to 90 min.
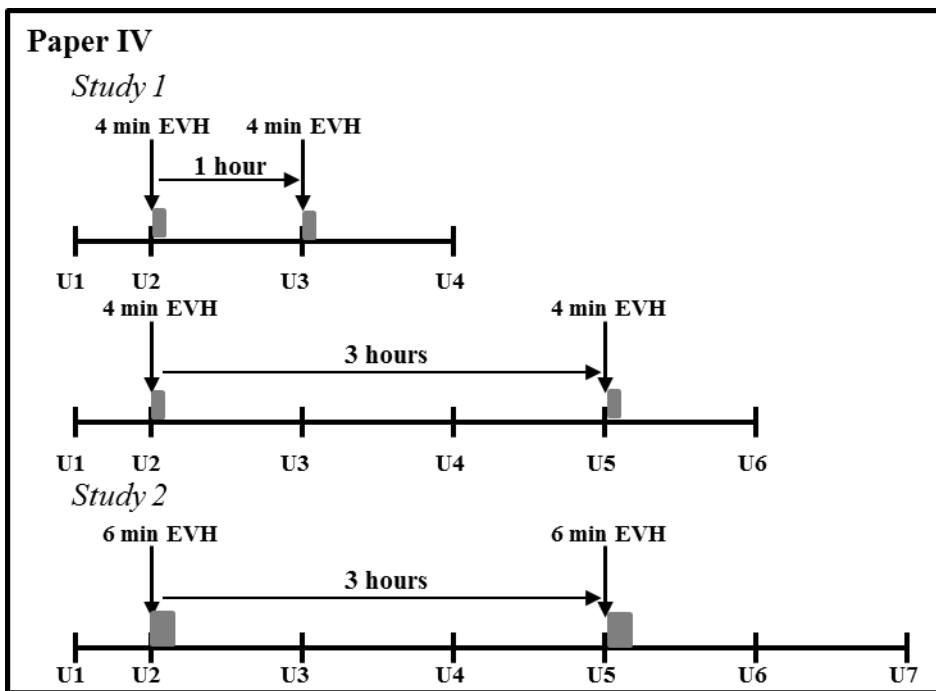
Figure 9 Study design Paper IV. U-urine sample.

The aim of **Paper IV** was to further characterise urinary mediator release during the refractory period. The urinary excretion of mediators is delayed compared to the airway response, requiring us to determine the optimal interval between challenges to detect refractoriness, while still being able to study urinary mediator release. All included subjects gave their written informed consent and the study was approved by the local ethics committee (Karolinska Institutet regional ethics committee Dnr 03-127, Ethics board Stockholm 2012/1277-32).

To determine the optimal interval between challenges, we performed an initial study (study 1) to compare two different intervals between challenges. In this first study 16 asthmatics were recruited to perform repeated 4 min EVH challenges, either 1 or 3 hours apart, in a randomised cross-over design.

During screening the subjects underwent a physical examination, skin prick testing, FeNO measurement, and spirometry. A 4 min EVH challenge was performed using a slightly modified protocol published by Smith et al (Smith et al. 1988). Subjects who met the inclusion criteria of a maximum fall in FEV1 ≥10% were included in the study. On the two study days, following baseline spirometry and measurement of FeNO, repeated challenges with 4 minutes of EVH (challenge I and challenge II) were performed. Urine samples were collected 30 minutes before, immediately before the start of the first challenge and then hourly until 240 minutes after the first challenge. Lung function was monitored repeatedly.

Based upon this initial range-finding study, study 2 was designed. Now, a 6 minute EVH challenge was performed and a fall in $FEV_1 \geq 15\%$ was a criteria for inclusion. Nine subjects were included, but one subject was excluded from analysis because of an asthma exacerbation between the screening visit and the study day, leading to 8 subjects being eligible for further analysis. The events of the screening day were the same as for study 1 with the exception of 6 min EVH instead of 4 min EVH. During the study day the subjects

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performed repeated challenge with 6 minutes of EVH 3 hours apart. Urine samples were collected 30 minutes before, immediately before the start of the first challenge and then every hour until 300 minutes after the first challenge.
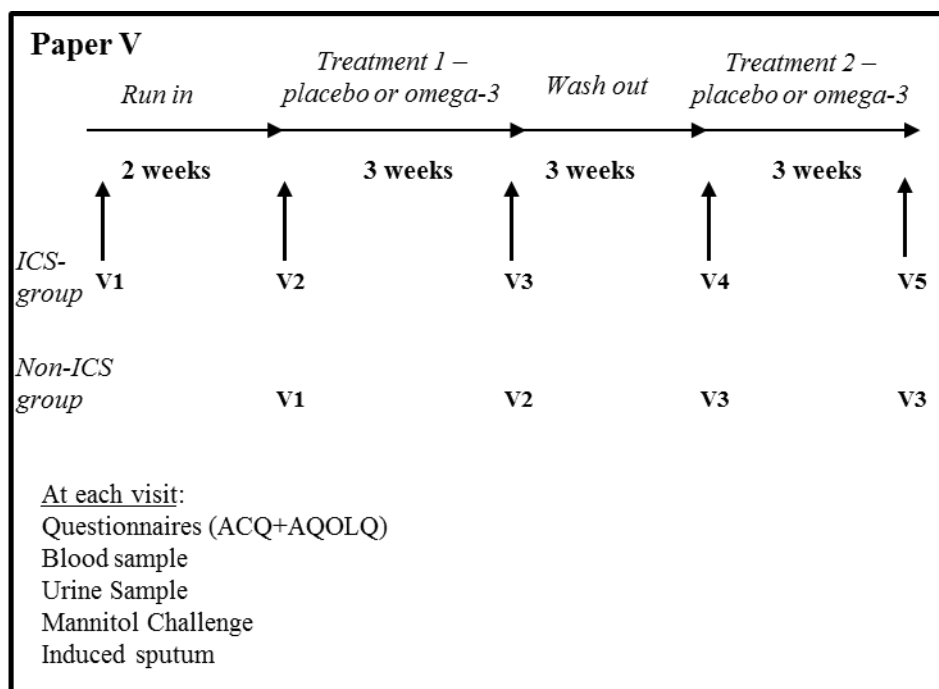

Figure 10 Study design Paper V. U-urine sample.

The aim of **Paper V** was to test whether dietary supplementation with omega-3 fatty acids could inhibit the bronchial response to mannitol challenge. Eleven asthmatic subjects with regular inhaled corticosteroid (ICS) treatment and 12 asthmatic subjects medicating only with short acting beta agonists (SABA) as needed, were recruited to Professor Paul O'Byrne's lab in Hamilton, Canada. Written informed consent was obtained. The protocol was approved by the ethics review board at St Joseph's Healthcare, Hamilton, Ontario, Canada (R.P.#06-2750) and Health Canada (Approval no. 120532). The trial was registered at ClinicalTrials.gov; No. NCT00526357.

The study used a randomised, double blind, placebo-controlled, cross-over design. The included subjects attended the lab at 4 occasions; at the beginning and at the end of each treatment period of 3 weeks. Those on regular ICS-treatment were required to undergo an extra visit 2 weeks prior to the first treatment period to confirm that they were responding to the mannitol challenge and that their disease was stable.

During each visit the subjects completed self-administered questionnaires (Asthma Control Questionnaire [ACQ] and Asthma Quality of Life Questionnaire [AQLQ]). Blood samples were drawn to assess fasting triglyceride, omega-3 and omega-6 levels. A urine sample was collected to assess eicosanoid levels. Then a mannitol challenge was performed which was followed by sputum induction.

The treatment periods lasted 3 weeks and were separated by a 3 week wash-out period. During the treatment periods the subjects were asked to take a daily dose of 10 capsules of either matched placebo, containing a mix of omega-6 and omega-9, or 10 capsules of the

active treatment, each capsule containing 400 mg of EPA or 200 mg of DHA; (10*40/20EE capsules; Ocean Nutrition Canada).
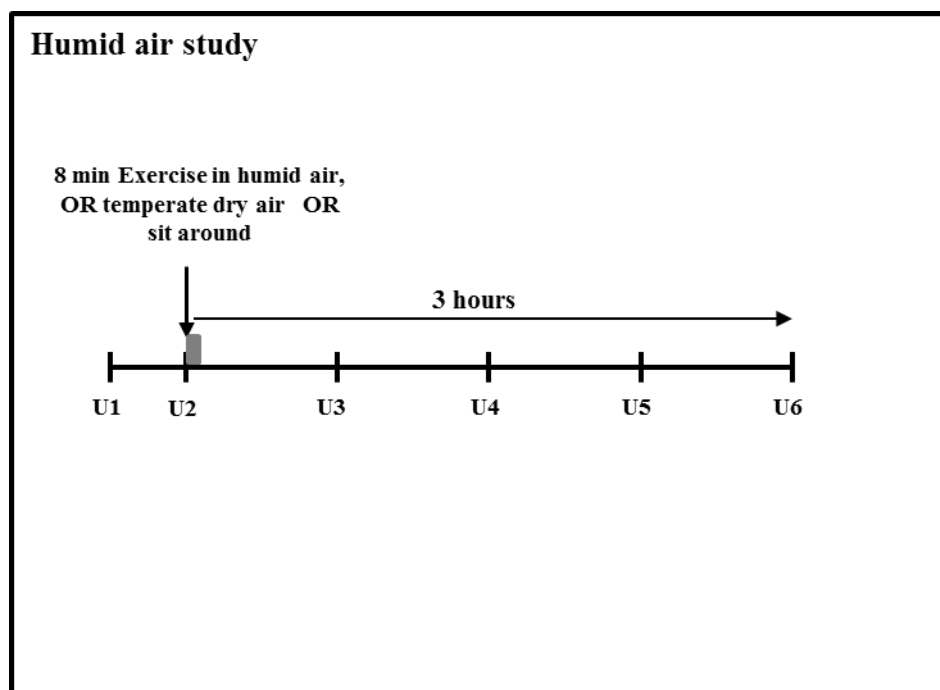
4.1.1 Ongoing studies:

Figure 11 Study design Humid Air study. U-urine sample.

In the Humid Air study we want to test the effect of inspired air conditions on urinary mediator release following EIB. This study is still ongoing. The protocol was approved by the Ethics Review Board at Brunel University, London, U.K. Subjects are being recruited to Pascale Kippelen's lab at Brunel University to perform an exercise challenge test on a cycle ergometer; if a fall >15% in FEV_1 is observed the subject is eligible for inclusion.

During the screening visit and during one of the experimental visits, participants exercise whilst breathing air that will be temperate dry (18^oC and < 8 mg H_2O/L). Fully saturated air at body temperature (37^oC and 100%RH) is inhaled during the exercise challenge in the last experimental condition. Participants breathe the test air for 4 minutes prior to exercise, for the entire duration of the exercise test, and for 2.5 minutes post-exercise. The air is delivered through a standard two-way non-rebreathing valve, with a nose clip in place. Participants' heart rate is monitored continuously by short-range radio telemetry using a chest belt. Ventilation is monitored by breath-by-breath gas analysis. Oxygen saturation (SpO_2) is monitored by pulse oximetry using forehead sensors. As a control condition the subjects are also to be called back to the lab for a study day during which they sit in the lab while monitoring lung function repeatedly. During the study days urine samples are collected twice at baseline and then at 45 min intervals following challenge until 180 min.

Figure 12 Experimental setup for the Humid Air study

4.1.2 Isolated human small airways

To be able to further characterise refractoriness, and try to define the key events involved, we set out to develop a new method in which we provoke isolated bronchial segments in an organ bath with repeated administration of mannitol solution. This study has been initiated and the methodology is still under development.

Macroscopically healthy human lung tissue was obtained by consent from patients undergoing lobectomy at the thoracic surgery unit at the Karolinska University Hospital, Stockholm, Sweden. Before being entered into the study the subjects give their written informed consent. The study is approved by the regional ethical review board in Stockholm (ref no. 2010/181-31/2).

Figure 13 An organ bath with a bronchial segment mounted between the metal prongs in the centre.

Within 3 hours of surgery, the lung tissue is processed at the Unit for Experimental Asthma and Allergy Research where bronchi with an inner diameter ≤1 mm are isolated using a dissection microscope. To reduce spontaneous activity the small airway segments are placed in culture plate wells containing Dulbecco's modified Eagle medium and kept overnight in a humidified incubator (37 °C at 95% O_2 and 5% CO_2). The next day functional experiments are performed using myographs to record isometric tension (ADInstruments Ltd., Hastings, U.K. and Organ Bath Model 700MO, DMT A/S, Aarhus, Denmark). The myographs contain Krebs-Ringer PSS and are kept at 37 °C, constantly bubbled with carbogen (5% CO_2 in O_2) to maintain a pH of 7.4. The preparations are then allowed to equilibrate for 30 minutes with the buffer changed every 15 minutes, this is then followed by a step-wise increase in tension over 60 min to a resting tension of 1.5 mN. The protocol is described in more detail by Säfholm et al (J Allerg Clin Immun *in press*).

Following the equilibration period, KCl (60 mM) is added twice with a washout in between, to ascertain bronchial reactivity and viability. This is followed by a 60 min equilibration, including several washes to allow the segment to return to baseline tension. Different agonists and interventions are then administered to the organ bath, to study the contractile response.

4.2 BRONCHIAL CHALLENGES

As discussed earlier, a feature of asthma that is frequently made use of in research, is the fact that an attack of asthma can be induced in the laboratory under controlled conditions. During my PhD we have focused on exercise or exercise-mimicking challenges. These exercise-mimicking challenges include EVH and mannitol which both have proven useful in assessments of EIB (Argyros et al. 1996; Brannan et al. 1998; Anderson et al. 2001).

4.2.1 Mannitol

Mannitol is a sugar alcohol that was originally isolated from the secretions of a medium-sized tree, the flowering ash (*fraxinus ornus*), and it was called 'manna' due to its resemblance to the biblical food. Mannitol is listed on the WHO model list of essential medicines as a diuretic. In Sweden it is approved for use as a diuretic in the treatment of e.g. oliguric renal failure and to reduce intracranial pressure (FASS).

Mannitol as an asthma provocation model was first suggested by Anderson and colleagues (Anderson et al. 1997). The rationale of using mannitol as a provocation is that it is thought to have a similar effect on submucosal osmolarity to that of exercise; upon inhalation a hyperosmolar environment favouring mast cell activation is created and a fall in lung function similar to that observed following exercise challenge is seen (Anderson et al. 1997; Brannan et al. 1998; Brannan et al. 2003; Brannan et al. 2006).

Figure 14 Mannitol capsuled and inhaler from the Aridol challenge kit

Mannitol was used as the provocation model in Paper I and in Paper V. During the challenge subject inhale Mannitol (Aridol, Pharmaxis, Frenchs Forest, NSW, Australia) from capsules using a dry powder inhaler (Plastiape, Osnago, Italy). The subject wears a nose-clip and is instructed to exhale, followed by a controlled deep inhalation of the powder and thereafter hold their breath for 5 s. The challenge is initiated with an empty capsule and FEV_1 is measured in duplicate 60 s later. If a fall in FEV_1 <10% from baseline is seen the challenge is continued with the inhalation of 5 mg mannitol, followed by FEV_1 measurements in duplicate 60 s later. If the fall in FEV_1 is <15% the next dose is doubled to 10 mg. If the fall is still <15% from the best FEV_1 following the 0 mg capsule baseline values, the dose is doubled to 20 mg and then to 40 mg and so on. This is repeated up to a maximum dose of 160 mg, which is given three times or until the fall in FEV_1 is > 15%. The maximum cumulative dose given is 635 mg.

The dose that elicits a 15% fall in FEV_1 (called PD_{15}) can be calculated and is used as an indicator of how sensitive a subject is to the challenge; the lower the PD_{15} value, the more responsive the subject is to the challenge.

4.2.2 Eucapnic voluntary hyperpnea (EVH)

As it became increasingly apparent that EIB is determined by the degree of ventilation achieved, challenges involving hyperventilation instead of exercise were developed. Initially the temperature of the air was thought to be an important factor (Phillips et al. 1985). However, when it was later shown that temperature was at most a marginal factor (Anderson et al. 1979), and the osmotic theory of EIB (Anderson and Daviskas 2000) became more popular, more standardised hyperventilation tests were developed (Argyros et al. 1996).

In Paper II and Paper III the protocol used is based on the one originally described by Argyros et al.(Argyros et al. 1996) requiring subjects to breathe at a target ventilation of 85% of the maximum voluntary ventilation ($30*FEV_1$) for 6 minutes.(Anderson et al. 2001) At room temperature a compressed dry gas mixture (~5% CO_2, 21% O_2, and balance N_2) flows from a cylinder through a rotameter (Fisher-Rosemount; Brooks Instruments, Hatfield, PA) via an open demand valve into a meteorological balloon (300 g, MFG No. 100 MRL; Kaysam Corp., Patterson, NJ) via a metal connector with tap (Morgan PKM 90750105) that allows simultaneous filling and emptying. The subject inhales the gas via the metal connector and breathing tube, via a two-way non-rebreathing valve (Hans Rudolph No. 2700) and a mouthpiece. The expired gas passes through a gas meter (American Dry Test Meters; American Meter Co., Horsham, PA), and the volume of air expired is then measured.

In paper IV a slightly different protocol was used. Hyperpnea with dry, room temperature air containing 5% carbon dioxide was performed through a low-resistance, one-way valve in the sitting position (Ailos Asthma Test®, Karlstad, Sweden).(Rosenthal 1984) The target ventilation was 35 x FEV1 x 0.75 (L/min) and was maintained for 4 min in study 1 and for 6 min in study 2.

4.2.3 Exercise

In the Humid Air study, which still is ongoing, we use exercise challenge to be able to study how the conditions of the inspired air affect urinary mediator release.

For this exercise challenge we use a cycle ergometer and the challenge is conducted according to the American Thoracic Society (ATS) guidelines for exercise challenge testing (Crapo et al. 2000). For this protocol an individual target workload is calculated as follows: Watts = (53.76 x measured FEV_1) -11.07. The subjects reach this workload after 3 min of exercise; the workload will be 60%, 75%, 90% and 100% of the target workload in the first, second, third and fourth minute of exercise. The subject then maintains the target workload for 5 minutes, making the total challenge time 8 min.

4.3 LUNG FUNCTION MEASUREMENTS

To monitor how lung function is affected by the various challenges, we use spirometry. The tests have been performed according to American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines (Miller et al. 2005) and involves the subject exhaling as fast and powerful as possible, following a maximal inspiration whilst connected to a mouthpiece and wearing a nose clip. In this manoeuvre we can record lung volume (forced vital capacity, FVC) and expiratory flow rates (forced expiratory volume in one second, FEV_1, forced expiratory flow between 25 and 75% of FVC [FEF25-75] and peak expiratory flow rate [PEFR]).

4.4 URINE SAMPLE COLLECTION AND ANALYSIS

Urine is the matrix we have used in all of my studies to investigate mediator release. The subject was asked to empty his/her bladder into a plastic beaker. The time and volume of the
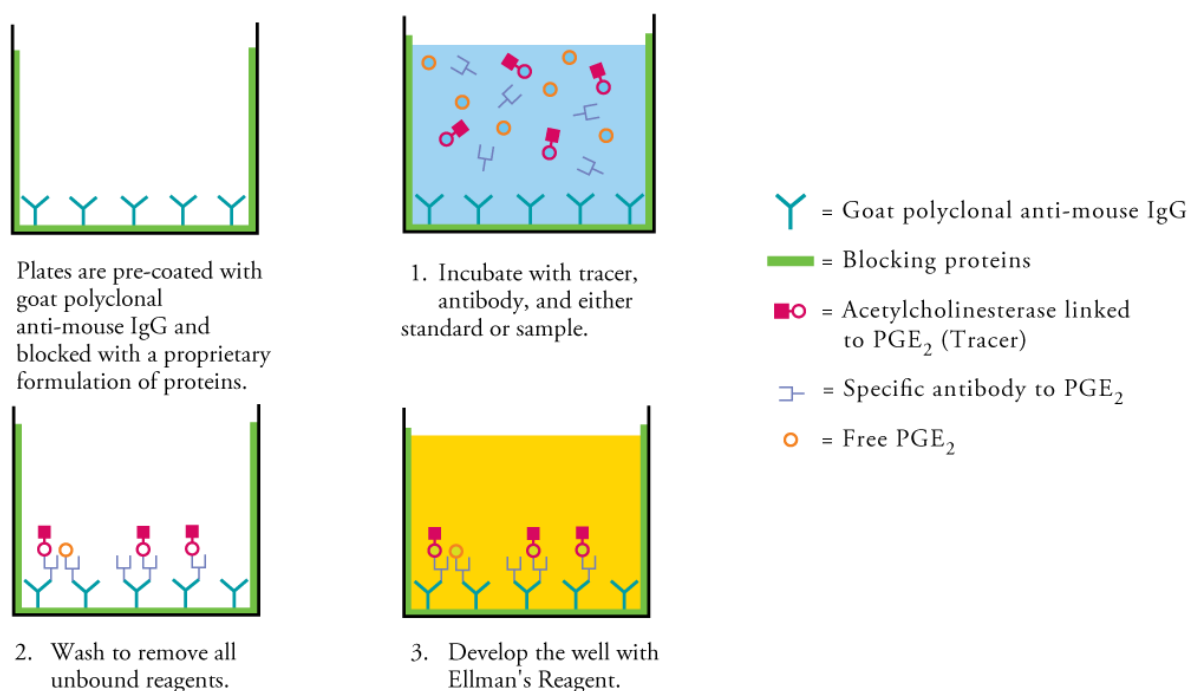
void was noted, and the samples were distributed into smaller plastic tubes, sealed and then stored at -20°C or -70°C until time of analysis.

4.4.1 Creatinine

To correct for dilution of the samples, creatinine was measured in all urine samples using a colorimetric assay. This method is a slight modification of the Jaffe method (from WHO's Blood Safety and Clinical Technology. Guidelines on Standard Operating Procedures in Clinical Chemistry). The method is based on that fact that the creatinine present in urine directly reacts with alkaline picrate resulting in the formation of a red colour. The intensity of the colour is then measured using a 505nm/green filter. A second absorbance reading after acidification with 30% acetic acid corrects for non-specific chromogens in the samples.

4.4.2 Enzyme immunoassay (EIA)

Enzyme immunoassay (EIA) was used in all studies to measure the urinary release of mediators. Commercially available kits were bought from Cayman Chemicals (Ann Arbor, Mi, USA). Urine samples were analysed un-extracted. The assays are based on the competition between free eicosanoid and acetylcholinesterase-linked eicosanoid for limited specific antiserum binding sites. The eicosanoid-antiserum-complex binds to IgG antibodies attached to the bottom of the well. The plate is then washed and Ellman's reagent added. In wells where a large proportion of the eicosanoid is acetylcholinesterase-linked there is a stronger colour shift. This is because the acetylcholinesterase cleaves the acetylthiocholine component of Ellman's to give free thiocholine. Thiocholine then reacts with 5-thio-2-nitrobenzoic acid, a second component of Ellman's, which gives a distinct yellow colour. The density of the colour is determined spectrophotometrically, and is proportional to the amount of eicosanoid-acetylcholinesterase bound to the well, which is inversely proportional to the amount of free eicosanoid in the original sample. This means the stronger the colour, the less eicosanoid present in the urine sample added to that well.

Plates are pre-coated with goat polyclonal anti-mouse IgG and blocked with a proprietary formulation of proteins.

1. Incubate with tracer, antibody, and either standard or sample.

2. Wash to remove all unbound reagents.

3. Develop the well with Ellman's Reagent.

Y = Goat polyclonal anti-mouse IgG

▬ = Blocking proteins

■O = Acetylcholinesterase linked to PGE_2 (Tracer)

⊐ = Specific antibody to PGE_2

O = Free PGE_2

Figure 15 Schematic description of the EIA. Reproduced with permission from Cayman Chemical s

To minimise errors in the analysis all samples are serially diluted in 3 different dilutions, and all are added to the plate in duplicate; therefore each sample ends up in 6 wells. We then discard the dilutions where the wells differ too much (SD >10 or CV >25%) and calculate the means of those considered acceptable.

4.4.3 Mass spectrometry (UPLC-MS/MS)

With EIA we can only analyse one mediator at a time per sample. Using paper 4 as an example where we examined 7 different mediators, this means we had to run 7 different EIA kits each time. To be able to simultaneously study several mediators in the same samples we participated in the development of a new ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method. Another advantage with the UPLC-MS/MS method is that it is very specific and we can therefore be more sure that we are actually measuring the intended compound. With EIA there is always a risk of cross-reactivity and you may not always actually be measuring what you think you are measuring; this is further discussed in the results section.

The UPLC-MS/MS analysis was performed in Craig Wheelock's laboratory at Karolinska Institutet. The method is described in greater detail by Balgoma et al (Balgoma et al. 2013). During the development of the method it became evident that the concentration of the urine sample significantly affected the results Therefore, the method includes a normalisation of the extracted urine volume by the ratio of absorbance at 300 nm to an optimised reference material. An internal standard for each compound to be measured is added to the sample. Following a solid phase extraction (SPE) procedure using HLB Oasis SPE cartridges (Waters, Milford, MA), the samples are analysed on the UPLC-MS/MS platform, which consists of 4 different methods that collectively measure 30 different eicosanoids and other lipid mediators. Prostanoids and isoprostanes are analysed by two methods, one for

compounds that undergo tautomerism and one for compounds that do not. A third method is applied to measure leukotrienes and a fourth to measure creatinine. The instrument used is a liquid chromatograph coupled to mass spectrometer (LC-MS/MS). Separation and quantification is performed on a UPLC Acquity-Xevo TQ mass spectrometer system (Waters).

5 RESULTS AND DISCUSSION

5.1 DEFINING THE AIRWAY RESPONSE AND LIPID MEDIATOR RELEASE FOLLOWING CHALLENGE

Studying mechanisms of bronchoconstriction due to exercise or exercise-mimicking challenges, such as EVH and mannitol, is complicated by the fact that many sampling methods can themselves interfere with the physiological response. This is true of induced sputum as well as bronhioalveolar lavage (BAL). Urine, on the other hand, is non-invasive and does not affect the physiological response. It is therefore attractive as a sampling method, however, it may be problematic due to the lag between the peak excretion of mediators in urine and the maximum pulmonary response. Therefore, to be able to study mediator release during refractoriness we first needed to better characterise the mediator release following exercise and exercise-mimicking challenges.

5.1.1 Mannitol (Paper I)

Paper I was initiated to study refractoriness following repeated mannitol challenge. The subjects performed repeated mannitol challenges, 90 min apart. The results after the first challenge confirmed earlier findings from our lab showing that mannitol challenge induces a decrease in lung function, which is associated with the release of mast cell-derived lipid mediators 11β-$PGF_{2\alpha}$ (previously also called $9\alpha,11\beta$-$PGF_{2\alpha}$) and LTE_4 (Brannan et al. 2003; Brannan et al. 2006).
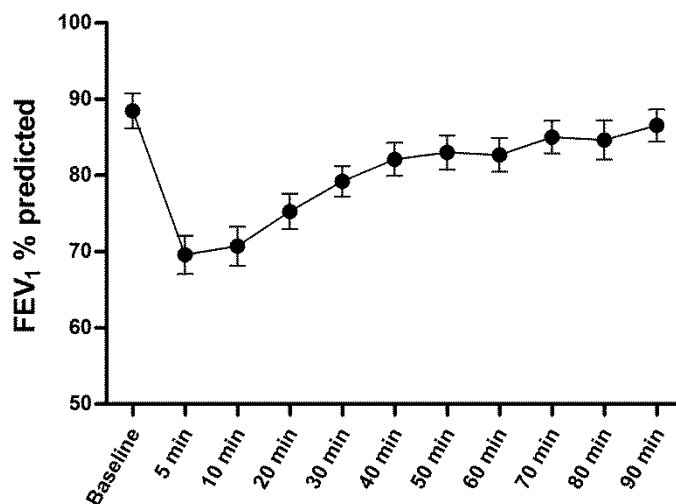

Figure 16 Airway response following mannitol challenge in 16 asthmatics
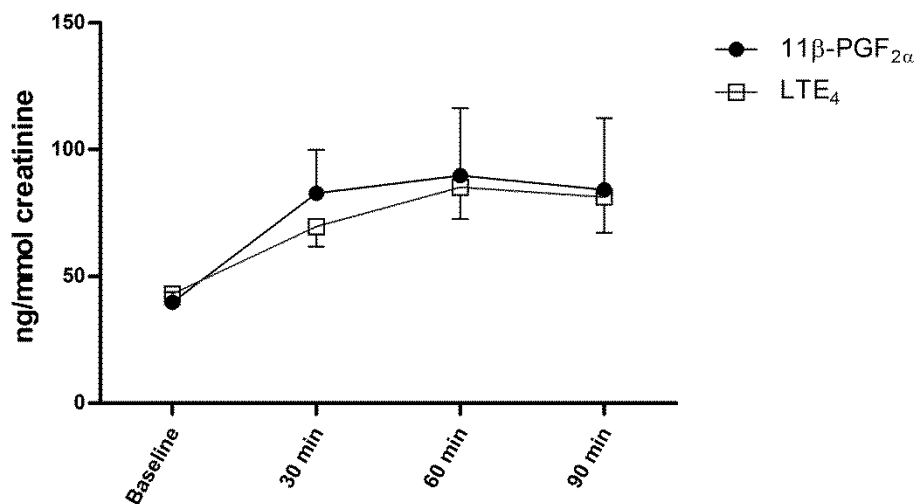
Figure 17 Urinary mediator release following mannitol challenge in 16 asthmatics

In the studies by Brannan et al, the release of 11β-$PGF_{2\alpha}$ had returned to baseline within 90 min, while the release of LTE_4 was still elevated. In our study however, the excretion of both 11β-$PGF_{2\alpha}$ and LTE_4 was still elevated at 90 minutes when the second challenge was initiated. The pattern of release of LTE_4, in both our and Brannan's studies, differed from that of 11β-$PGF_{2\alpha}$ in that the peak increase in the urine occurred later. There was however a correlation between the peak levels of the two mediators. This suggests that they originate from the same source, or that they are released simultaneously from different sources. The mast cell is known to be the main source of 11β-$PGF_{2\alpha}$, which is why this mediator can be used as a marker of mast cell activation (Dahlen and Kumlin 2004). CysLTs may originate from other inflammatory cells such as eosinophils and alveolar macrophages. However, previous findings from our lab confirm that mast cells *in vitro* release both PGD_2 and CysLTs upon hyperosmolar stimulation (Gulliksson et al. 2006).

5.1.2 Eucapnic Voluntary Hyperpnea (EVH) (Paper II + III)

Study I provided evidence that mannitol causes a very similar reaction to exercise, both regarding the airway response, as well as the release of lipid mediators into the urine. Next, we wanted to confirm these findings using EVH for which no previous reports of urinary mediator release exist. We also wanted to compare the response between healthy subjects, asthmatics, healthy elite athletes (EIB-) and elite athletes with bronchoconstriction upon exercise (EIB+) to get a better understanding of what determines whether a subject responds to EVH challenge.

In **Paper II** the athletes and untrained subjects with asthma responded similarly to EVH challenge with a maximum drop in lung function of about 22 % and 23 % respectively.
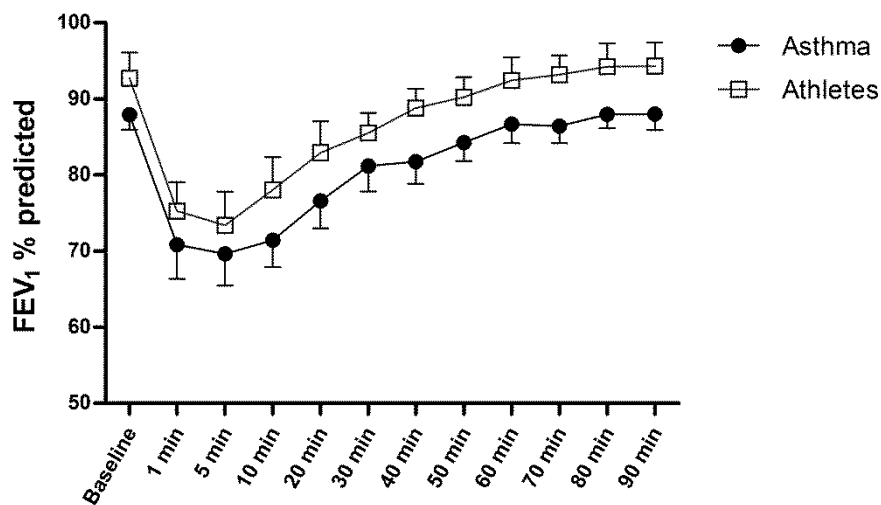

Figure 18 Airway response to EVH challenge in 8 subjects with asthma and 7 athletes with EIB

At baseline, there were no differences in mediator excretion, but following EVH, the asthmatics displayed 50% higher levels of both 11β-$PGF_{2\alpha}$ and LTE_4. An increase in 11β-$PGF_{2\alpha}$ levels was seen in both groups; however LTE_4 only increased significantly in the asthmatics. In the athletes, 11β-$PGF_{2\alpha}$ returned to baseline within 90 minutes, whereas in asthmatics the excretion was still elevated.
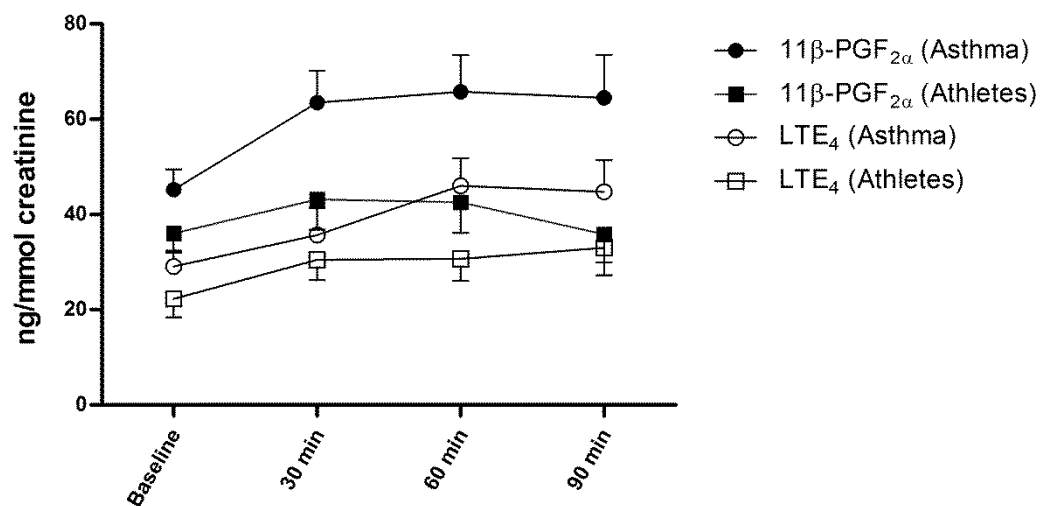

Figure 19 Urinary mediator release following EVH challenge in 8 subjects with asthma and 7 athletes with EIB

The mediator excretion was correlated with the maximum fall in lung function. It is not known why the asthmatics displayed higher mediator excretion but this might be an effect of increased numbers of mast cells in the airways (Brightling et al. 2002; Lai et al. 2014). Also, it could be that the mast cells of asthmatics to some extent behave differently than those of athletes, either by being more easily triggered to release, or exhibiting a defect in the termination of the secretory response. Another possibility is that water transport is somehow impaired in asthmatics, leading to the EVH having greater effects on the osmolarity of the

airways than what is seen in athletes (Park et al. 2008; Loughlin et al. 2010). Athletes with EIB often have sputum neutrophilia in contrast to asthmatics who more often present with sputum eosinophilia (Bonsignore et al. 2001). This difference in inflammatory cell composition may explain why the athletes did not show increased LTE_4, which is known to be produced in higher amounts by eosinophils than neutrophils.

In **Paper III** we compared the response to EVH challenge in subjects classified as EIB+ (>10% fall in FEV_1 at screening) and those classified as EIB-. The post-challenge fall in lung function in the EIB+ group was reproduced during the study day, whereas no significant fall in lung function was seen in the EIB- group.
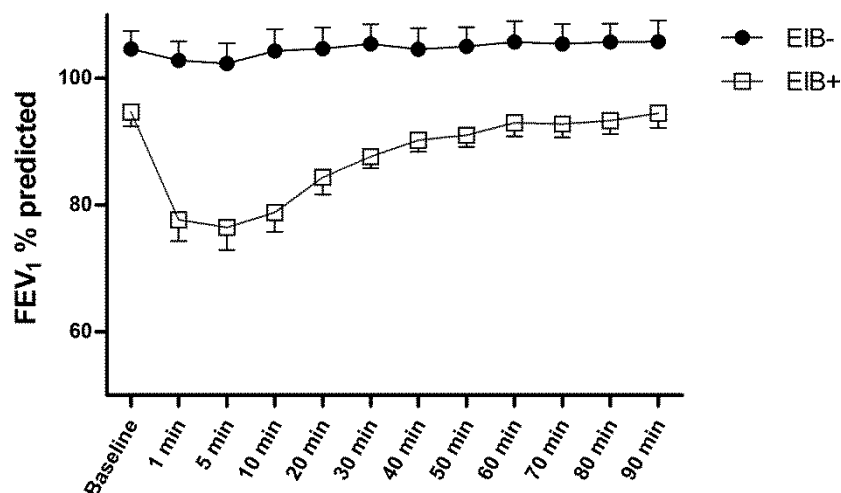

Figure 20 Airway response to EVH in 11 athletes with EIB (EIB+) and 11 athletes without EIB (EIB-)

For the mediators, the baseline levels of 11β-$PGF_{2\alpha}$ were higher in EIB+, whereas LTE_4 was found to be lower in this group. An increase of 11β-$PGF_{2\alpha}$ was seen in both groups following challenge, with the EIB+ having a higher peak excretion compared to EIB-. LTE_4 increased in EIB+ but not in EIB-.
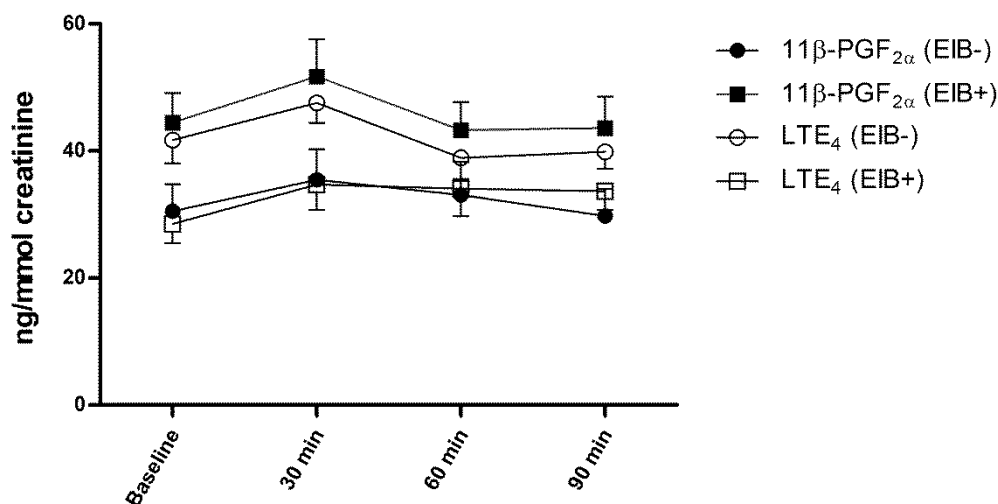

Figure 21 Urinary mediator release following EVH in 11 EIB+ and 11 EIB-

Similarly to study II, there was an association between the fall in lung function and the excretion of 11β-PGF$_{2α}$, however in this study no such association was found for LTE$_4$.

Interestingly, the EIB- subjects also excreted mediators following challenge, however not to the same extent as the EIB+. This finding suggests that mediator release is a necessity for EIB to occur, however the airways also need to be sensitive to the mediators excreted. This finding is in line with studies showing an increased reactivity to methacholine in athletes with EIB compared to healthy athletes, confirming EIB+ have more sensitive airways compared to EIB- (Anderson and Kippelen 2008).

5.1.3 Exercise and the effect of conditions of the inspired air

The studies showing no fall in lung function when performing exercise challenge while breathing fully saturated humid air, all support the osmotic hypothesis of EIB (Strauss et al. 1978; Henriksen et al. 1981; Hahn et al. 1984a; Wilson et al. 1990). The absence of a pulmonary response is thought to be a consequence of the fact that there is no mediator excretion during the humid conditions, however this has never been confirmed by actual measurements. We therefore initiated a study to investigate mediator release following exercise, either performed breathing room air or hot, humid air.

This study is currently ongoing and preliminary results confirm the earlier finding that no fall in lung function is apparent while breathing hot, humid air.
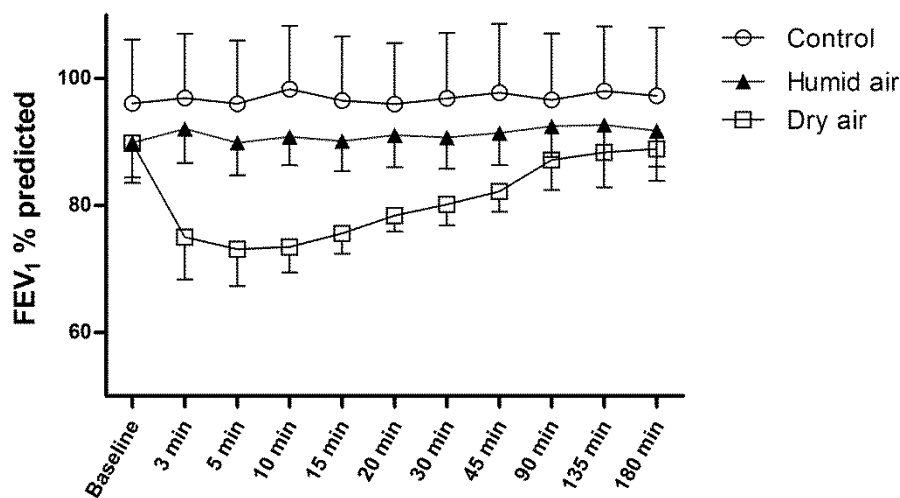

Figure 22 Airway response to exercise challenge during different conditions in 7 asthmatic subjects
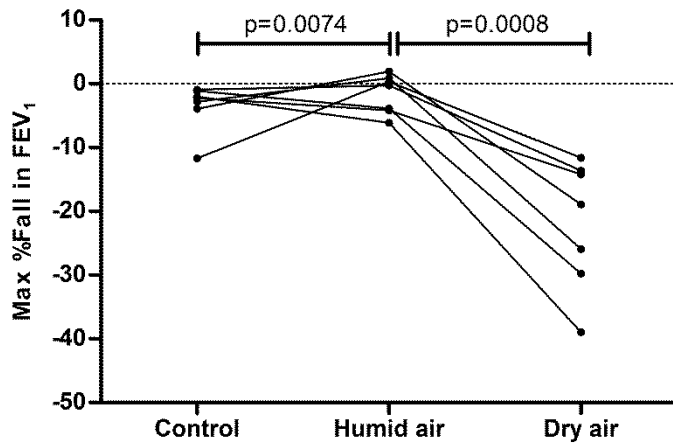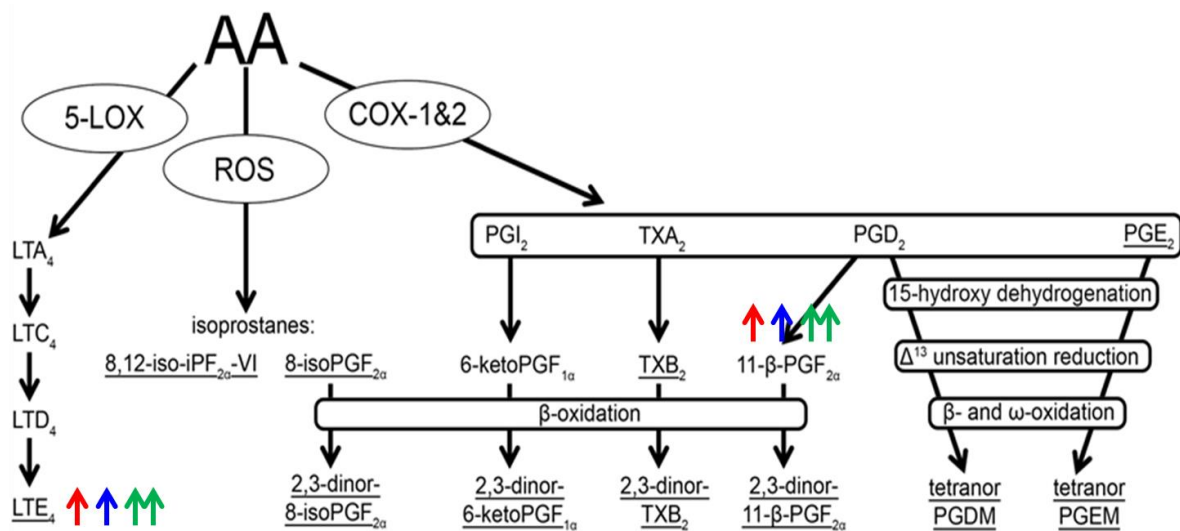
Figure 23 Maximum fall in lung function following exercise challenge during different conditions in 7 asthmatics

Interestingly, bronchoconstriction was observed in one subject during the control study day when the subjects were just sitting still at the lab. However, comparing the maximal fall during each of the study days revealed clear differences; with no fall in FEV_1 following exercise in humid air, while about a 20% fall was seen following exercise in dry temperate air. This is well in line with previous findings (Strauss et al. 1978; Anderson et al. 1979).

Regardless of the clarity of the airway data so far, the urinary mediator results have been inconclusive. More subjects need to be included before any conclusions can be drawn.

5.1.4 Summary

To summarise the initial data on mediator excretion, the pattern of mediator release appeared to be very similar in exercise (O'Sullivan et al. 1998b), mannitol and EVH challenges, lending strong support to the view they act via the same mechanisms. However, comparing the magnitude of the mediator release induced by EVH and mannitol, the latter increased release by about 100%, whereas EVH increased the release by about 50%. Also, following mannitol, the excretion was increased for a longer period. These differences suggest that mannitol is a more powerful and long-lived stimuli compared to EVH.

Figure 24 Overview of urinary mediator release in paper I-III

Another conclusion that can be drawn is that to elicit bronchoconstriction following exercise, mediator excretion needs to take place and the individual needs to be somewhat sensitive to the mediators released. However, we still did not have a full understanding of how long it takes before the excretion returns to baseline, which of course is of vital importance when moving on to study mediator release during the refractory period, i.e. what happens to the mediator excretion following a second challenge.

Humid air protects against bronchoconstriction following exercise, but it is still unclear why this is the case. When patient inclusion has been completed and all urine samples have been analysed we will most likely have a better understanding of this.

5.2 THE EFFECT OF INTERVENTIONS ON LIPID MEDIATOR RELEASE AND LUNG FUNCTION

To further understand the involvement of mediators in the development of EIB we also tested the effect of various treatments on the lung function and dynamics of mediator excretion following challenge.

5.2.1 Inhaled corticosteroids (Paper II)

Chronic treatment with inhaled corticosteroids can inhibit EIB in asthmatics (Weiler et al. 2005; Subbarao et al. 2006). Also, there is evidence that single high doses of ICS can be effective in inhibiting the response to exercise (Thio et al. 2001). In study II we tested the effect of hydroflouralkane (HFA) beclomethasone diproprionate (BDP), as this is a potent glucocorticosteroid with a small particle size, resulting in a greater peripheral deposition (Leach et al. 2002). The peripheral airways are of importance since the recruitment of the small airways into the conditioning of the inspired air during exercise is thought to be a determinant for the severity of EIB (Anderson and Holzer 2000). Also, the small airways

have a high content of mast cells in both healthy and asthmatic subjects (Carroll et al. 2002; Andersson et al. 2009).

Treatment with one single dose (1500 µg) of BDP significantly inhibited the airway response in both groups. In both groups, a small bronchodilatory effect was seen after the administration of the drug and before the EVH was initiated. BDP also reduced the post-EVH maximal change as well as the AUC_{1-90} for 11β-$PGF_{2\alpha}$ and LTE_4, in both asthmatics and athletes.
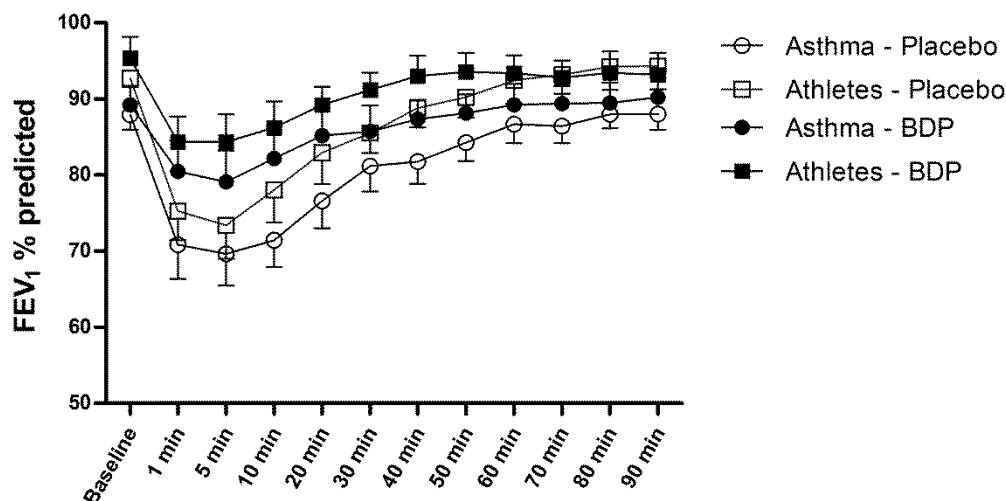

Figure 25 The effect of 1500 µg compared to placebo, on the airway response to EVH challenge in 8 asthmatic subjects and 7 athletes with EIB
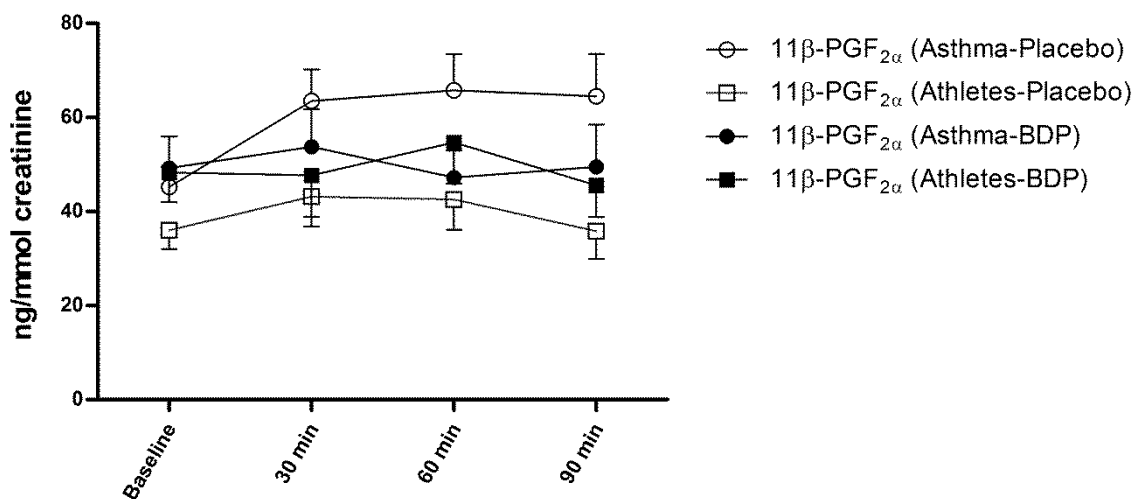

Figure 26 The effect of 1500 µg compared to placebo, on the urinary 11β-$PGF_{2\alpha}$ release following EVH in 8 asthmatic subjects and 7 athletes with EIB
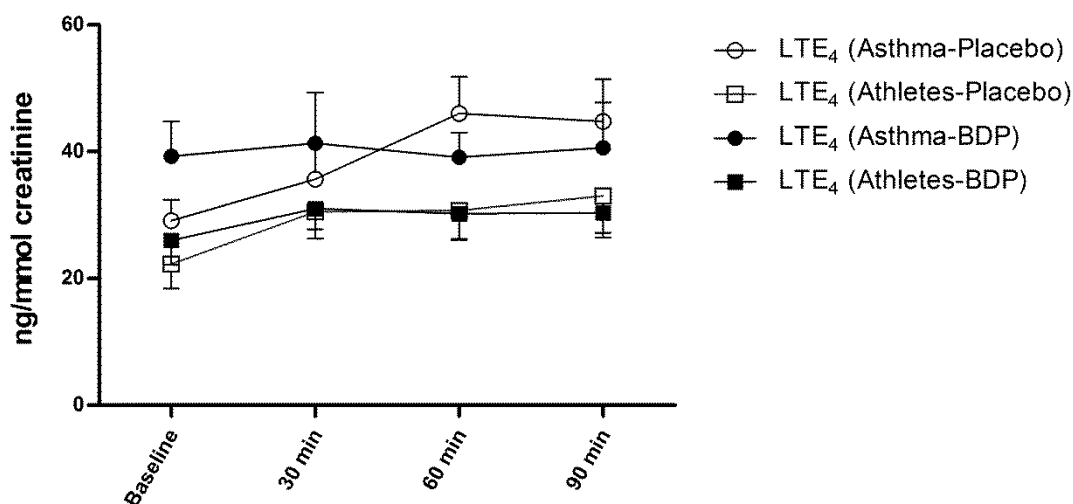
Figure 27 The effect of 1500 µg compared to placebo, on the urinary LTE$_4$ release following EVH in 8 asthmatic subjects and 7 athletes with EIB

What needs to be taken in to account however, is that the baseline level of mediators was elevated in the afternoon when the BDP treatment was given; this could partly explain the differences seen in mediator excretion between treatments. Nevertheless, since there was a clear relationship between the fall in lung function and mediator excretion it can also be argued that the treatment lead to a real decrease in mediator excretion.

The mechanism of the inhibitory effect of one single high dose of BDP is unclear. The effect is too rapid to be explained by genomic effects on nuclear glucocorticoid receptors. Previously, a single inhalation of ICS has been shown to have no effect on the response to direct challenges such as histamine (Ketchell et al. 2002), whereas a protective effect has been observed following indirect challenges such as AMP (Ketchell et al. 2002), hypertonic saline (Gibson et al. 2001), exercise (Thio et al. 2001), and allergen (Parameswaran et al. 2000). Together this suggests that the treatment affects the inflammatory cells via which the indirect challenges alter the lung function. However, it is unlikely, and there is no evidence that a single dose of ICS will reduce the number of mast cells in the airways or that their function is acutely altered (Gibson et al. 2001). There is also evidence that glucocorticoids do not directly inhibit the biosynthesis of leukotrienes (Gyllfors et al. 2006).

ICS have also been shown to be potent vasoconstrictors (Kumar et al. 2000; Mendes et al. 2003; Ewing et al. 2010), and these vasoconstrictive properties could have counteracted the reactive hyperaemia that follows hyperpnea (McFadden et al. 1986). It should be noted however that the reactive hyperaemia is thought to comprise only a minor part of EIB (Anderson and Daviskas 2000). Also, it is not clear how long-lasting the vasoconstrictive effects of ICS are (Kumar et al. 2000; Mendes et al. 2003). It is therefore unclear whether this can explain the protective effect of BDP seen 4 h after inhalation.

Taken together, the studies mentioned above suggest that there is some kind of effect on mast cells, although not a direct effect on their numbers. It may be that BDP prevents mast cell mediator release by changing the fluid balance in the epithelium and thereby preventing the creation of a hyperosmolar milieu which is normally observed following EVH. In fact, a

rapid effect of ICS has been seen on the anti-secretory response of the airway epithelium which would support such a phenomenon (Verriere et al. 2005; Urbach et al. 2006). Another plausible mechanism, and perhaps more appealing since it also provides an explanation of the protection against AMP, is that BDP inhibits mediator release through a reduction in intracellular calcium which has been reported in guinea pigs (Zhou et al. 2008).

5.2.2 Sodium cromoglycate (Paper III)

We then went on to study the effects of sodium cromoglycate (SCG) which is thought to exert its effects by stabilising mast cells and preventing their mediator release. In our study 40 mg SCG, administered 10 min prior to EVH challenge, decreased the maximum fall in lung function by approximately 50% in the EIB+ group. In the EIB- group, as expected, there was no significant fall in lung function on either of the study days. SCG blocked the increase in 11β-$PGF_{2\alpha}$ in both groups, and blocked the increase in LTE_4 in the EIB- group.
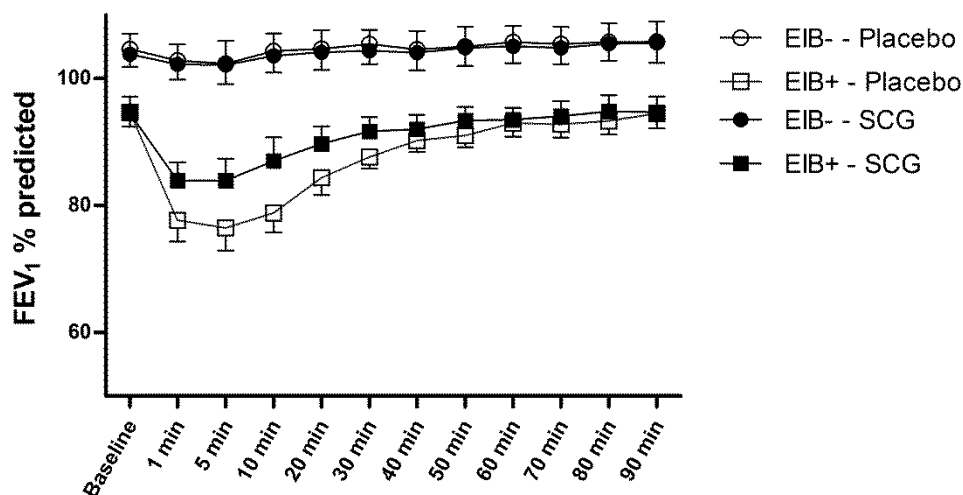

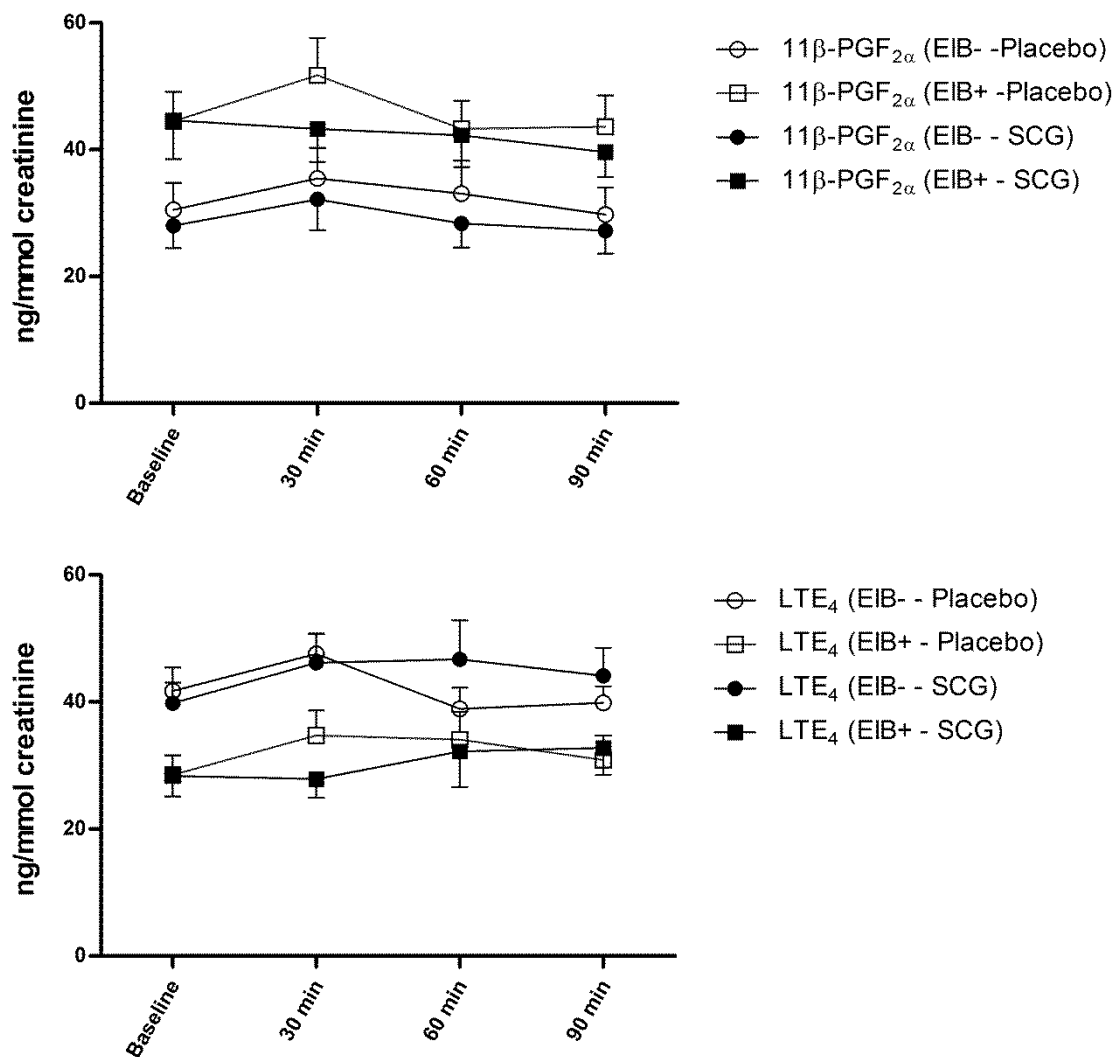**Figure 28 The effect of 40 mg SCG compared to placebo on the airway response to EVH in 11 EIB+ and 11 EIB-**

Figure 29 The effect of 40 mg SCG compared to placebo, on the urinary mediator release following EVH in 8 asthmatic subjects and 7 athletes with EIB

The finding that excretion of 11β-$PGF_{2\alpha}$ was inhibited by SCG is in keeping with earlier findings from our lab, studying the effect of SCG on mannitol-induced bronchoconstriction (Brannan et al. 2006). In that study, there LTE_4 release was observed both during placebo and SCG treatment, suggesting that LTE_4 originates from inflammatory cells other than the mast cell, most likely the eosinophil (Moloney et al. 2003). There is however evidence that SCG can also inhibit the activation of eosinophils (Moqbel et al. 1986; Kay et al. 1987). The differences in the effects of treatment on the response to challenge may also be explained by differences in how powerful a stimulus each of the challenges is. It may be speculated that EVH is less powerful than mannitol, thereby exerting a relatively smaller effect on eosinophils than mast cells, which is supported by the finding that EVH seems to give a smaller and more short-lived increase in mediator release compared to mannitol.
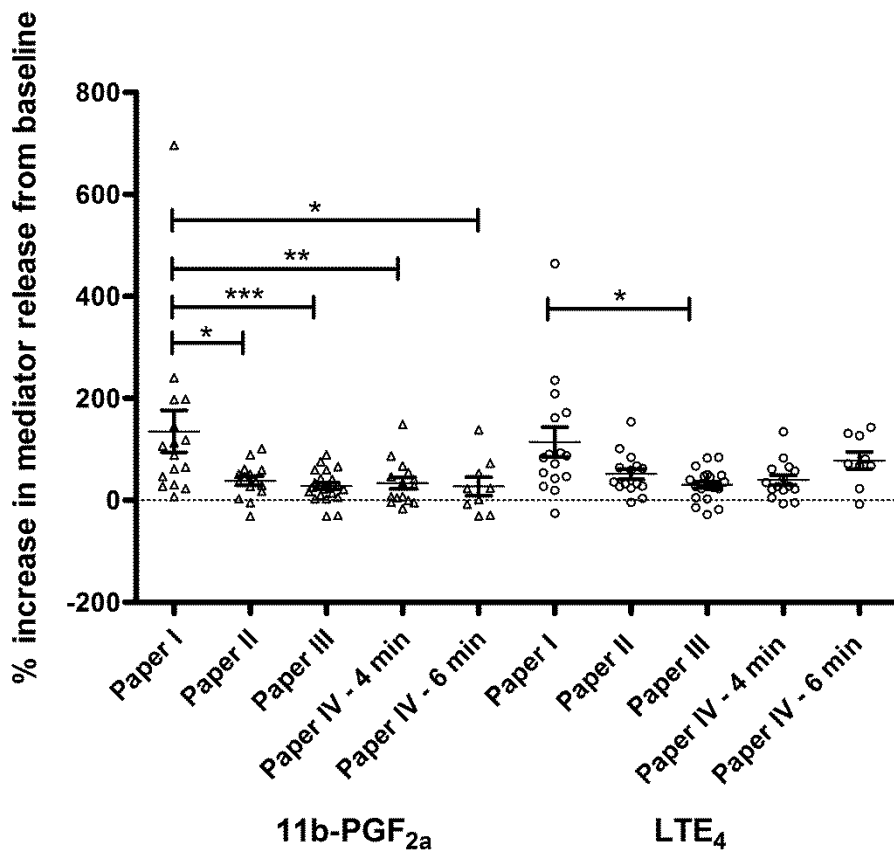
Figure 30 Comparison of the urinary mediator release in paper I-IV

The fact that mannitol seems to induce a more pronounced mediator release might be due to the fact that the stimulus of the EVH challenge subsides immediately when the challenge is stopped. For mannitol, however, it may take some time before the inhaled mannitol is cleared from the airways and is stopped from exerting its osmotic effects. Of course, the studies are not directly comparable as they do not include the same individuals; however it is a similar group of patients in all of them.

Another interesting aspect is that in paper I-III, the challenges were performed in Australia, whereas in paper IV they were performed in Sweden. In Australia the prevalence of house dust mite allergy is high, and the majority of the included subjects were sensitised. This means that the subjects undergo challenge during a period when they are exposed to an allergen they are sensitised to. This is very different from the situation in Sweden where pollen allergies are most common and we do not include patients during the pollen season. This means that the subjects are not exposed to an allergen they are sensitised to when performing the challenge. The fact that the subjects in Australia may have a more active inflammation could explain why the mediator release in paper I was so high. On the other hand, the results from the EVH studies were comparable between Australia and Sweden. It would be interesting though, to compare EIB and mediator release during and outside the pollen season in allergic subjects.

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5.2.3 Fish oil (Paper V)

Considering the growing interest and also promising data regarding dietary supplementation with omega-3 fatty acids (Mickleborough et al. 2003; Mickleborough et al. 2006; Tecklenburg-Lund et al. 2010), we also wanted to examine the effect of pre-treatment with omega-3 on the response to mannitol challenge.

Three weeks treatment with omega-3 supplements (4.0 g/d eicosapentaenoic acid and 2.0 g/d docosahexaenoic acid) had no effect, compared to placebo, on the bronchoconstriction following mannitol. This was true regardless of whether the participants were treated with ICS or not.
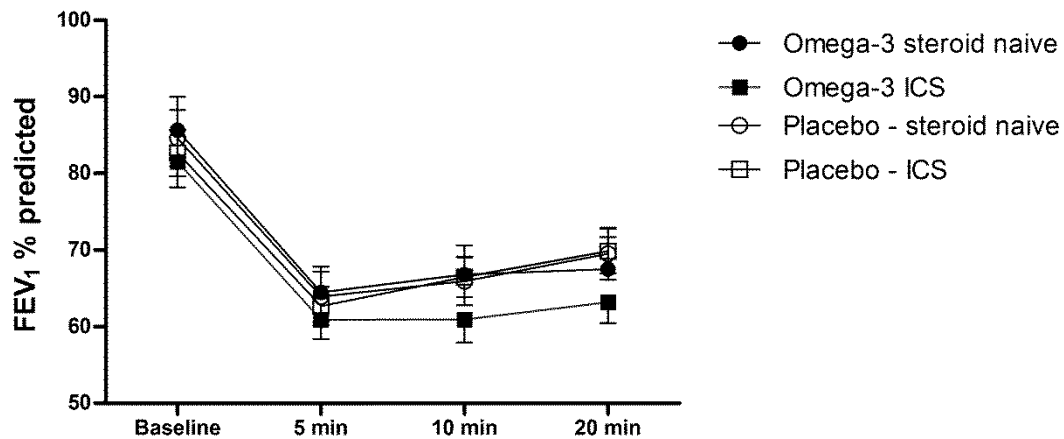
Figure 31 The effect of omega-3 supplementation on the airway responsiveness to mannitol challenge in 12 steroid naïve asthmatic subjects and 1 asthmatic subjects on regular treatment with ICS
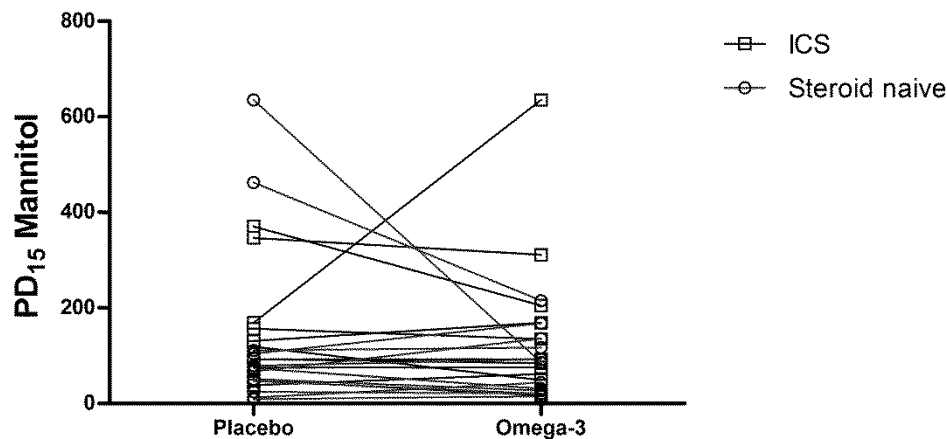
Figure 32 The effect of omega-3 supplementation on the PD_{15} mannitol in 12 steroid naïve asthmatic subjects and 1 asthmatic subjects on regular treatment with ICS

In a subgroup of patients, sputum was also analysed and we did not see any differences in eosinophil counts between treatments. Neither did the treatment have any beneficial effects on the baseline lung function nor asthma symptom score. The omega-3 did cause a lowering of blood triglyceride levels, and as expected, shifts in serum fatty acids and eicosanoid metabolites, confirming that the participants were taking their supplements. However, no effects were observed on urinary mast cell mediators.

The mechanism thought to explain the suggested positive effects of omega-3, is a diversion from the production of bronchoconstricting prostaglandins and leukotrienes containing the omega-6-substrate arachidonic acid, towards mediators biosynthesised from omega-3 substrates. This of course necessitates that these alternative mediators are less potent in causing inflammation and bronchoconstriction. However there is evidence that 5-series leukotrienes derived from omega-3 are just as potent as the 4-series that are derived from arachidonic acid (Dahlen et al. 1982). The lack of effect of omega-3 may also be explained by a failure of omega-3, even in high doses, to alter the substrate flow in tissue residing cells, whereas in blood, there are sufficient amounts to alter metabolism. In our study, we saw a decrease in serum triglycerides, and it may be that the omega-3 can alter metabolism in blood cells and other cells exposed to circulating omega-3, such as endothelial cells and renal tubular cells. In support of this, is our finding of increased urinary PGE_2 which is thought to originate from the kidney (Hamberg and Samuelsson 1971).

Our findings support the studies by Arm and colleagues who found no positive effects of omega-3 supplements in asthma (Arm et al. 1988). The same authors later saw a small effect on the late, but not the early, phase following allergen-induced bronchoconstriction(Arm et al. 1989a). Our data is however in conflict with the results of Mickleborough et al (Henriksen and Nielsen 1983; Carlsen et al. 2000; Mickleborough et al. 2006), in that we saw no beneficial effect of the omega-3 supplement. In the studies by Mickleborough et al they used the same type of treatment over the same period of time. The most obvious difference is the choice of challenge protocol where we used mannitol as compared to exercise or EVH. The studies showing beneficial effects on the response to exercise, were performed until volitional exhaustion instead of the recommended standard 8 min protocol in the ATS guidelines (Parsons et al. 2013). Another difference is in the study populations, where the subjects in the studies by Mickleborough et al, used a high degree of rescue medication with SABA (up to 10 times daily) without being on ICS, indicating that their symptoms were uncontrolled. This might represent a different group of asthmatic subjects compared to the subjects included in our study.

One weakness in our study is the lack of urine samples following challenge, which prevents us from drawing conclusions regarding the effect of changes in mediator release caused by the challenge. However, considering no effects at all were seen on the baseline excretion along with the absence of effects on the airways, there is little reason to believe there to be any effects on the excretion following challenge.

In summary, our findings suggest that it is difficult to alter the metabolic profile of tissue residing cells such as lung mast cells, and also, that there is most likely still enough arachidonic acid available as the preferred substrate for the *de novo* synthesis of leukotrienes and prostaglandins following challenge. Upon stimulation, arachidonic acid is liberated by phospholipase A_2, which is known to have a preference for phospholipids containing arachidonic acid; this in turn may explain why omega-3 has no effect on the response to challenges such as mannitol (Leslie 1997).

5.3 THE DEVELOPMENT OF REFRACTORINESS FOLLOWING CHALLENGE AND ITS RELATION TO RELEASE OF LIPID MEDIATORS

Following the initial studies focusing on mediator excretion after single challenge, we wanted to further study excretion during the refractory period which was also the main purpose of the thesis. Exercise-induced bronchoconstriction gained increased interest in the 60's and 70's, and in the 80's the mechanisms began to become identified (Anderson and Daviskas 2000). Early in this search, refractoriness was found to occur with repeated challenges, and the potential to take advantage of this natural protection in the search for new treatments was quickly identified. Several suggestions for the mechanism of refractoriness were put forward, with the most popular being depletion of mast cell mediators (Edmunds et al. 1978), and the release of protective prostaglandins, the latter supported by the finding that NSAIDs abolish refractoriness (O'Byrne and Jones 1986; Margolskee et al. 1988).

5.3.1 Mannitol (Paper I)

First, we wanted to test whether mannitol-induced bronchoconstriction is followed by a period of refractoriness, and secondly we wanted to test whether this refractoriness is associated with a decreased release of mast cell mediators.

Following the second challenge the maximum fall in lung function was about 50% less than the fall following the initial challenge. The degree of protection was individual and continuous with a variation from 0 to 88%.

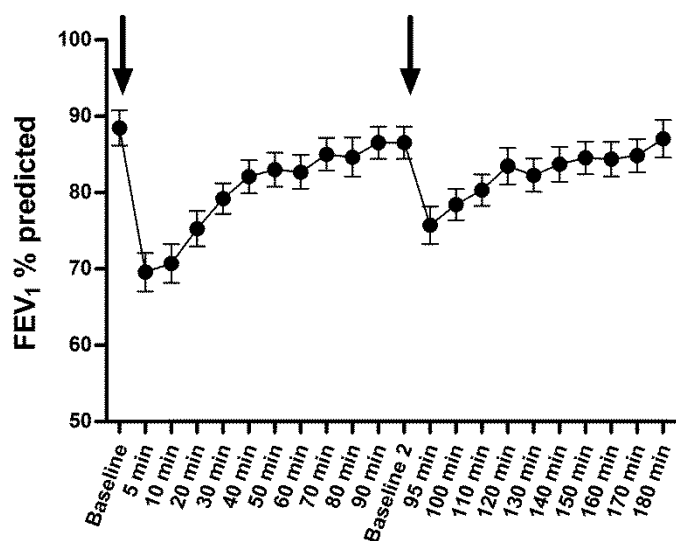**Figure 33 Airway response to repeated mannitol challenge in 16 asthmatics. Arrows indicate time of mannitol challenge.**

The release of both 11β-PGF$_{2\alpha}$ and LTE$_4$ was increased both after the first and the second challenge. Mediator excretion did not however return to baseline levels before the second challenge was initiated which complicates the interpretation to some extent.
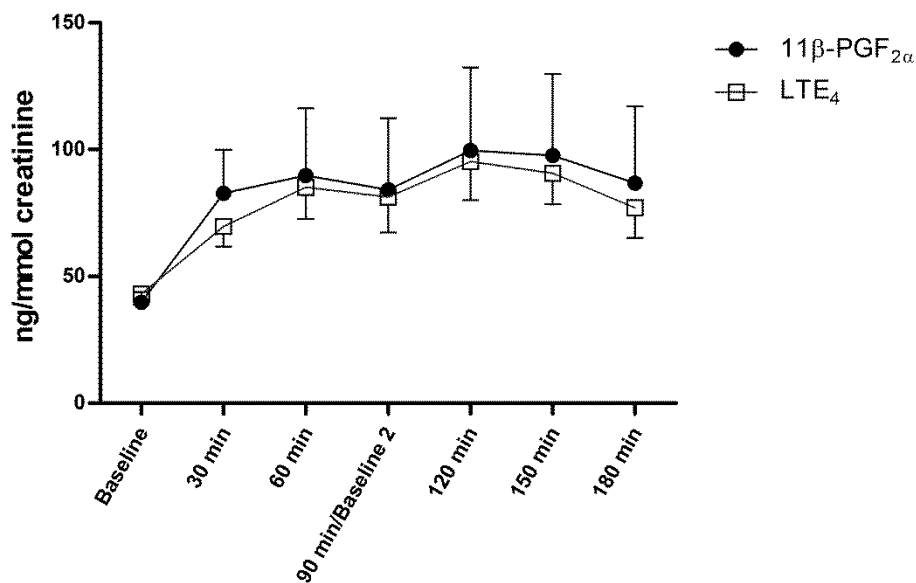
Figure 34 Urinary mediator release following repeated mannitol challenge in 16 asthmatics

Nevertheless, previous studies (Brannan et al. 2003; Brannan et al. 2006) have shown that mediator excretion returns to baseline within 90-120 minutes, suggesting that the increase following the second challenge is a true increase. These findings are in conflict with mediator depletion being the mechanism of refractoriness.
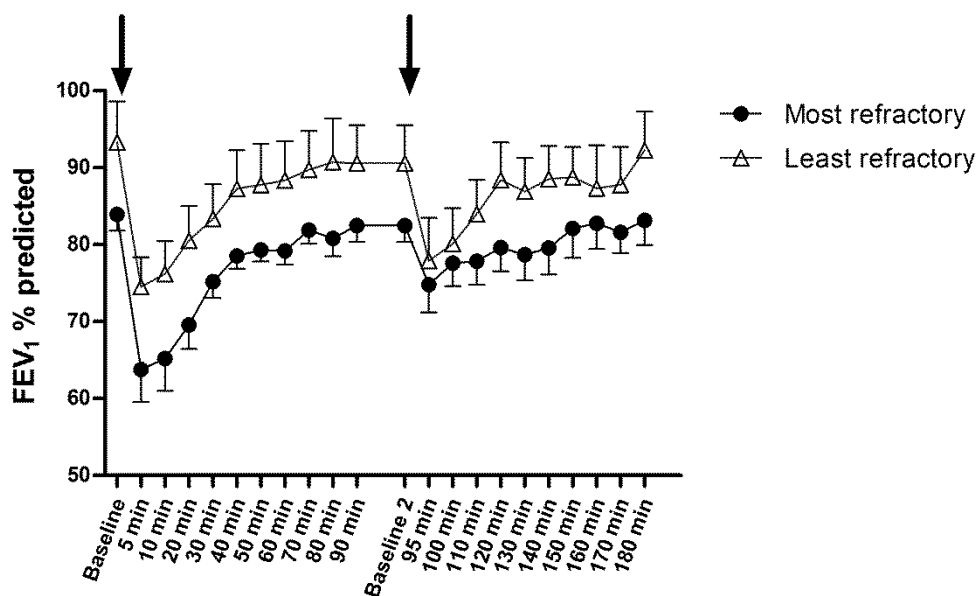
Figure 35 Comparison airway response to repeated mannitol challenge in the 6 most refractory and the 6 least refractory subjects

Figure 36 Comparison of urinary mediator release following repeated mannitol challenge in the 6 most refractory and the 6 least refractory subjects

Comparing the 6 most refractory with the 6 least refractory subjects revealed a clear pattern with the most refractory subjects displaying greatest mediator excretion. This suggests that the levels of mediators are important in the development of refractoriness and lead us to suggest that the decreased responsiveness occurs at the level of the airway smooth muscle.
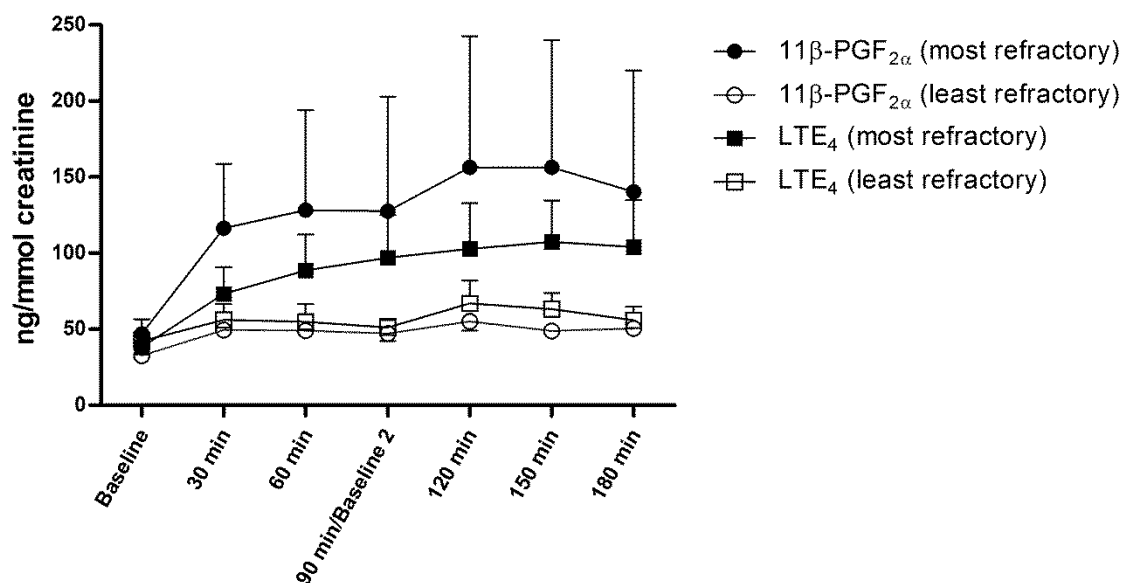
5.3.2 EVH (Paper IV)

From the results of study I, where excretion was found to be elevated at 90 min, it was obvious that we still needed to define the optimal time between challenges in order to study mediator release during refractoriness, while making certain not to extend the interval too much and thereby missing the refractory period completely. We decided to first compare the use of 60 and 180 min intervals between repeated 4 min EVH challenges.

In general, the 4 min challenge rendered rather weak responses in the 16 subjects included in the study, and several subjects did not reach the maximum fall of >10% in FEV_1 following the first challenge. Refractoriness was seen however, yet only accounting for absolute differences of a few percent in the maximal fall in lung function. Also, mediator excretion did not increase to any great extent following challenge. Nevertheless, the data did not reveal evidence of a decreased release following the second challenge. It was clear that a 60 min interval is insufficient since this does not allow enough sampling times for the urine. For the 180 min interval, the mediators had returned to baseline which makes interpretation of mediator release following the second challenge more straightforward.

Having obtained these results from the first part of the study, we decided to initiate a second part using 6 min EVH challenges to optimise the conditions and achieve stronger responses, both concerning the airway response and the mediator release. We chose the 180 min interval between the challenges to be certain that mediators would return to baseline before the second challenge. The 6 min challenge did indeed render stronger responses, and we found a variable degree of protection in the 8 subjects included. Regarding mediator

excretion, the group mean showed an increase in LTE_4 but not $11\beta\text{-PGF}_{2\alpha}$ after the first challenge, and vice versa after the second challenge. The patterns displayed by the individual graphs, however, showed a strong tendency towards an increase of both mediators following both challenges in line with the findings of study I. Presumably the study was slightly underpowered to adequately reveal group effects.

To get a better understanding of the specific mediators released, we decided to further analyse the urine samples using a UPLC-MS/MS panel, enabling us to study a wider range of lipid-derived mediators. Of the 30 metabolites included in the panel, we found measurable levels of 13. The most abundant mediators in descending order were tetranor-PGEM (~3500 ng/mmol creatinine), the main isoprostane metabolite $8,12\text{-iPF}_{2\alpha}\text{-VI}$ (~500 ng/mmol creatinine), tetranor-PGDM (~200-250 ng/mmol creatinine), and $PGF_{2\alpha}$ (~100 ng/mmol creatinine). Metabolites of thromboxane were slightly less abundant, and CysLTs were found at the lowest concentrations of all measured compounds. To compare methods, EIAs for a range of the mediators found in the UPLC-MS/MS analysis were also used. Comparing EIA and UPLC-MS/MS results, the levels detected by EIA were generally higher compared to the levels detected by UPLC-MS/MS. There was however a good correlation between the EIA and UPLC-MS/MS results. Bland-Altman plots,(Bland and Altman 1986) and correlations for the measured mediators are found in paper IV, online supplement figure 2. Graphs showing the individual mediators can be found in paper IV, figure 4 and online supplement figure 1.

PGD_2 metabolites – the peak excretion of $11\beta\text{-PGF}_{2\alpha}$ (EIA) compared to baseline did not reach significance following challenge I, but there was a significant increase following challenge II. The excretion returned to baseline before the start of the second challenge. By UPLC-MS/MS, $11\beta\text{-PGF}_{2\alpha}$ was below the detection limit, however its metabolite 2,3-dinor-$PGF_{2\alpha}$ was found at similar concentrations to the $11\beta\text{-PGF}_{2\alpha}$ values indicated by EIA, and an increase was observed following both challenges. The EIA results for tetranor-PGDM, a later and more abundant metabolite of PGD_2, showed increased levels following challenge I, but failed to reach significance following challenge I. Conversely, by UPLC-MS/MS, the increase in tetranor-PGDM failed to reach significance following challenge I, but increased significantly following challenge II.

Cysteinyl leukotrienes – there was an increase in LTE_4 (EIA) from baseline following challenge I, but not following challenge II. The excretion was still elevated before the start of the second challenge which may explain why no increase was observed. By UPLC-MS/MS most of the subjects displayed increased levels following challenge I, with a strong tendency for the whole group although the difference was not statistically different. The levels returned to baseline before challenge II, and significantly increased levels were also seen following challenge II.

Thromboxanes, $PGF_{2\alpha}$ and Isoprostanes – TXB_2 and its metabolites, the isoprostanes and $PGF_{2\alpha}$ failed to display consistent increases, irrespective of whether analysed by EIA or UPLC-MS/MS.

Prostaglandin E_2 – The EIA for PGE_2 showed increased concentrations following challenge I, but not following challenge II. By UPLC-MS/MS the levels of PGE_2 were significantly

increased following both of the challenges. Tetranor-PGEM increased significantly following challenge I, whereas the increase following challenge II failed to reach significance. There were no differences in the levels of urinary PGE_2 between male and female subjects, whereas the levels of PGEM were significantly higher in male subjects. The peak levels of PGE_2 or PGEM after the first or second challenge did not correlate with the degree of refractoriness.

Prostacyclin – Using EIA, clear increases in the prostacyclin metabolite 6-keto-$PGF_{1\alpha}$ were observed following both challenges.
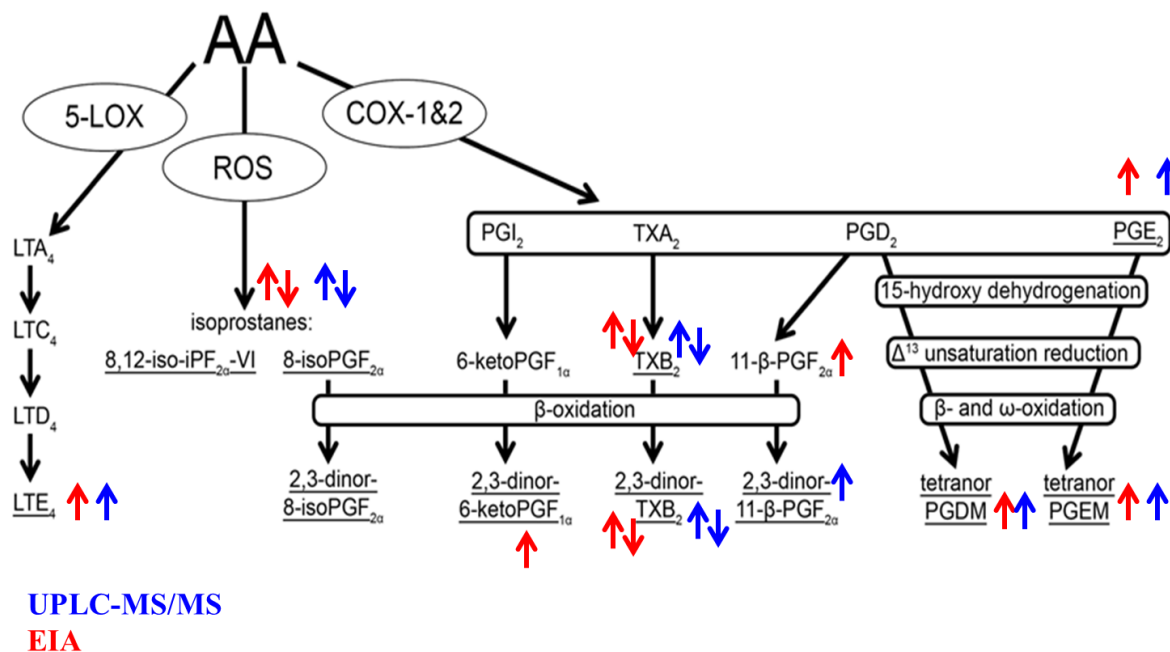
Figure 37 Overview of mediator release in paper IV. Blue arrows indicate data from UPLC-MS/MS analysis; red arrows indicate EIA data. Two way arrows indicate that the data was not consistent for that mediator.

Regarding CysLTs and PGD_2 metabolites, the current results confirm findings from our previous studies, where increased release was observed following exercise and exercise mimicking challenges. We also show for the first time that metabolites indicating the release of PGE_2 and PGI_2 were increased after both challenges. The finding of increased PGE_2 and PGI_2 is interesting since these two mediators are known to be bronchoprotective (Woodward et al. 2011). This finding further supports the notion that protective prostaglandins are involved in the development of refractoriness, and may explain why pre-treatment with NSAIDs blocks the development of refractoriness.

We observed increases in both primary PGE_2, and its most abundant metabolite tetranor-PGEM after EVH. This is distinctly different to what has been seen following allergen challenge, where no increases in urinary PGE_2 or PGEM were seen (Daham et al. 2014). Release of PGE_2 following EVH, but not allergen challenge may be explained by the different mechanisms by which the challenges elicit bronchoconstriction. Both challenges are mast cell dependent, but whereas allergen-induced bronchoconstriction is triggered by IgE cross-linking, EVH activates mast cells via changes in osmolarity (Anderson and Daviskas 2000). It is possible that the changes in osmolarity may induce release of PGE_2 from other cells in the airways, such as the epithelium (Hjoberg et al. 2000; Harrington et al. 2008). This

is the first demonstration of increased primary PGE_2 following airway challenge, and the question arises concerning the source of the PGE_2. From studies of PGE_2 metabolism, it is known that PGE_2 is rapidly metabolised, and urinary PGE_2 excretion following the infusion of PGE_2 was only detectable when given as a renal artery infusion, but not as a brachial vein infusion (Zipser and Martin 1982). The current concept is therefore that the kidney itself is the exclusive source of urinary PGE_2. We think our finding of increased urinary PGE_2 could be due to the systemic load of PGE_2 following a massive release from the airways of which a small proportion is excreted in the urine un-metabolised. Another explanation for increased urinary PGE_2 could also be that the CysLTs induce release of PGs when circulated in the renal circulation. Contradicting the latter is the fact that a release of PGE_2 should also have been observed following allergen challenge, since this challenge caused increases in CysLTs.

Another interesting finding were the undetectable levels of $11\beta\text{-}PGF_{2\alpha}$ following analysis by UPLC-MS/MS, whereas the levels of its metabolite $2,3\text{-}dinor\text{-}PGF_{2\alpha}$ corresponded well to the $11\beta\text{-}PGF_{2\alpha}$ detectable by EIA. This suggests that what we actually measure with the EIA is $2,3\text{-}dinor\text{-}PGF_{2\alpha}$ and not $11\beta\text{-}PGF_{2\alpha}$. This has previously been noted in work from our group (O'Sullivan et al. 1999), though the commonly used term has still been $11\beta\text{-}PGF_{2\alpha}$ since this is the name of the commercially available kit. When testing the cross-reactivity, we found that the antibody in the $11\beta\text{-}PGF_{2\alpha}$ kit recovered 88% of $11\beta\text{-}PGF_{2\alpha}$, and 11% of $2,3\text{-}dinor\text{-}PGF_{2\alpha}$. This indicates a substantial cross-reactivity between the antibody and $2,3\text{-}dinor\text{-}PGF_{2\alpha}$. Since the two metabolites originate from the same primary mediator, this does not have any practical implications for the monitoring of mast cell activation. However, the varying terminology may cause confusion and we therefore suggest that, in the future, PGD_m could be a more useful way to describe these early metabolites of PGD_2.

5.4 ORGAN BATH EXPERIMENTS TO UNDERSTAND MECHANISMS

Mechanistic studies are often difficult to perform *in vivo* because certain specific agonists and antagonists cannot be administered to humans. As an alternative, we can stimulate human airways in an organ bath to study the contractile responses to various stimuli. We therefore designed a new model of mannitol stimulation in the organ bath.

Jongejan et al. have previously described the effects of hyperosmolar challenge on isolated human bronchi (Jongejan et al. 1991). They found that contraction was induced with increased osmolarity after the addition of mannitol to the organ bath. These contractions were however insensitive to pre-treatment with a histamine antagonist, CysLT synthesis inhibitor, the COX-inhibitor indomethacin, as well as the mast cell stabilising drug, sodium cromoglycate. Taken together, this would suggest that the bronchoconstriction is an effect of osmolarity changes that directly affect the bronchial smooth muscle.

Other interesting studies of the effects of hyperosmolarity on isolated tissue were performed by Hjoberg et al, who in a series of papers describe the effect of hyperosmolarity on relaxing factors in the guinea pig trachea (Hjoberg et al. 1999; Hjoberg et al. 2000; Hjoberg et al. 2003; Hjoberg et al. 2005). Considering EIB is thought to be caused by

changes in osmolarity in the airways upon exercise, and that we believe PGE_2 plays a role in the development of refractoriness, we wanted to test this further in the human bronchi.

Adding Mannitol at different concentrations up to 850 mOsm in the bath fluid caused a contraction. However this contraction seemed to be caused by a disruption of the membrane potential since no further contraction could be elicited. Also, pre-treatment with antagonists of PGs, CysLTs and histamine had no effect on the bronchoconstriction, indicating that this reaction was not in any way mirroring what we see *in vivo* following mannitol inhalation.

We then went on to test what happened if, following a short exposure to mannitol, we changed the media to wash the mannitol away.
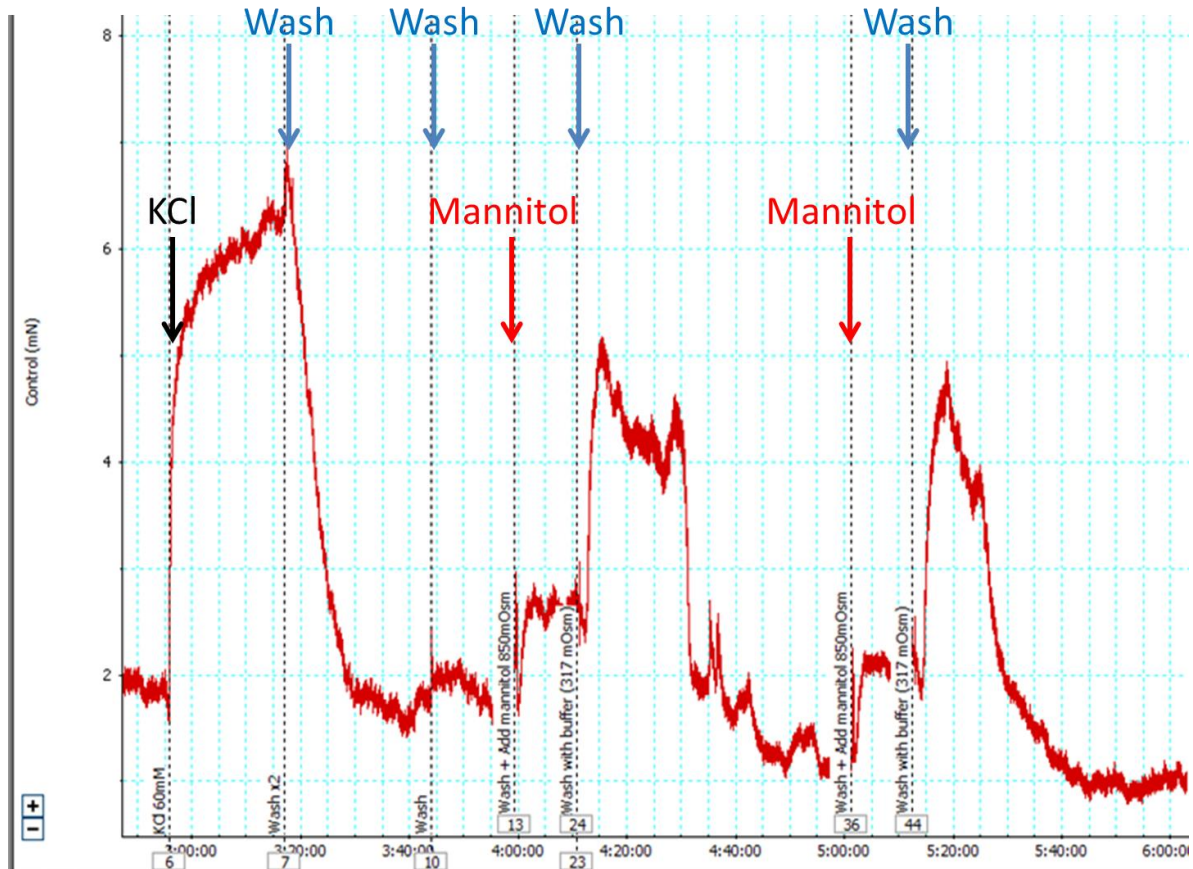
Figure 38 Trace of the contractile response in isolated human bronchi in the organ bath. The first peak represents the contractile response to potassium chloride (KCL). The contraction then returns to baseline following a wash. Mannitol (850 mOsm) is added and a small contraction is seen. Following wash a more powerful contraction is seen; this returns to baseline spontaneously after about 20 min. The response to mannitol is the repeatable.

The observed effect is demonstrated in the traces above. After adding 850 mOsm we saw a small contraction. Washing away the mannitol after 10 minutes results in a small relaxation, after which a strong contraction is seen. We hypothesised that this second contraction is caused by the mediators released from the mast cell, mediators which are bound to their respective receptor on the bronchial smooth muscle. When the osmolarity is normalised the muscle regains its ability to contract and the mediators can then induce the contraction we see. To investigate this theory we then went on to test the effect of different antagonists on the second contraction.
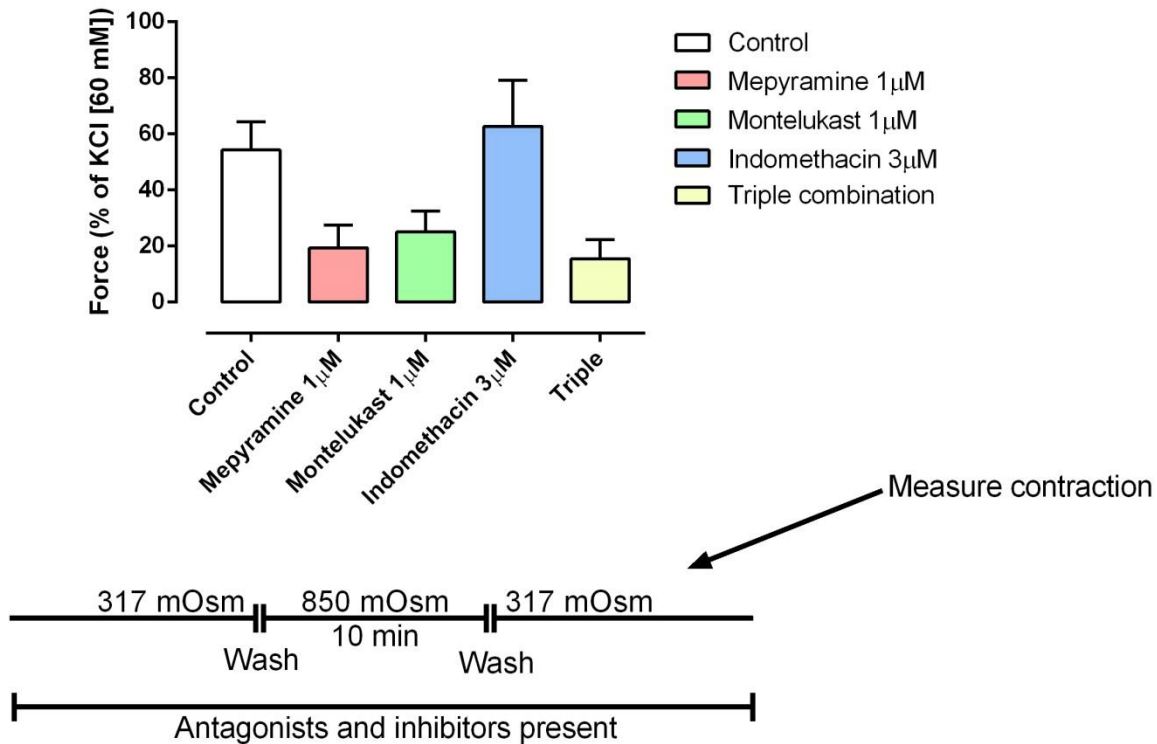
Figure 39 The effect of interventions on the airway response to mannitol in isolated human bronchi in the organ bath

Pre-treating the segments with the histamine H_1 receptor antagonist mepyramine decreased the contraction, and the same was true when pre-treating with the $CysLT_1$ antagonist montelukast. Pre-treatment with indomethacin did not decrease the contraction. When pre-treating with a triple combination, antagonism with mepyramine, montelukast and the TP receptor antagonist SQ-29,548, no further was decrease seen, compared to single treatment with mepyramine or montelukast.

The above described findings are only from one patient, and of course need to be replicated before drawing firmer conclusions. However, it is intriguing that we are able to induce a response that is similar to EIB in that it is sensitive to histamine and leukotriene antagonism. It should be noted, however, that upon inhalation, mannitol affects the osmolarity of the submucosa by forcing water across the epithelium; and the smooth muscle or the mast cells are most likely never directly exposed to the mannitol itself. In the organ bath setup, the epithelium as well as the underlying tissue is directly exposed which of course can have great effects.

Apart from replicating the findings above, we also need to evaluate the effects of different concentrations of mannitol, and different exposure times to fine-tune the system. We then want to take it further to try to study refractoriness. From what we have seen this far the response is repeatable when the second exposure to mannitol occurs 60 min after the first. Perhaps the fact that all the tissue is exposed to mannitol is the explanation for not being able to observe refractoriness, and when we wash away the mannitol we may also be washing

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away important factors involved in the induction of refractoriness. In the future it would be interesting to test a setup similar to the one used by Hjoberg et al (Hjoberg et al. 2003), and expose only the epithelial surface to the mannitol.

6 CONCLUSIONS

The main conclusions of this thesis are:

- The mast cell mediators PGD_2 and CysLTs are released following exercise, EVH, and mannitol challenge.

- The airway response and mediator release is qualitatively similar for the above mentioned challenges. Quantitatively, mannitol induced greater mediator release compared to EVH.

- The release of mediators occurs in athletes both with and without EIB, indicating that airway hyperreactivity towards the released mediators is a key determinant of positive EIB.

- SCG and BDP block the airway response following EVH. This was accompanied by a decreased release of mast cell mediators indicating these as being a contributing mechanism to the decreased airway response after the drugs.

- Treatment with omega-3 fatty acids for 3 weeks had no effect on the airway response to mannitol or the urinary release of mast cell mediators.

- The use of EIA is an attractive alternative to UPLC-MS/MS, giving similar mechanistic information regarding measurements of CysLTs, metabolites of PGD_2, PGE_2, TXB_2 and PGI_2.

- Refractoriness after exercise challenge is not caused by a decreased release of mast cell mediators following the second challenge.

- Subjects that are more refractory tend to release more mediators, suggesting the mechanism of refractoriness involves desensitisation to the released mediators at the level of the airway smooth muscle.

- PGE_2 and PGI_2 are released during the refractory period following EVH, lending support for prostaglandins as being of importance to the development of refractoriness.

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7 GENERAL DISCUSSION

Defining mediator release following single challenge

A prerequisite to be able to define mediator release during the refractory period is to better understand the dynamics of mediator release following one single challenge. In our studies, as well as in earlier studies (O'Sullivan et al. 1998b; Brannan et al. 2003; Brannan et al. 2006), we saw increased urinary excretion of CysLTs and PGD_2 following different exercise-type challenges. Urine is attractive in that it is a non-invasive sampling method and does not affect the response to the challenge itself. The lag time between the release in the lung and the excretion into the urine, may however complicate the interpretation of data. At the time when our studies were performed, urine was the only available non-invasive choice considering the uncertainty of sampling methods such as exhaled breath condensate (EBC) and saliva (Gaber et al. 2006). Invasive methods such as induced sputum are poorly suited for repeated measurements following challenge because the collection procedure itself could be considered a challenge to the airways.

To better characterise the urinary release, to get a better understanding of what happens, and when, we compared the use of different challenges, as well as studied the effects of different interventions. In this thesis, we describe for the first time the nature of mediator release following EVH. Comparing the excretion following challenge, it was obvious that mannitol gave a stronger and more long-lived signal regarding mediator release. This may be explained by the fact that an EVH challenge lasts for 6 min and thereafter the stimuli ceases abruptly. Mannitol on the other hand is given as a step-wise, increasing dose, meaning that the challenge itself often takes longer. Also, there is reason to believe that the inhaled mannitol takes some time for the lung to eliminate. There is previous data from allergen challenges showing that cumulative challenges produce a greater increase in urinary excretion of LTE_4 than a single-dose challenge (Kumlin and Dahlen 2000).

Considering the treatment responses, both the airway response and the release of mediators were inhibited by inhalation of SCG, as well as one single high dose of ICS. SCG is known to act on mast cells, and probably also on eosinophils, and it is therefore expected that we see a decreased response and mediator release. For the high single-dose ICS on the other hand, the mechanism of inhibition is less clear, but genomic effects are highly unlikely, considering the short time between administration of the drug and the challenge. Since we saw decreased mast cell activation, this indicates that mast cells were somehow affected, even though little can be said about the mechanisms involved. It may very well be that the ICS changes the local milieu of the airways, and thereby simply prevents the osmotic changes that otherwise are seen following exercise. Following pre-treatment with omega-3 fatty acids, no effect was seen on the bronchial responsiveness to mannitol, or the airway inflammation measured by sputum eosinophils. In this study it is unfortunate that no samples were collected after the mannitol challenges. However, since there were no effects at all, it is very likely that the mediator release after challenge was unaffected.

A big step forward in our understanding of the mediators released was the application of a recently developed mass-spectrometry method to identify a wider range of eicosanoids and

other lipids (Balgoma et al. 2013). It was only possible to apply this method to Paper IV within the scope of this thesis. The future analysis of more samples will of course further increase our knowledge. This was the first time the release of a wider range of eicosanoids in urine was described following EIB. It was striking that even though samples from only 8 subjects were analysed, the increase in excretion of some of the mediators was very pronounced. Another important finding was the, in general, good agreement between EIA and UPLS-MS/MS when the methods were compared. Numerically the results were, as expected, not always the same, but the pattern of release remained the same. This makes EIA an attractive methodological alternative that is more easily available and also less labour intensive.

Our experiments are designed to assure that each subject is their own control, minimising the risk of drawing false conclusions from the data due to variations in the analysis. Given that we see some variation in baseline samples, on different days, one single urine sample says very little about what is going on. Using creatinine to correct for dilution of the urine samples is a generally accepted method and we have used this throughout to be able to compare the data. However, we have also started to look into other ways of presenting the data; for example calculating excretion per hour instead. This gives slightly different results and what should be the method of choice remains to be established. For the time being, we continue to normalise in relation to creatinine, one advantage being that this enables comparisons with older results.

More recently, new non-invasive methods have been developed measuring exhaled volatile and non-volatile particles (Fens et al. 2011; Almstrand et al. 2012). There is also a new method of bronchosorption where airway lining fluid is absorbed via a device that is inserted into the airways through bronchoscopy (Jackson et al. 2014). Urine is still very attractive since it is non-invasive and does not affect the challenge itself. The data we have on urinary excretion is also robust. However, we do get a reflection of whole body release, and even though the vast majority of mediator release occurs in the lung after airway challenge, the contribution from the rest of the body remains to be determined. Therefore it is of importance to study local release in the airways which may be performed by the methods described above. Applying these new techniques could provide a better understanding of how local release affects urinary excretion.

Differences in sensitivity to the released mediators explains EIB
In paper III, we found that both the EIB+ and the EIB- group demonstrated mediator release following EVH. The fact that the mediators released only induced bronchoconstriction in the EIB+ subjects raises the question of what differs between these two groups. From previous studies, it is known that EIB+ are more sensitive to methacholine challenge, indicating that their airways are more hyperreactive and this probably explains why they also respond with bronchoconstriction following mediator release. However, it should be noted that we only measured CysLT and PGD_2 in this study and we don't know anything about the other mediators released. For example, it would be interesting to see if the release of PGE_2 differed between EIB+ and EIB- subjects. PGE_2 inhalation has been shown to protect against allergen,

and metabisulphite-induced bronchoconstriction, but no effect has been seen on direct stimuli such as methacholine (Pavord et al. 1991; Pavord et al. 1993; Melillo et al. 1994; Gauvreau et al. 1999). Also there is evidence suggesting that epithelial injury occurs following exercise or exercise mimicking challenges (Bolger et al. 2011a; Bolger et al. 2011c; Kippelen et al. 2013; Simpson et al. 2013). Furthermore, it is thought that this injury leads to a decreased release of PGE_2 (Hallstrand et al. 2005a; Hallstrand et al. 2005b). An alternative explanation of the bronchoconstriction in EIB+, but not in EIB-, could therefore involve differences in the ability to release PGE_2 and protect against the EVH.

The role of PGE_2 in the development of refractoriness

The inhibitory effect of pre-treatment with NSAIDs on refractoriness is well established (O'Byrne and Jones 1986; Mattoli et al. 1987a; Margolskee et al. 1988; Hawksworth et al. 1992; Manning et al. 1993; Wilson et al. 1994). This strongly suggests that a cyclooxygenase product is involved in the development of refractoriness. Of the prostaglandins, PGI_2 and PGE_2 are known to be bronchoprotective, and PGE_2 inhalation has been shown to inhibit EIB (Melillo et al. 1994). However, no one has previously been able to confirm release of these two mediators following EIB. In paper IV we were however able to demonstrate release of both PGE_2 and PGI_2 following EVH for the first time. It remains unclear exactly how the protective prostaglandins would exert their actions in EIB. The action of PGE_2 has been extensively studied and it has been shown that PGE_2 exerts its effects via the EP_{1-4} receptors; EP_1 and EP_3 were thought to mediate bronchoconstriction, whereas it was thought EP_2 and EP_4 caused bronchorelaxation (Woodward et al. 2011). From recent studies however, it seems that the situation is more complex. In organ bath experiments, we have established that PGE_2 at lower concentrations relaxes human bronchi, but at higher concentrations causes constriction (Säfholm et al, J Allerg Clin Immun *in press*). The bronchodilation was mediated through the EP_4 receptor. Moreover, PGE_2 inhibited anti-IgE induced bronchoconstriction by reducing mast cell mediator release. This effect was shown to be EP_2 mediated.

These findings are of interest, because they provide a mechanism that could be of importance in EIB considering the central role of mast cells. This would suggest that PGE_2 causes refractoriness by inhibiting the release of mast cell mediators, which we however have not observed, rather, the release has been the same following both challenges. Also, it is not clear how long-lasting this mast cell inhibiting effect is. The urinary excretion of both PGE_2 and PGI_2 returned to baseline within 90 minutes, suggesting the release in the lung had ceased some time before that. This would seem to make it difficult to explain why refractoriness can last as long as 4 hours. Interestingly, in the recent study of PGE_2 receptors in human bronchi (Säfholm et al), it was found that PGE_2 turns on a long-lasting down-regulation of mast cells. Irrespective of whether this mast cell inhibition plays a role in refractoriness or not, it could also have a vital role in controlling the response to challenge itself, as a self-limiting mechanism to stop the reaction from deteriorating. There is evidence to suggest that CysLTs themselves can activate mast cells (Paruchuri et al. 2008). This would create a self-activating system considering the release of mediators following EIB. Mechanisms such as PGE_2-induced inhibition of mast cell activation as well as

desensitisation of receptors may be vital mechanisms to even survive an exercise challenge. Interestingly, considering the "self-activating loop" between CysLTs and mast cells, it is known that leukotrienes are important in sustaining EIB (Dahlen et al. 2002; Anderson 2004). In general, a lot of attention is often paid to the appearance of a reaction, whereas there is little focus is on why it disappears.

The mystery of humid air

Another missing link, in the understanding of EIB and refractoriness, is the effect of humid air, where a period of tolerance has been noted by some but not by others, when an exercise challenge breathing hot humid air is performed before performing an exercise challenge breathing room air (Anderson et al. 1979; Ben-Dov et al. 1982; Hahn et al. 1985; Wilson et al. 1990). It is interesting that this tolerance after a non-bronchoconstriction challenge is sensitive to NSAIDs which suggests similar mechanisms of refractoriness. Generally, it is believed that when exercise is performed breathing hot humid air, there is no mediator release which would explain the absence of bronchoconstriction. However, it is interesting that if humid air is breathed during the recovery phase following challenge, the recovery to baseline is much more rapid (Hahn). This suggests that the humid air itself can induce the release of something protective (Johnston et al. 1992). We have initialised a study to compare urinary excretion following exercise challenge in either room air or hot humid air, but the data so far is inconclusive regarding the mediators released. A further step would then be to examine mediator release during the refractory period and the effect of the condition of the inspired air.

A proposal for the mechanism of refractoriness

In paper I and IV, more emphasis was put into understanding the mechanisms of refractoriness. In paper I, we could for the first time demonstrate refractoriness to repeated mannitol challenge. This was shortly thereafter replicated by another group (Suh et al. 2011). In our study, the release of mediators was increased after both challenges; and the most refractory subjects displayed the greatest mediator release. This was then replicated in paper IV, where we also saw increased release following the two EVH challenges. From the results of Paper I and IV, decreased mediator release seems unlikely as the mechanism, since the release of mediators also remained the same after the second challenge. The fact that the most refractory in paper I also displayed the highest mediator release, lends support to the notion that refractoriness occurs at the level of the airway smooth muscle.

Our *in vivo* studies in intact humans do not permit conclusions about this mechanism to be drawn at a molecular level, but the rationale behind this suggestion comes from studies of G-protein coupled receptors (GPCRs), a family of receptors to which the eicosanoid receptors belong. In studies of GPCRs, a very common finding is agonist-promoted desensitisation, namely, that with increased or prolonged exposure to an agonist, the receptor of that agonist is down-regulated to balance the response (Perry and Lefkowitz 2002). This has been extensively studied with the β_2-adrenergic receptor which is the target of β_2-agonists commonly used in asthma treatment (Cazzola et al. 2013). With acute stimulation, this desensitisation of the receptor is associated with phosphorylation of the receptor by kinases

(McGraw et al. 2003). The receptors are then internalised into the cell by arrestins (Perry and Lefkowitz 2002). With more chronic stimuli the expression of the receptor is down-regulated and thereby the number of receptors is decreased. In our studies we see an increase in urine within 30 minutes, and excretion usually peaks within 60-90 minutes before returning to baseline; therefore the dynamics of this response should be considered an acute rather than chronic stimulus.

From the studies of GPCRs it is obvious that the receptors do not work in isolation but rather exhibit extensive cross-talk in a complex manner (McGraw et al. 2007). For example, it has been shown that activation of the PGE_2 receptor EP_1 greatly reduces the bronchodilating action of β_2-agonists through this cross-talk (McGraw et al. 2006). Similar findings exist for the CysLTs (Rovati et al. 2006).

In Paper I-IV we found release of CysLTs and PGD, two mediators that are known to be involved in EIB. These eicosanoids exert their action via the DP_1 and TP receptors, and CysLT receptors respectively (Boyce 2007). It is likely that desensitisation occurs similarly to what is observed for other GPCR agonists. *In vitro* LTD_4 has been shown to induce phosphorylation and internalisation of the $CysLT_1$ receptor (Naik et al. 2005). It is not clear if this process of desensitisation occurs at all following exercise challenge. If desensitisation does occur, it could also be an explanation of recovery after challenge. Also, it is not clear how long-lasting this process is, and if it could explain the development of refractoriness. In intestinal epithelium, *in vitro*, there is evidence that the process of internalisation upon LTD_4 stimulation is very rapid and that the receptors are recycled back to the surface within 15-20 minutes (Parhamifar et al. 2010). Since refractoriness is also a time-limited response, usually not lasting more than 2-3 hours, this internalisation-recycling theory would fit well and also explain why the largest degree of refractoriness is seen the closer to the first challenge that the second challenge is performed (Edmunds et al. 1978). In our studies, we observe elevated urinary excretion for as long as 120 minutes, or even more after challenge (Paper I-IV), however, we do not know how long the elevation of mast cell mediator release in the lung actually lasts. Also, there is a great variability in the urinary release between different subjects which could explain why some are rendered refractory whereas others are not.

To summarise the findings of this thesis, I suggest an alternative explanation for the development of refractoriness, where PGE_2 and/or PGI_2 may play a vital role. The fact that mediators are released to the same degree after both challenges in papers I and IV suggests that the airways are somehow less reactive to the mediators released. Desensitisation is a general mechanism seen in GPCR signalling (McGraw et al. 2003), and there is little reason to believe this would not also occur in the airways. The effect of NSAID pre-treatment may be explained by prostaglandins playing a vital role in the cross-talk between the receptors to induce phosphorylation and internalisation. In the absence of prostaglandins, the leukotrienes themselves would not be able to induce enough internalisation to decrease the responsiveness to further challenge. Whether mediators other than the leukotrienes and prostaglandins are involved in this process remains to be established. Also, which receptors are responsible is unclear.
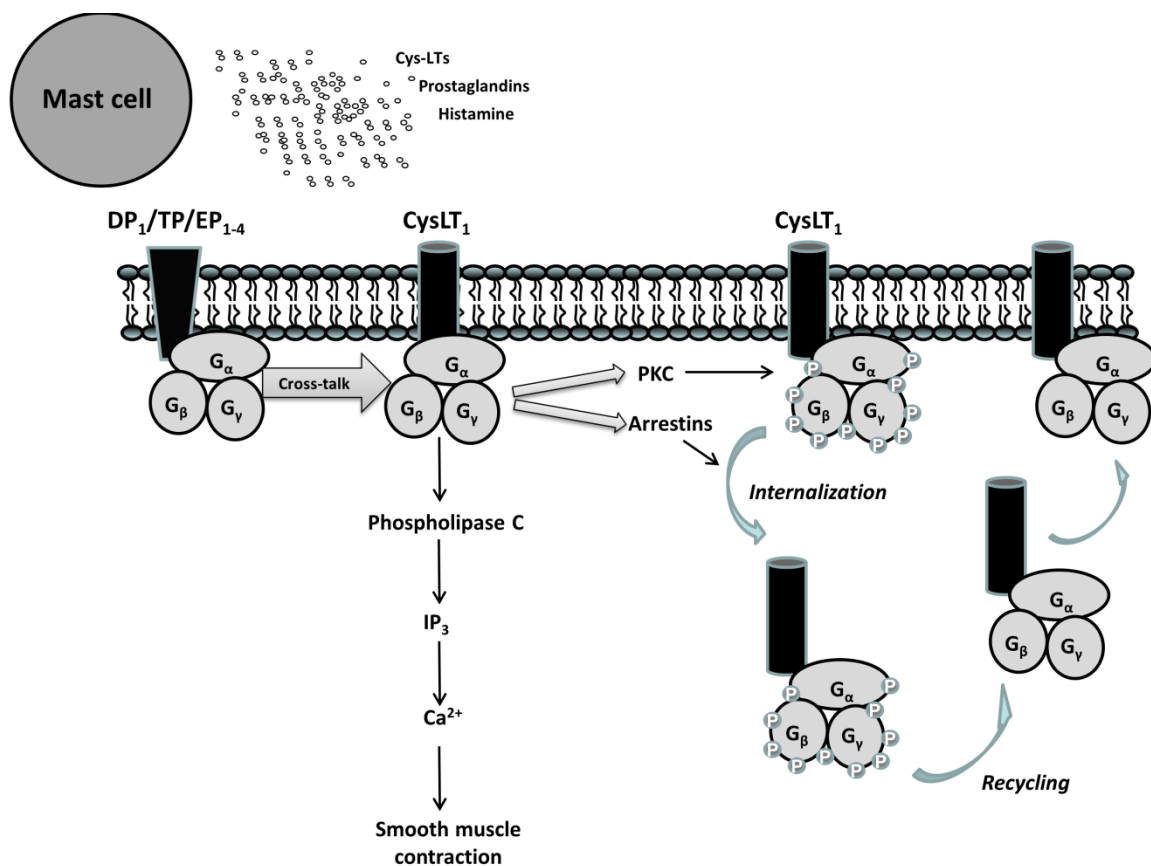
Figure 40 Desensitisation of the cysteinyl-leukotriene receptor1 as a mechanism of refractoriness.
With exercise there is release of cysteinyl leukotrienes from mast cells that stimulate the GPCR $CysLT_1$ causing contraction of the muscle and exercise-induced bronchoconstriction. The prostaglandins generated during the same exercise could, via stimulation of the GPCRs $DP_1/EP_{1-4}/TP$ lead to cross talk so that the cyLT1 is desensitised though phosphorylation and internalisation. Refractoriness to a second exercise challenge within a short period occurs as a result. When the levels of mediators return to baseline values the $CysLT_1$ receptors are recycled back to the cell surface and the muscle becomes sensitive to further stimulation. When generation of prostaglandins is inhibited by agents such as indomethacin and flurbiprofen then there is limited desensitisation of the $CysLT_1$ and therefore limited refractoriness.
GPCR – G-protein coupled receptor, $CysLT_1$ – cysteinyl leukotriene receptor 1, DP_1 – prostaglandin D receptor 1, EP_{1-4} – prostaglandin E receptor 1-4, TP – thromboxane receptor
Reproduced with permission (Larsson et al. 2013)

In the search to define the molecular mechanisms involved, we have started to develop a method for mannitol challenge in the organ bath. Although we only have preliminary results it is a promising method that will hopefully enable us to examine the effect of specific agonists and antagonists to more clearly define the role of each mediator. Another interesting opportunity is the analysis of histamine in urine (Kolmert et al. 2014) which, in combination with the UPLC-MS/MS method, will provide a better understanding of all the mast cell mediators involved.

The overall aim of the project was to define the mechanisms that explain why an episode of EIB is followed by refractoriness. Looking back at the results one can say that we made several new discoveries which provide the basis for further research on the exact mechanisms. The consistent findings of increased mediator release following both challenges, and the finding of PGE_2 and PGI_2 release during refractoriness are two important discoveries. Also, the findings will help us to design future studies; perhaps mannitol is a better challenge

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than EVH if we want a more powerful response when it comes to mediator release. We have gained knowledge of the optimal time interval between challenges and this, together with the newly developed UPLC-MS/MS platform, has improved our knowledge of what is released and when. Considering urinary mediator excretion, I think we have come as far as possible when it comes to studying the mechanisms of refractoriness. Apart from measuring local mediator release by new methods, another important step in furthering our understanding will be to use specific interventions to see how they affect refractoriness. Such interventions can be carried out in a clinical setting, but to further elucidate the molecular mechanisms we will also have great use of the newly developed model for mannitol challenge of human bronchi in the organ bath.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Astma är en vanlig luftvägssjukdom som drabbar 1 av 10 i befolkningen. Astma kännetecknas av varierande grad av andningssvårigheter, pipande andning, och att luftvägarna är hyperreaktiva och inflammerade. Ett astmaanfall kan utlösas av en mängd faktorer; allt från kall eller varm luft till allergen och läkemedel. En av de vanligaste orsakerna, som kan utlösa astmaanfall är dock ansträngning. När man anstränger sig andas man mer, vilket leder till att luftvägarna torkas ut. Denna uttorkning i sin tur leder till att mastceller som finns i lungorna frisätter substanser som får luftvägarna att dra ihop sig. Exempel på sådana substanser är leukotriener och prostaglandiner. Dessa substanser som frisätts i lungorna transporteras med blodet till njurarna och hamnar så småningom i urinen.

Något som ses hos astmatiker med ansträngningsutlösta besvär, är att om de först får springa för att framkalla ett astmaanfall och sedan återhämta sig, så blir astmaanfallet lindrigare om de får springa en andra gång. Att luftvägarna är mindre reaktiva andra gången kallas för refraktäritet, och denna effekt kan vara i upp till 4 timmar. Målet med det här avhandlingsarbetet var att definiera mekanismerna vid refraktäritet och på så sätt identifiera möjliga mål för ny behandling av astma.

I den här avhandlingen ingår 5 olika studier där vi provocerat fram astmaanfall genom att försökspersoner antingen fått hyperventilera torr luft eller andas in mannitol som är ett pulver som torkar ut luftvägarna. Dessa två metoder har tidigare visat sig vara mycket lika ansträngning i sättet som de utlöser astmaanfall.

Både hyperventilering av torr luft och inandning av mannitol utlöste astmaanfall och gav ökade nivåer av prostaglandiner och leukotriener i urin. När provokationerna gjordes upprepat blev astmaanfallet andra gången lindrigare, d.v.s. de blev refraktära. Refraktäriteten sågs trots att det frisattes lika mycket prostaglandiner och leukotriener. Detta skiljer sig från det man tidigare trott; att refraktäriteten beror på att det frisätts mindre under andra provokationen. Tvärtom var det så att de med högst grad av refraktäritet (störst skillnad mellan astmaanfallen efter första, respektive efter andra provokationen) var de som hade de högsta nivåerna av prostaglandiner och leukotriener i urinen.

I en av studierna mätte vi ett bredare spektrum av substanser och fann då att en viss typ av prostaglandiner, som anses skydda mot astmaanfall, frisätts efter att försökspersonerna hyperventilerade torr luft. Det skulle kunna tyda på att denna typ av prostaglandiner är viktiga för utvecklingen av refraktäritet.

Vi testade även effekten av olika behandlingar, dels på hur kraftigt astmaanfall som utlöses av provokationerna, men även hur det påverkar frisättningen av substanser till urinen. Inhalationskortison, som är en vanlig medicin vid astma; i en hög dos hindrade det astmaanfall och minskade också mängden prostaglandiner och leukotriener i urinen. Kromoglikat, som man tror verkar genom att hindra mastcellerna från att frisätta substanser, förhindrade även det astmaanfall efter torrloftsprovokation. Ingen ökning av mängden prostaglandiner och leukotriener i urinen sågs efter förbehandling med kromoglikat. Slutligen testade vi effekten av 3 veckors behandling med fiskleverolja som vissa tror kan ha gynnsamma effekter, vi såg däremot ingen som helst effekt av fiskleveroljan.

Sammanfattningsvis tyder våra resultat på att refraktäritet beror på att luftvägarna är mindre reaktiva för de substanser som frisätts när försökspersonerna provoceras för andra gången. Exakt vad denna minskade känslighet beror på går inte att säga utifrån de nuvarande resultaten men detta kommer undersökas vidare. En viss typ av prostaglandiner som kan skydda mot astmaanfall verkar ha en viktig roll.

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