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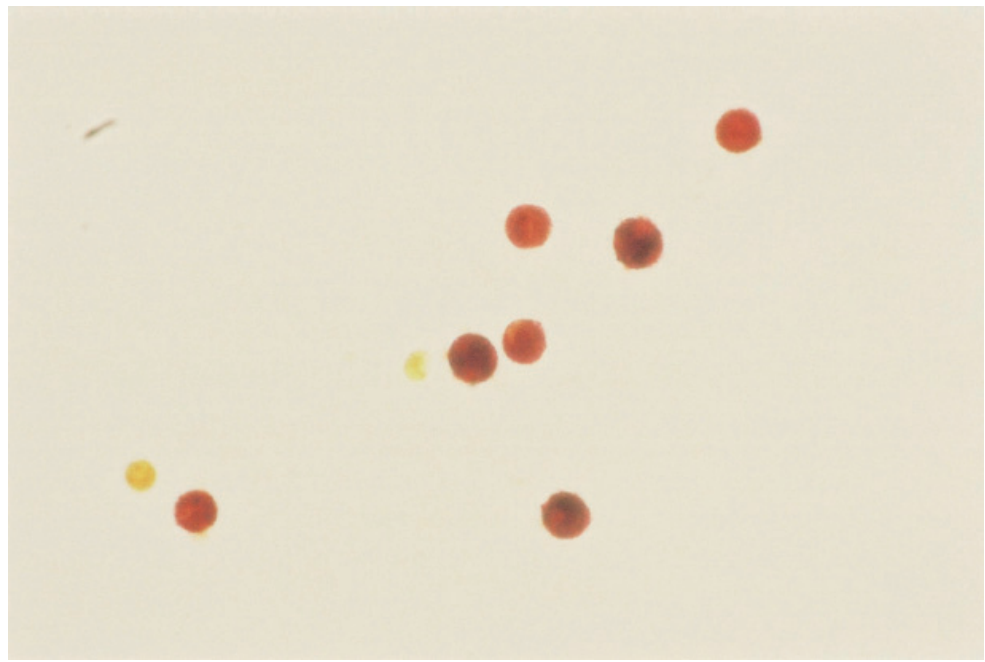
Mast Cell Activation in Response to Osmotic and Immunological Stimulation with Focus on Release of Eicosanoid Mediators



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MAST CELL ACTIVATION IN RESPONSE TO OSMOTIC AND IMMUNOLOGICAL STIMULATION WITH FOCUS ON RELEASE OF EICOSANOID MEDIATORS

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ABSTRACT

Mast cells are important in asthma and other inflammatory diseases. Subjects with asthma have been found to have an increased number of mast cells in their airway smooth muscle and this was related to airway sensitivity. Normally harmless stimuli may trigger bronchoconstriction in subjects with asthma and exercise can generate airway constriction in subjects with asthma. The mechanism for exercise-induced bronchoconstriction (EIB) has been suggested to be related to an increased airway fluid osmolarity. This may activate mast cells with subsequent release of mediators acting on bronchial smooth muscle leading to bronchoconstriction. Mannitol inhalation causes bronchoconstriction, and the mechanism is probably by increasing airway fluid osmolarity. The aim of this thesis was to establish whether hyperosmolar stimulation activates human mast cells *in vitro* and *in vivo* with focus on the release of biologically active mediators. Human cord blood derived mast cells (CBMC) were used for studies on mediator release in response to immunological and osmotic activation *in vitro*. Bronchial provocation by mannitol inhalation was used to mimic EIB for studies *in vivo* on airway reactivity and urinary excretion of mediators.

For the first time, mannitol was found to induce the release of PGD_2 and LTC_4 in CBMC *in vitro*. Prostaglandin D_2 was formed both via the COX-1 and COX-2 pathways in CBMC. The late response after stimulation with the combination of anti-IgE and IL-1β was more COX-2 dependent. Further, the pro-inflammatory cytokine IL-1β induced the expression of COX-2. In addition to COX derived PGD_2, CBMC was found to release TXB_2 and occasionally also PGE_2 after stimulation with IL-1β, anti-IgE or their combination. Hypoxia (4% O_2) was not found to increase the release of mediators as compared to normoxic (21% O_2) conditions. Interleukin-4 induced the expression of 15-LO in CBMC and the main 15-LO derived metabolite was 15-KETE followed by 15-HETE in IL-4 treated CBMC stimulated with arachidonic acid. The release of 15-HETE was also induced by mannitol

Both asthmatic and control subjects had an increased urinary excretion of the PGD_2 metabolite 9α,11β-PGF_2 as well as LTE_4 after mannitol challenge *in vivo*. The increase in 9α,11β-PGF_2 was related to bronchoconstriction since only the asthmatic subjects responded to mannitol. Further, the mast cell stabiliser sodium cromoglycate (SCG) and the β_2-agonist formoterol protected from mannitol-induced-bronchoconstriction in asthmatic subjects with 63% and 95%, respectively. In addition, both inhibitors dampened the mannitol-induced urinary 9α,11β-PGF_2 excretion compared to placebo treatment.

In conclusion, mast cells release PGD_2 after mannitol stimulation *in vitro* and *in vivo* and treatment with a mast cell stabiliser further supports the mast cell involvement in mannitol-induced bronchoconstriction *in vivo*. Both COX-1 and COX-2 enzymes were involved in PGD_2 formation and mast cells were unaffected by hypoxic environmental changes *in vitro*. The expression of 15-LO in mast cells *in vivo* and *in vitro* support that these cells can contribute to the formation of novel metabolites with unknown functions. The mediator formation in mast cells seems to be important for subjects with EIB since their airways respond more easily with bronchoconstriction. Inhibition of PGD_2 formation protects from bronchoconstriction in subjects with EIB. The physiological effect of some mast cell mediators remains to be elucidated however PGD_2 appear to have a central role in the airway response to mannitol.

Key words: exercise-induced bronchoconstriction, cyclooxygenase, cord blood derived mast cells, mannitol, prostaglandin D_2, leukotriene C_4, leukotriene E_4, histamine and 15-lipoxygenase.

LIST OF PUBLICATIONS

The results in this thesis are based on the following publications, which will be referred to in the text by their roman numerals.

I. **Gulliksson M**, Palmberg L, Nilsson G, Ahlstedt S and Kumlin M.
Release of prostaglandin D_2 and leukotriene C_4 in response to hyperosmolar stimulation of mast cells.
Allergy 2006;61(12):1473-9.

II. **Gulliksson M**, Nold-Petry C, Dahlén S-E, Nilsson G, Pfeilschifter J, Palmberg L, Ahlstedt S and Kumlin M.
Cyclooxygenase (COX) isoenzyme participation in release of PGD_2 from human cord blood derived mast cells in normoxic and hypoxic environment.
Manuscript

III. **Gulliksson M**[#], Brunnström Å[#], Johannesson M, Backman L, Nilsson G, Harvima I, Dahlén B, Kumlin M and Claesson HE.
Expression of 15-lipoxygenase type-1 in human mast cells.
Submitted

IV. Brannan JD, **Gulliksson M**, Anderson SD, Chew N and Kumlin M.
Evidence of mast cell activation and leukotriene release after mannitol inhalation
Eur Respir J 2003;22(3):491-6.

V. Brannan JD[#], **Gulliksson M**[#], Anderson SD, Chew N, Seale JP and Kumlin M.
Inhibition of mast cell PGD_2 release protects against mannitol-induced airway narrowing.
Eur Respir J 2006;27(5):944-50.

[#] Equal contribution

CONTENTS

LIST OF ABBREVIATIONS

5-LO	5-Lipoxygenase
12-LO	12-Lipoxygenase
15-LO	15-Lipoxygenase
15-HETE	15-hydroxy-eicosatetraenoic acid
15-HPETE	15-hydroperoxy-eicosatetraenoic acid
15-KETE	15-keto-eicosatetraenoic acid
AA	Arachidonic acid
$CBMC^{MNC}$	Cord blood mast cell derived from mononuclear cells
$CBMC^{SC}$	Cord blood mast cell derived from CD34 selected cells
CysLT	Cysteinyl leukotriene
BAL	Bronchoalveolar lavage
EIB	Exercise induced bronchoconstriction
ELISA	Enzyme immunoassay
EVH	Eucapnic hyperventilation
FEV_1	Forced expiratory volume in one second
HIF	Hypoxia-inducible factor
IgE	Immunoglobulin E
IL	Interleukin
LT	Leukotriene
PG	Prostaglandin
PCR	Polymerase chain reaction
SCF	Stem cell factor
SCG	Sodium cromoglycate
SEM	Standard error of the mean
SD	Standard deviation
TX	Thromboxane
MS/MS	Mass spectrometry
MC_T	Tryptase positive mast cell
MC_{TC}	Tryptase and chymase positive mast cell
NSAID	Non steroidal anti-inflammatory drug
NAL	Nasal lavage
HPLC	High pressure liquid chromatography

INTRODUCTION

Mast cells are key effector cells in inflammatory diseases such as bronchial asthma, immediate and delayed hypersensitivity reactions, atopic eczema, drug and food allergy, hay fever and respiratory inflammation.[1, 2]

The mechanism for antigen induced bronchoconstriction can be explained by the involvement of IgE cross-linking leading to mast cell activation and release of mediators acting on bronchial smooth muscle and other effectors leading to asthma attacks.[3, 4]

Another trigger of bronchoconstriction in patients with asthma is exercise. The mechanism behind exercise induced bronchoconstriction (EIB) has been debated. The hyperosmolar theory has been proposed for explaining the mechanism behind EIB.[5] During exercise the ventilation rate increases and inspired air is humidified causing dehydration of the airway surface liquid. The increased airway osmolarity is thought to cause cell activation with subsequent release of mediators.

The aim of this thesis was to establish whether hyperosmolar stimulation will activate human mast cells *in vivo* and *in vitro*. For studies in healthy volunteers and subjects with asthma, provocation by inhalation of mannitol was used to mimic EIB. In the experimental studies, human cord blood derived mast cells (CBMC) preparations were used. In addition to hyperosmolar stimulation of CBMC, the studies included characterisation of arachidonic metabolism and involvement of different enzymes.

BACKGROUND

MAST CELL CHARACTERISTICS

Mast cell origin and maturation

Mast cells were first identified by Paul Ehrlich. He identified the cytoplasmic granules and described the cells in 1878 and named these cells "mastzellen", which can be translated to "well fed cells" for their rich cytoplasmic granulae content.[6] Mast cells were identified by staining with a methachromatic dye demonstrating that the cells contained methachromatic cytoplasmic granules. In their granules two well defined structures were early recognized as histamine and heparin.[7]

Mast cells are of hematopoietic origin derived from the pluripotent cells that reside in bone marrow[8, 9] and foetal liver.[10] In peripheral blood, CD34-, c-kit-, CD13-positive and FcεRI-, FcγRII-, CD14-, CD17- negative mast cell progenitors circulate as precursor cells that matures first when entering the tissue.[11-13] It was concluded that mast cells originate from a specific linage of hematopoietic progenitors based on the CD14 and CD17 negative precursor phenotype that differed from circulating basophils or monocytes.[12]

The differentiation into mature mast cells is dependent on different growth factors and the most important factor for growth, differentiation, survival, adhesion and degranulation of human mast cells is stem cell factor (SCF).[14, 15] The lifespan of mast cells are long compared to other inflammatory cells. They can survive in tissues for several months after which they undergo apoptosis.[16] Under normal conditions, mast cells are distributed in all vascularised tissue and they are particular abundant in tissue that interferes with external environment such as skin, gastrointestinal tract and respiratory system. They are also found under the epithelial surface of the skin as well as near blood vessels, nerves, smooth muscles and in the central nervous system.[17, 18]

Mast cell heterogeneity

The tissue microenvironment determines maturation and phenotype development of mast cells.[19] Human mast cells exhibit different characteristics such as cell size, cytokine production and protease expression.[16] They can be divided into two groups according to their neutral protease content. MC_{TC} contains tryptase and chymase and MC_T mainly contains tryptase.[20] Human lung mast cells and intestinal mucosal mast cells belong to the tryptase positive MC_T subgroup of mast cells with 90 % tryptase positive cells.[21, 22] This population is dynamic as the number of mast cells in these locations can be increased by mucosal inflammation.[23] In the lung, MC_T type of mast cells predominate the alveolar wall and the epithelium. There is also a subpopulation of MC_{TC} cells close to bronchial airway smooth muscle and in glandular regions (lymphoid follicles).[24] Skin and intestinal submucosal mast cells belong to the tryptase and chymase MC_{TC} positive subgroup. This population resides relatively constant in tissue, where it can be activated.[23] It is not known whether the ratio of MC_{TC} and MC_T are changed in the asthmatic lung, however other conditions, such as fibrosis, can shift the MC_T phenotype towards MC_{TC} type.[25]

MAST CELL FUNCTIONS

Since mast cells are distributed in tissues that are exposed to the external environment, they are in close contact with antigens, pathogens and other factors invading mucosal surfaces and skin.[17] Mast cells are involved in host defence against foreign compounds via the innate and adaptive immune responses as in clearance of parasite infections, phagocytosis of particles, antigen processing and cytokine production.[26-29]

Mast cells are key effector cells in allergic reactions and these reactions may have lethal outcome as anaphylactic reactions. Allergy is often triggered by an IgE overproduction stimulated by environmental allergens that are normally harmless such as pollen or house dust mite. For some people, allergies start with eczema or gastro-intestinal problems evolving into asthma with symptoms such as hyperreactivity and airway bronchoconstriction.[30, 31] The role of the mast cell in inflammatory reactions relates to their ability to synthesise, store and/or release pro-inflammatory mediators upon stimulation. Mediator release may cause acute and late phase allergic reactions and chronic inflammation.[17]

In subjects with asthma, the total number of mast cells in the superficial bronchial mucosa does not seem to differ much compared to control subjects.[2] It has been found that mast cells accumulate in bronchial smooth muscle and this was related to airway hyperresponsiveness in subjects with asthma.[32] Another study demonstrated that mast cells were localised to three distinct sites in the bronchial mucosa of asthmatic subjects *i.e.*, the airway smooth muscle, the airway mucosal glands and the bronchial epithelium.[2] The mast cell accumulation in airway smooth muscle may be due to the chemotactic effect of stem cell factor produced by smooth muscle cells.[33]

MAST CELL ACTIVATION

Immunological activation

Mast cell activation may be initiated by an allergen (multivalent antigen) via cross linking of IgE antibodies on the cell (**Fig 1)**. The high affinity FcεRI receptors are attached to the membrane and bind IgE with high affinity in a 1:1 ratio.[3] IgE-dependent activation may lead to acute allergic reactions such as acute asthma, anaphylactic reactions and allergic rhinitis.[3] Mast cells possess approximately 300 000 high affinity receptors per cell. Only a few of these IgE bound receptors need to be cross-linked for activation.[16]

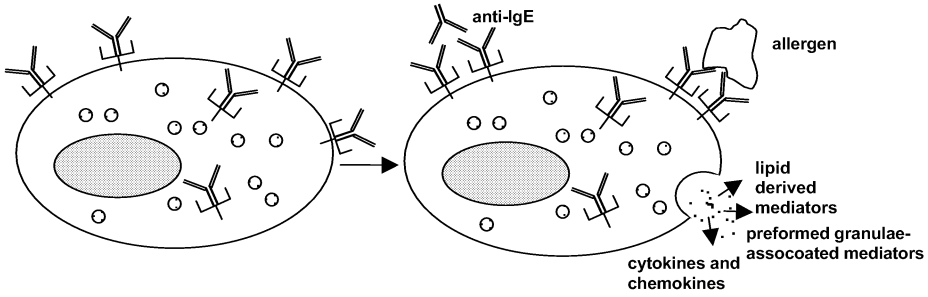

Figure 1. Mechanism of mast cell IgE activation via antigen (allergen) cross-linking of IgE bound to FcεRI. The interaction of allergen/antigen with IgE brings the receptor into juxtaposition and initiates mast cell activation and mediator release.[3, 34]

Osmotic activation

Mast cells may also be activated via non-IgE associated reactions, *e.g.* osmotic activation caused by non-permeable particles. Osmotic activation of cells is caused by the movement of water crossing the cell membrane from a region of low solute concentration to a region of high solute concentration for equalization of solute concentration. If the solute outside the cell cannot cross the cell membrane this event may result in cell dehydration as water is transported out of the cell. Increase in ion concentration inside the cell may lead to activation and mediator release.[35, 36] Human lung mast cells were found to be activated by small changes in osmolarity causing release of histamine *in vitro*.[37] Prior to this study, it was however unknown if hyperosmolarity stimulated *de novo* synthesis of leukotrienes and prostaglandins in mast cells.

Other non-IgE stimuli activating mast cells besides osmotic agents are cytokines, calcium ionophores, neuropeptides, basic compounds, complement factors, cytokines, dextrans, lectins, emotional stress, temperature changes, contrast media and opiates.[16, 27, 37-40] all leading to signal transduction, formation and release of a range of bioactive products.

MAST CELL MEDIATORS

The consequences of mast cell mediator formation and release are immediate responses, late phase responses and sometimes chronic inflammation.[16] These events are the result of mast cell mediators, exerting their effects on target cells within the tissue, where they can recruit other inflammatory cells as well as being inactivated. Mast cell activation can result in the release of three different types of mediators (**Fig 1**):

Enzymatically *de novo* synthesised lipid mediators named eicosanoids which are derived from arachidonic acid stored in the cell membrane. These are prostaglandins (PGs), leukotrienes (LTs), tromboxanes (TXs), monohydroxy acids (HETEs) and lipoxins (LXs). Eicosanoids are synthesised within minutes and can be released for a substantial time. Therefore, this class of mediators may contribute to acute as well as in late inflammatory responses.[16, 41] Platelet activating factor (PAF) is also produced via phospholipid metabolism in mast.[42]

Preformed secretory granulae associated mediators are released via exocytosis. For example, histamine, proteases (tryptase, chymase), proteoglycans (heparin, chondroitin sulphate E), peptidases (carboxypeptidase) and certain cytokines belong to this group. These substances are released within seconds or minutes and hence, they are important in an early phase of an acute allergic inflammation such as immediate hypersensitivity reactions.[16]

Cytokines and chemokines such as TNF-α, IL-4, IL-5, IL-6, IL-13, TNF-α, macrophage inflammatory protein (MIP)-1α, MIP-1β are secreted. These mediators may be both preformed and newly synthesised and they are important both in early and late inflammatory responses orchestrating leukocyte infiltration.[16, 43]

Eicosanoids

Membrane phospholipids sustain a pool of fatty acids and upon cell activation esterified arachidonic acid can be hydrolysed from membrane phospholipids by the enzyme phospholipase A_2.[44] Eicosanoids, "eikosi" meaning 20 in Greek are a family of polyunsaturated fatty acid metabolites with 20 carbon atoms. The phospholipases responsible for arachidonic acid hydrolysation can be activated by different stimuli.[45]

Prostanoid formation

Prostaglandins were named from the prostate gland and were first isolated from seminal fluid.[46, 47] Prostanoids include prostaglandins (PG) and thromboxanes (TX) and they are formed when arachidonic acid is presented to prostaglandin endoperoxide synthase (PGHS) (also known as cyclooxygenase) at the nuclear envelope or at the endoplasmatic reticulum (ER). Prostaglandin endoperoxide synthase converts arachidonic acid to the unstable metabolite prostaglandin G_2 (PGG_2) with insertion of two oxygen molecules. Prostaglandin G_2 is subsequently reduced to PGH_2.[48] Prostaglandin endoperoxide synthase is a heme containing dioxygenase with two catalytic activities, cyclooxygenase and peroxidase. It exists in two isoforms COX-1/PGHS-1 and COX-2/PGHS-2.

The two isoforms, COX-1 and COX-2 share 65% amino acid sequence homology and they catalyse the same reactions. Despite this, the enzyme expression and function differ. Cyclooxygenase-1 is expressed in most organs and considered to be responsible for the constitutive basal prostanoid biosynthesis. Cyclooxygenase-2 is almost undetectable in most cells at rest but, it is upregulated in inflammatory conditions.[49] Inflammation is in part mediated by the production of prostaglandins such as PGE_2, PGI_2 and TXB_2 produced by the COX enzymes. Thus, both enzymes are targets of the non steroidal anti-inflammatory drugs (NSAIDs) and together with aspirin these compounds acts as anti-inflammatory, antipyretic and analgesic drugs.[49]

Aspirin (acetylsalicylic acid) was synthesised in 1870 and Bayer launched Aspirin® in 1898. Aspirin inhibit the formation of COX-1 related (TXB_2) products and modifies COX-2 related products causing side effects such as gastrointestinal bleeding and ulceration. In 1971 it was found that NSAIDs inhibited the formation of prostaglandins and this could be associated with the side effects.[50] Shortly thereafter, prostaglandins were found to be protective for the stomach.[51] In 1994 the three dimensional structure of COX (now named COX-1) was found.[52] In 1996 another COX enzyme COX-2 was characterized independently by two different research groups.[53, 54] There is one major difference between the enzymes that allows for selective inhibition, the substitution of an amino acid in the COX-2 side pocket. This allows access to a wider side-pocket for substrate binding.[54] Drugs binding to this pocket are considered to be selective inhibitors of the COX-2 enzyme. Thus, since there are two different enzymes and COX-2 is upregulated in inflammatory conditions, selective COX-2 inhibitors ("coxibs") are developed with the thought of dampening the side effects caused by the unselective inhibitors. The first selective COX-2 inhibitors were celecoxib and rofecoxib.[55]

Prostaglandin H_2 is an unstable cyclic endoperoxide and a key mediator in the formation of biologically active prostanoids such as prostaglandin (PGD_2), prostacyclin (PGI_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane A_2

(TXA_2) (**Fig. 2**). These conversions are performed via enzymatic reactions, catalysed by respectively synthase.[56, 57] Prostaglandins are formed by almost all cells in the body but, there is often only one dominating product in each cell type.[41]

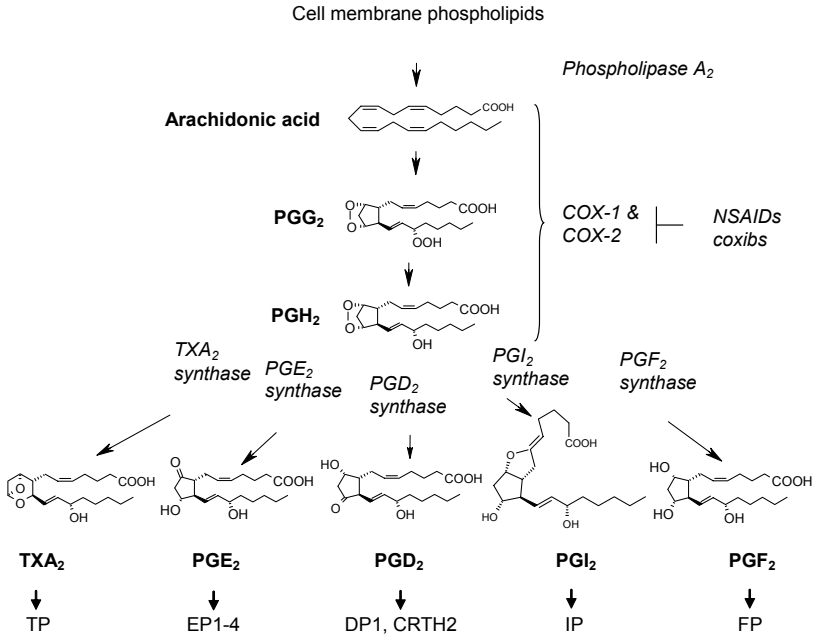

Figure 2. Biosynthesis of prostanoids via the cyclooxygenase pathway. Specific G-protein coupled receptors and COX inhibitors (NSAIDs and coxibs) are included in the figure.

In mast cells, PGD_2 is the dominating COX-derived product.[58] Prostaglandin D_2 is synthesised via conversion of PGH_2 by prostaglandin D synthase (PGDS). PGD synthase is predominantly found in the cytosol as in contrast to the cyclooxygenase which is found close to cell membranes.[57] In addition to mast cells, basophils, T-lymphocytes, platelets and macrophages are also reported to produce PGD_2 though in 100-1000 times lower amounts as compared to mast cells.[59]

Thromboxanes are formed via conversion of PGH_2 into TXA_2 catalysed via thromboxane synthase. Thromboxane A_2 is a very unstable metabolite and is rapidly converted to TXB_2.[56] Thromboxane synthase has been found in platelets and macrophages.[41] In humans, TXB_2 is mainly produced by activated platelets causing platelet aggregation and contraction of vascular and bronchial smooth muscle.[60, 61]

Prostaglandin E_2 is formed from PGH_2 via the action of three possible PGE synthases. Microsomal prostaglandin E synthase-1 (mPGES-1) is the dominating enzyme in PGE_2 formation, however there are also other PGE producing enzymes such as mPGES-2 and cytosolic PGE synthase. Prostaglandin E_2 mediates pain and is considered as immunomodulatory, bronchoprotective and also protects stomach and intestine.[41, 51, 62, 63] It is primary formed from airway epithelium and bronchial smooth muscle.[64] Inhaled PGE_2 inhibit allergen induced bronchoconstriction.[64, 65] PGE_2 has been reported to inhibit histamine release from human lung mast cells.[66]

Prostanoid catabolism

Prostaglandins are rapidly degraded and unmetabolised prostaglandins have a half life of less than 1 min in the circulation.[67] Most of the prostaglandins undergo degradation accomplished by cytosolic 15- hydroxyprostaglandin dehydrogenase (15-PGDH) acting on the 15-OH group with formation of the unstable 15-keto prostaglandins.[57] Secondly, a 13-reductase (Δ 13-reductase) reduces the 13-trans double bound and together with 15-PGDH form 15-keto-13,14-dihydroprostaglandins.[57] Thirdly, the resulting inactive metabolites are often further processed by β- and ω-oxidation with shortening of the carbon chain before they are excreted by the kidneys.

The profile of PGD_2 metabolites excreted into the urine has been studied by intravenous injection of ^{3}[H]-PGD_2 in human.[68] The majority of the PGD_2 was metabolised to prostaglandin F-ring structures. The urinary metabolite 9α, 11β-PGF_2 represented 0.3% of the radioactivity and was the major C-20 metabolite. No intact PGD_2 was found in urine.[68] In human liver and lung, PGD_2 can be metabolised to 9α, 11β-PGF_2 through the action of a NADP-dependent 11-ketoreductase[69](**Fig. 3**). In human lung 9α, 11β-PGF_2 can be further metabolised via the PGDH/Δ13 pathway to 15-keto and 15-keto-13,14-dihydro-9α, 11β-PGF_2 in addition to 9α, 11β-PGF_2 formation.[70] Since, 9α, 11β-PGF_2 is the main 20 carbon PGD_2 metabolite found in urine it is a valuable marker of mast cell released PGD_2.[68]

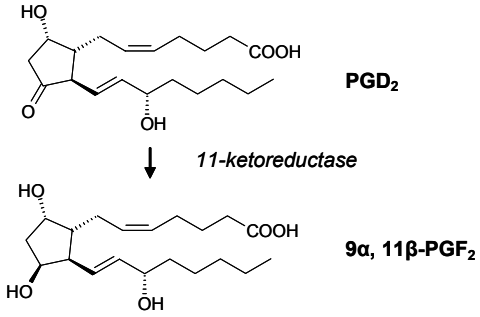

Figure 3. Biosynthesis of 9α, 11β-PGF_2 from PGD_2

In a study, two methods were used to analyse the amount of urinary 9α, 11β-PGF_2 excretion; Enzyme immunoassay (ELISA) and gas chromatography-mass spectrometry (GC-MS). It was found that the values found by GC-MS were in the same range but consistently lower compared to those found by ELISA. Purification of samples led to the finding of two related dinor compounds.[71] Thus, the amount of urinary 9α, 11β-PGF_2 measured by ELISA may represent the sum of three different compounds.[71] ELISA was found to be fast, sensitive and sufficiently specific for monitoring the PGD_2 metabolite 9α, 11β-PGF_2 in urine samples.

Thromboxane B_2 is further converted to urinary metabolites for clearance by the kidneys.[72] The major TXB_2 metabolite in circulation was found to be 11-dehydro-TXB_2, formed by a dehydrogenation at C11.[73, 74] The fractional conversion of TXB_2 after i.v injection of TXB_2 showed an equal ratio between 11-dehydro-TXB_2 and 2,3 dinor-TXB_2 in urine.[75]

Leukotriene formation

The leukotrienes were discovered in 1979.[76, 77] The name "leukotriene", comes from two words, leukocyte and triene (for the conjugated double bounds). Leukotrienes are derived from arachidonic acid in response to cell activation (**Fig. 4**). 5-Lipoxygenase reversibly translocates from either nucleoplasm or cytoplasm to the perinuclear region. Here, 5-LO activating protein FLAP[78] together with 5-LO convert arachidonic acid to the unstable intermediate 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) and further to the epoxide intermediate leukotriene (LT)A_4.[79] Thus, 5-LO is the key enzyme in leukotriene biosynthesis and it is expressed in myeloid cells.[41, 80]

Leukotriene A_4 can be converted to either LTB_4 by LTA_4 hydrolase.[81] or is conjugated to reduced glutathione by LTC_4 synthase to form LTC_4.[82-84] Leukotriene A_4 hydrolase has been found in both cytosolic and intranuclear compartments. The microsomal glutathione-s-transferase type 2 (MGST2) can conjugate LTA_4 with GSH producing LTC_4 in mast cells.[85]

Leukotriene A_4 formed in activated myeloid cells can be further metabolised via transcellular metabolism by leukocytes, endothelial cells and platelets with no 5-LO activity with subsequent formation of LTC_4.[80] LTC_4 is transported out of the cell by a distinct cellular export mechanism "the multidrug resistance-associated protein, MRP.".[86] Thereafter, cleavage of glutamic acid by extracellular γ-glutamyl traspepeptidase (GGT) will form LTD_4 which can be further metabolised via cleavage of glycine by a dipeptidase to provides LTE_4.[83, 87] Leukotriene B_4, on the other hand, is transported out of the cell via an uncloned transporter named LTB_4 transporter where it can act on BLT_1 or BLT_2 receptors.[41]

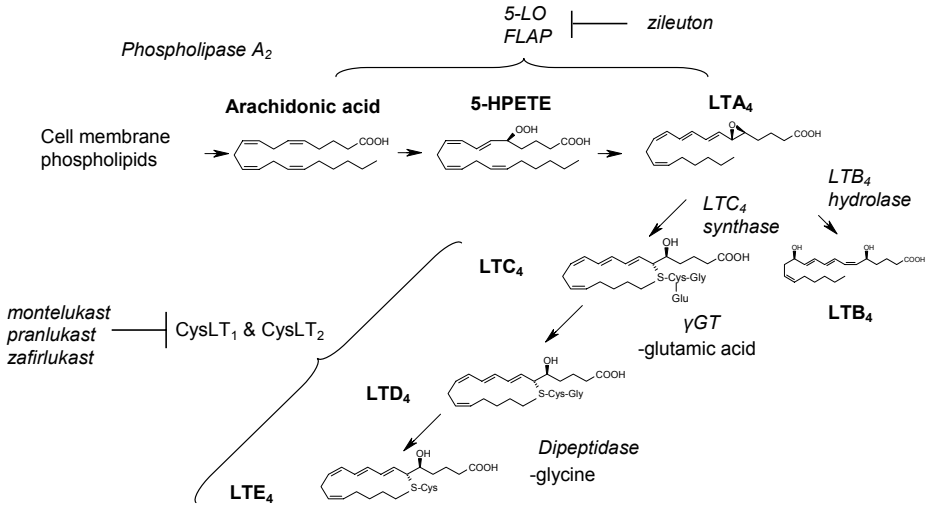

Figure 4. Biosynthesis of leukotrienes from arachidonic acid. Biosynthesis inhibitors (zileuton) specific G-protein coupled receptors and the related receptor inhibitors (montelukast, pranlukast and zafirlukast) are also included in the figure.

Together LTC_4, LTD_4 and LTE_4 are referred to as the cysteinyl leukotrienes (Cys-LTs) since they all contain a cystine group. The amount of CysLTs have been found elevated in acute severe asthma, after allergen challenge of atopic asthmatics and

in aspirin induced asthma.[88, 89] Leukotriene E_4 is the first metabolite with reduced biological activity of the cysteinyl leukotrienes and can thus be considered as the first "metabolite". Leukotriene E_4 is the end metabolite in human lung.[90] Cysteinyl leukotrienes are eliminated via excretion into urine or bile.[91] The majority is processed by the hepatic route whereas the renal route is more rapid, as LTE_4 appeared in urine after a few minutes.[92] In human a substantial 13% of infused radiolabelled ^{3}[H]-LTC_4 was converted and excreted into urine as LTE_4.[92] Cysteinyl leukotrienes are mainly produced by mast cells, eosinophils and to a lesser extent by monocytes.[41]

Other 15-lipoxygenase products

The most abundant eicosanoid derived metabolite, produced from arachidonic acid in human lung is 15-hydroxy-eicosatetraenoic acid (15-HETE)[93] (**Fig 5**). 15-Lipoxygenase first converts arachidonic acid to 15-hydroperoxy-eicosatetraenoic acid (15-HPETE) which is further metabolised to 15-HETE. There are two types of 15-LO in humans; 15-LO type-1, mainly expressed in airway epithelial cells, eosinophils, reticulocytes and in monocytes [94-98] and 15-LO type-2, expressed in hair roots, cornea, lung, skin and in prostate gland.[99] 15-LO-1 appears to be found almost exclusively in humans where it is expressed in low levels in most cells under resting conditions.[98] However, during anaemia the expression is upregulated in lung, spleen, kidney and liver, and certain cytokines (IL-4 and IL-13) also upregulates the expression.[98]

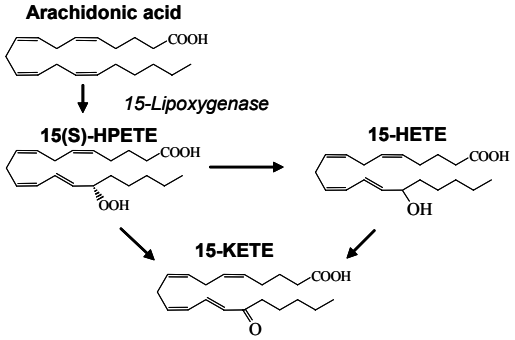

Figure 5. Biosynthesis of 15-KETE from arachidonic acid

The corresponding enzyme in most other species is the so called leukocyte type 12-LO.[98]

15-Lipoxygenase is also responsible for the formation of lipoxins and resolvins via the 15-LO and 5-LO pathway.[100] Lipoxins are formed by cell-to-cell interaction via the action of two or more lipoxygenase enzymes in response to inflammation. For example, 15-LO derived 15-HPETE or 15-HETE in epithelial cells or monocytes can serve as a substrate for neutrophil or monocyte 5-LO with subsequent LXA_4 or LXB_4 formation via the action of LXA_4 or LXB_4 hydrolase, respectively. Lipoxins can also be formed from LTA_4 with insertion of molecular oxygen at C15 via the action of 12-LO or 15-LO. Thus, cell-to-cell interaction of human neutrophil 5-LO and platelet 12-LO can also form LXs. Lipoxygenase A_4 and LXB_4 are vasodilatory. In addition, LX formation down regulates leukotriene synthesis in leukocytes, therefore causing anti-inflammatory responses.[100]

Granulae stored mediators

Histamine is a hydrophilic chemotactic amine and it is the main amine stored in mast cells and basophils.[101] It is formed via decarboxylation of histidine by L-histidine decarboxylase found in mast cells and basophils.[101] Once formed, histamine can be either rapidly inactivated or stored in cytoplasmic granules bound to anionic side chains of the proteoglycans that make up the matrix (in human cells, heparin and chondroitin sulfate).[101] Besides mast cells and basophils, histamine may also be released from neurons, lymphocytes and gastric enterochromaffin-like cells.[102] Only a small part (2-3%) of released histamine is excreted as intact histamine.[103] In the body, histamine is methylated by *N*-methyltransferase with formation of N^{τ}-methylhistamine, which is the major metabolite excreted into urine. Further, 50-70% of histamine in the body is transformed to N^{τ}-methylhistamine. N^{τ}-methylhistamine can be further metabolised to N-methylimidazoleacetic acid by a monoamine oxidase. The rest, 30-40% of histamine is metabolised to imidazoleacetic acid by a diamine oxidase, also called histaminase.[101]

Human lung mast cells contain tryptase and chymase as the two major granular neutral proteases, though tryptase is the major one.[104, 105] Mast cell granulae has a pH value regulated to approximately 5.5. This ensures that the protease activity is low. Optimum for activation of proteases lies between pH 6-9 for chymase and is neutral for tryptase.[106, 107] Tryptase and chymase bind to proteoglycans with attached heparin or chondroitin sulfate glycosaminoglycan chains and forms separate complexes. Tryptase is synthesised as a precursor protein with an N-terminal signal peptide followed by a propeptide.[107] There are four different types of tryptase, α, β, γ and δ. The main form stored in granulae is β-tryptase and CBMC were found to express both the α and the β form.[107, 108]

Mediator functions in asthmatic responses

The biological effects of mast cell mediators depend on the stimulus and the "net effect" of produced and secreted metabolites and also on the intracellular events caused by binding to different receptors. The type of activation depends on receptor expression, ligand affinity, signal transduction pathway and the cellular context. The G-protein coupled receptors have a seven transmembrane spanning protein. The receptors are generally located in the plasma membrane and sometimes also in the nuclear envelope.[109] Activation can lead to bronchoconstriction, increased vascular permeability, mucous secretion and changes in blood vessel tone which are cardinal symptoms of asthmatic responses.[110]

There are at least nine known prostaglandin receptors in humans, they are named by the letter "P" and a prefix of "D", "E", "F", "I" or "T", corresponding to preference for prostanoid ligands[111], and they all belong to the G-protein-coupled receptors with exception of the DP_2 (CRTH2).[110] Prostaglandin D_2 binds to DP_1, CRTH2 and TP receptors.[111] The leukotrienes also bind to G-protein-coupled receptors and the cysteinyl leukotrienes binds to two known receptors, the $CysLT_1$ and $CysLT_2$.[112] Histamine bind to four different G-protein coupled receptors H_1, H_2, H_3 and H_4. Symptoms associated to allergic diseases are generally mediated via binding to H_1 receptors.[102]

Bronchoconstriction

Both PGD_2 and its metabolite 9α, 11β-PGF_2 are potent bronchoconstrictors [113, 114] acting on the TP receptor.[114-116] In control subjects inhaled PGD_2 was 10-times more potent than histamine.[113, 117] In asthmatic subjects PGD_2 and its metabolite 9α, 11β-PGF_2 were almost 30-times more potent than histamine in causing bronchoconstriction.[113, 117] Bronchoconstriction caused by inhaled PGD_2 was reversed to two thirds by a TP antagonist in asthmatic subjects.[116] PGD_2 may also cause vasodilation of vascular smooth muscle by acting on bronchial DP_1 receptors.[111, 118] Thromboxane may also induce presynaptic release of acetylcholine from cholinergic nerves in airways.[119]

In healthy subjects, LTC_4 and LTD_4 were found to be 1000 and 700 times more potent than histamine in causing bronchoconstriction, respectively.[120] Bronchoconstriction is mediated via the Cys-LT_1 receptor on the bronchial smooth muscle.[121] Leukotriene C_4 may act synergistically with histamine or PGD_2 in causing bronchoconstriction in asthmatic subjects.[122]

Histamine causes bronchoconstriction via binding to H_1 receptors on the airway smooth muscle [101] where reflex stimulation of vagal afferent nerve fibres also may contribute to the bronchoconstriction.[123, 124] Histamine can also generate prostaglandin formation [101] and induce proliferation of cultured airway smooth muscle cells.[125]

Mast cell tryptase may degrade neuropeptides that mediates bronchodilation with subsequent increased bronchial responsiveness and this might be a part of the mechanism behind tryptase induced hyper-reactivity.[107] Tryptase can also cause activation of the G-coupled protease activated receptor-2 (PAR-2). Activation can lead to increased sensitization of methacoline and infiltration of eosinophils.[107] Furthermore, PAR-2 receptor binding may also potentiate contractile responses to histamine in subjects with asthma.[126, 127]

Microvascular permeability

Microvascular permeability causes airway oedema in humans.[128] Mast cells release a variety of pro-inflammatory mediators acting on endothelial cells, stimulating them to separate. Plasma will leak and the increased flow of plasma and protein may act on the epithelial cells disturbing the barrier to the environment causing the epithelial cells to separate, leading to loss of protection of the tissue. The unfiltered plasma will reach the lumen and plasma proteins will come in contact with any activating factor being in the environment.[129] Subjects with asthma have an increased number of damaged epithelial cells compared to control subjects and the mucociliary clearance has been found to be disrupted.[130] The epithelial cells are in different stages of damage and mast cells are present in damaged areas of epithelium.[130] Normally, nerves are seen close to basal lamina. However, superficial localisations of nerves are in the bronchial epithelium of asthmatic subjects.[130]

Mast cell tryptase has been suggested to form bradykinin from kinogen.[131] Bradykinin is 100 -fold more potent than histamine in causing vascular permeability. It is also a vasodilator and increases capillary blood flow.[132]

Prostaglandin D_2 does not trigger vascular leakage itself[133] but rather a vasodilation and thus it might lead to plasma exudation in skin.[134]

Histamine binding to the H_1 receptor causes vascular endothelial cell leakage, vasodilation, and stimulates the release of neuropeptides from sensory nerves which also may cause vascular permeability.[101, 135, 136] Histamine is known to induce expression of intracellular adhesion molecule (ICAM-1), vascular cellular adhesion molecule (VCAM-1) and P-selectin on endothelial cells and can thus consequently induce leukocyte rolling.[102]

The cysteinyl leukotrienes, LTC_4 and LTD_4 are 1000 times more potent than histamine on a molar basis on inducing vascular permeability in the postcapillary venules.[137] Furthermore, they are also potent vasoconstrictors.[137] For comparison, leukotriene B_4 causes plasma leakage since it is chemoattractant for neutrophils and thus, causes neutrophils to cross the endothelial barrier.[137-139] Another mast cell mediator, platelet activating factor (PAF) also causes vascular leakage and the PAF induced response was inhibited by the selective PAF inhibitor.[139, 140] Another important mediator causing endothelial leakage is the cytokine TNF-α.[16]

Mucus secretion

Under normal conditions goblet cells comprises a small part of the columnar ciliated epithelial cells lining the airway. However, in subjects with asthma, 20-25% of epithelial cells are goblet cells with subsequent increased mucus production. Mucus may also have effect on ventilation and perfusion, cause hypoxemia leading to wheezing and dyspnea.[141] Potent mucus stimulating mast cell products are histamine, PGD_2 and $PGF_{2\alpha}$. Prostaglandin D_2 and $PGF_{2\alpha}$ are equally potent, whereas PGE_2 significantly reduces mucus production in human lung fragments.[142] Leukotrienes are the most mucus stimulating mediators derived from mast cells.[143] Histamine may cause lower airway mucus secretion by binding to H_2 receptors on submucosal glands.[101]

MAST CELL MODELS

Previously, mast cells have been obtained from skin [144], intestinal tract [145] and lung.[146] Despite the fact that mast cells are abundant in tissue their numbers are relatively limited and they are difficult to isolate. Consequently, for mast cell studies, development of human mast cells *in vitro* has been achieved using different sources of progenitors and culture conditions and the cells have been developed from peripheral blood [147] and cord blood.[148, 149] Cord blood is a rich source of stem cells and for maturation of these undifferentiated cells into tryptase positive mast cells they need to be cultured with stem cell factor (SCF) and IL-6.[108] Mast cells derived from different sources can be stimulated *in vitro* for investigation of activation and mediator release.

Mediator release can also be inhibited via different pharmacological interventions inhibiting either receptor binding or the mediator synthesis. Mast cells from different anatomical places in the body have different response to non-immunological stimulation *in vitro* and their mediator formation is affected differently by mast cell stabilisers.[150] For example, it is known that MC_T types of mast cells are less responsive to non-IgE dependent activation as in contrast to MC_{TC} types of mast cells.[22, 151] Mast cells may also be unresponsive to different inhibitors. For example, MC_{TC} are known to be unresponsive to cromones such as disodium cromoglycate and nedocromil sodium.[22] CBMC were found to express tryptase and chymase [107, 108] however, they can be cultured by different protocols making them more MC_{TC} or MC_T -like, and thus, this may also provide them to be more or less responsive to mast cell stabilisors.

ASTHMA AND AIRWAY HYPERRESPONSIVENESS

According to The Global Strategy for Asthma management and prevention, supported by GINA (Global initiative for Asthma), asthma is defined as a chronic disorder of the airway in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is reversible, either spontaneously or with treatment.[152]

Different tests can be used *in vivo* for demonstrating airway hyperresponsiveness and airway inflammation. These are divided into two categories of provocation tests depending on their airway smooth muscle action; the "indirect" and the "direct" tests.[153] The stimuli used in indirect tests are physical stimulus such as exercise, osmotic challenge as hyperpnea of dry air, hypertonic saline, distilled water, adenosine monophosphate and mannitol.[153] They are predictors of currently active asthma since well controlled asthmatics on steroids, cromones, frusemide and/or heparin may not respond to these stimuli.[153] Indirect stimuli causes release of endogenous mediators that trigger bronchial smooth muscle contraction and thus a positive test reflects an ongoing airway inflammation.[153] The direct tests are *i.e*. histamine and methacoline challenge however hyperresponsiveness to these agents is not specific for asthma.

EXERCISE-INDUCED BRONCHOCONSTRICTION

In the early 1970's, exercise was introduced as the first standardised indirect challenge test for laboratory use.[5, 154, 155] Exercise was recognized as the most common stimulus for provoking bronchoconstriction and the constriction could be prevented by certain drugs.[5] Among subjects with untreated asthma exercise-induced bronchoconstriction (EIB) occurred in up to 90 % of the patients.[156] Often EIB in children can precede the development of asthma, representing an early stage of the disease.[30] Elite athletes can ventilate more than 200 ml/min, cross country skiers develop asthma like symptoms and this is most probably due to the high exposure of cold and dry air.[157] In fact, long time repeated exposure to insufficiently conditioned air may lead to airway inflammation and remodelling in skiers.[158]

Following exercise, the workload causing tension in muscles and a rise in body temperature will lead to increased breathing. Since inhaled air is humidified during respiration this increase in breathing will cause loss of airway surface liquid, lining the airways (**Fig. 6**).[159] It has been calculated that the fluid lining the ten first airway generations is less than one ml.[160] The dehydration causes water to cross the epithelium into the lumen with resulting dehydration, as the cells loses volume and instead an increase in osmolarity occurs with a higher concentration of calcium and inositol triphosphate inside the cell.[159, 161] This might cause an increase in the formation of inflammatory mediators with subsequent constriction of the bronchial smooth muscle.[162] In line with this, exercise induced bronchoconstriction in asthmatic patients with EIB was significantly reduced when breathing air at 37ºC and 100% humidity.[163] The same mechanism is thought to be caused by mannitol inhalation, though water is transported across the epithelium in response to the composition of the surrounding solute.

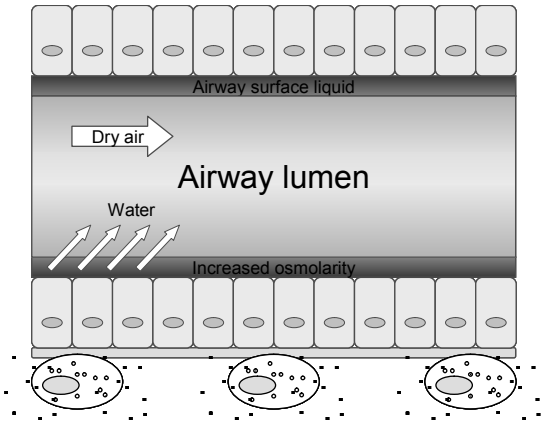

Figure 6. The osmolarity hypothesis. As inhaled air is humidified by the airway surface liquid, a transiently increased osmolarity is created which may affect cells close by. This will activate the cells and subsequently lead to mediator release.

In 1997 provocation with a new hypertonic challenge method was developed for identifying patients with EIB where inhalation of a dry powder of mannitol was used.[164] This method can be used as a surrogate for exercise to identify patients with EIB.[165, 166] Mannitol has also been used to monitor acute and chronic treatment of patients with asthma to determine the severity of the disease and current treatment effectiveness.[167, 168] It has been reported that human lung mast cells release histamine in response to hyperosmolar mannitol stimulation.[169]

AIMS

The general aim of this thesis was to increase the knowledge about formation and release of prostaglandins, leukotrienes and other arachidonic acid derived metabolites in mast cells, and in particular the role of mast cells and their mediators in mannitol-induced bronchoconstriction.

Specific aims

I. To explore if mast cells are activated with release of PGD_2 and CysLTs in response to mannitol stimulation *in vitro*.

II. To study if PGD_2 in mast cells is formed via COX-1 or COX-2 pathway.

III. To investigate if 15-lipoxygenase is expressed in mast cells, and if so which products that are generated.

IV. To examine if mannitol-induced bronchoconstriction is associated with mast cell mediator release as assessed by urinary excretion of the PGD_2 metabolite 9α, 11β-PGF_2.

V. To investigate if the effects of the β_2-adrenoreceptor agonist (formoterol) and disodium cromoglycate (SCG) on mannitol-induced bronchoconstriction can be explained in terms of inhibition of mast cell mediator release.

METHODS

Methods used in this thesis are described in the referred papers as indicated below. Methods not described in detail in **Paper I-V** is presented here.

Table. I.

Method and study design	**Paper**
Preparation of CBMC	I, II, III
RP-HPLC	II, III
Mass spectrometry	III
Enzyme immunoassay	I, II, III, IV, V
Radioimmunoassay	IV
Immunocytochemistry	I, II, III
Immunohistochemistry	III
Western blot	II, III
PCR	III
Subjects and study design	IV, V
Mannitol challenge	IV, V

PREPARATION OF CORD BLOOD DERIVED MAST CELLS (CBMC)

Human cord blood derived mast cells were developed essentially as described in **Paper I, II and III**. All cord blood donors were anonymous and thus, no individual data or information regarding atopy status or family history was available.

ANALYTICAL METHODS

Analysis of cell culture supernatants were performed with reverse-phase high performance liquid chromatography (RP-HPLC), mass spectrometry as described in **Paper II and III**.

In order to confirm the identity of immunoreactive PGD_2 and TXB_2 cell supernatants from CBMC were analysed with RP-HPLC. The samples were injected into a silica based steel cartridge C_{18} HPLC column (3.9 x 150 mm,) eluted with acetonitrile/water/acetic acid (29/71/0.01) for separation of metabolites with an isocratic flow rate of 1ml/min. UV absorbance was monitored at 210 nm for PGD_2 and 205 nm for TXB_2 analyses using a tunable absorbance detector (Waters 386) and metabolites were identified by the retention time of authentic standards. Fractions (1ml) were collected and the organic phase was evaporated before analysis of PGD_2-MOX or TXB_2 with enzyme immunoassay **(Paper II and additional unpublished data)**.

Enzyme immunoassays were used for analysis of LTC_4, PGD_2, 9α,11β-PGF_2, LTE_4, TXB_2, 15-HETE and histamine content in samples. A radioimmunoassay was used for analysing the N^{τ}-methylhistamine content. These assays were performed as described in **Paper I-V**.

CBMC were subjected to cytospin preparations and stained for tryptase enzyme-histochemically as previously described.[170](**Paper I, II**). The G3 monoclonal antibody against tryptase was also used for CBMC tryptase staining (**Paper III)**. Human lung biopsies were stained immunohistochemically with the AA1 monoclonal antibody against tryptase and with the anti-15-LO-1 polyclonal antibody (made in house) (**Paper III**). Skin biopsies were enzyme-histochemically stained for tryptase [171] and immunohistochemically with the anti-15-LO-1 polyclonal antibody (made in house) (**Paper III**).

Molecular biology techniques as western blot were performed on CBMC enzyme expression according to **Paper II and III**. PCR analyses of CBMC mRNA expression were performed as described in **Paper III**.

Subjects and study design

All subjects with asthma had a clinical diagnose of asthma and showed a positive skin prick test. Asthmatic subjects were required to have a baseline forced expiratory volume in one second (FEV_1) ≥70% of predicted, control subjects were required to have a normal spirometry before entering the study. All subjects had to be without any respiratory infection in the 4-week period prior to the study. All subjects were non-smokers (**Paper IV and V)**. The mannitol challenge was performed as described in **Paper IV and V**.

Statistical analysis

For normally distributed unpaired data comparisons between more than two groups were made with parametric tests (One Way Analysis of Variance), further pair wise comparisons were performed with Student's t-test. For non-normally distributed unpaired data, comparisons between more than two groups were made with nonparametric tests (Kruskal-Wallis One Way Analysis of Variance on Ranks). If significant, further pair wise comparisons were performed with Mann-Whitney Rank Sum Test.

For normal distributed paired data, comparisons between more than two groups were made with parametric tests (One Way Repeated Analysis of Variance). Further pair wise comparisons were performed with Student's paired t-test. For non-normally distributed paired data differences between more than two groups were determined with Friedman Repeated Measures Analysis of Variance on Ranks. The difference between two groups was determined by Wilcoxon Signed Rank Test. Correlation was calculated with Spearman's Rank Order.

The geometric mean (Gmean) and 95% confidence interval (CI) for the provoking dose required to cause a 15% fall in FEV_1 (PD_{15}) were calculated using log transformed values and the values were normally distributed. The areas under the mediator excretion curves (AUC; ng or µg per mmol of creatinine vs time) were made from individual data points using the trapezoidal rule for integration. The values were then converted to AUC/h. Sample size requirements were calculated using the data from.[172, 173] (**Paper I-V**).

Difference was regarded as significant if $P < 0.05$.

ETHICAL APPROVAL

Ethical approval regarding the collection of cord blood was given by the ethical review board at Karolinska Institutet (Dnr: 01-374). Ethical approval for mannitol provocation was issued by the Central Sydney Area Health Service Ethics Committee (Protocol No. X99-0089 and X02-0171). All subjects gave written consent form.

RESULTS AND DISCUSSION

MAST CELL MEDIATOR RELEASE IN RESPONSE TO MANNITOL STIMULATION

Since mannitol, as an osmotic stimulus, was shown to induce bronchoconstriction in subjects with EIB [165] the aim in **Paper I,** was designated to explore if CBMC could be activated by mannitol with release of PGD_2 and LTC_4. In this study, CBMC were stimulated with increasing doses of mannitol for 0.5h and supernatants were analysed for content of PGD_2, LTC_4 and histamine.

Mannitol stimulation resulted in release of PGD_2 and LTC_4 as well as histamine. For PGD_2 and histamine release, there was a peak at 0.7M (950 mOsm) mannitol, whereas the release of LTC_4 was further increased by 1.0M (1284 mOsm) mannitol. Despite the profound release (70% of total) of histamine, no lactate dehydrogenase was detected and thus, no cytotoxic effect was demonstrated. In relation to this, it has been reported that the airway surface liquid may reach an osmolarity of 900 mOsm/l H_2O after exercise.[174] Thus, all three mediators were released *in vitro* at a level of osmolarity that is in the same range as reported for EIB *in vivo*.

For comparison, CBMC were also subjected to immunological stimulation. Challenge with anti-λ, an antibody against the λ-chain of the IgE immunoglobulin provoked the release of PGD_2, LTC_4 as well as histamine. These results obtained by anti-λ stimulation and release of PGD_2 and LTC_4 confirm previous studies on CBMC and human lung mast cells.[169, 175-177] In our study, immunological stimulation was a rather weak stimulus for histamine release with 10% and 17% of total histamine release after 2 and 20 μg/ml anti-λ, respectively. Similar amount of released histamine have previously been reported from CBMC with approximately 7-20 % of total histamine after anti-IgE stimulation.[175, 178, 179] For comparison, human lung mast cells released approximately 20% of total histamine after immunological stimulation.[37, 58, 169]

CBMC were also stimulated with the combination of mannitol (0.7M) and anti-λ (2 μg/ml) (**Fig. 7**). The combined stimulation significantly increased the release of LTC_4 in CBMC compared to mannitol alone. This is in contrast to previous results in human lung mast cells where significantly decreased levels of both PGD_2 and LTC_4 were found after combined stimulation compared to anti-IgE alone.[169] However, as previously reported in human lung mast cells [169] we found a synergistic effect of stimulation with anti-IgE in a hyperosmolar solution for histamine release.

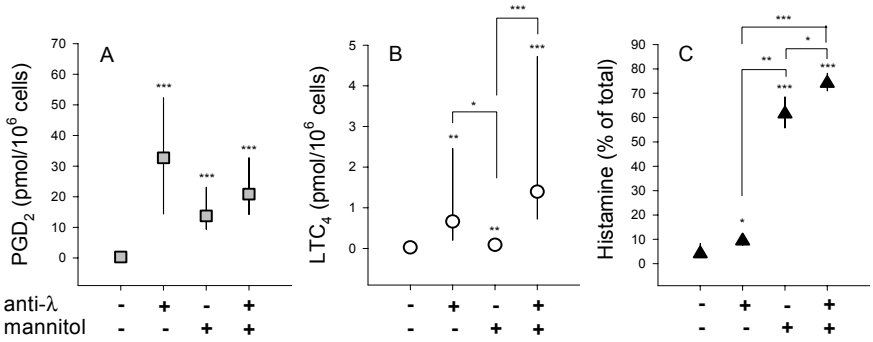

Figure 7. CBMC were stimulated with anti-λ (2 µg/ml), manntiol (0.7M) or their combination for 30 min. Mediator release of A) PGD_2 (square), B) LTC_4 (circle) and C) histamine (triangle) were analysed. Values are presented as median (25^{th}, 75^{th} percentile) and represents eight (A, B) and seven (C) individual experiments (n = 10-15 and n = 8-12 respectively). *P <0.05, **P < 0.01, ***P < 0.001 when compared with control level or as indicated.

To investigate a possible stimulus-dependent mediator release we examined whether there was a correlation between the released mediators. We found a positive correlation between the release of PGD_2 and LTC_4 after anti-λ stimulation but not after mannitol. Thus, mast cells can release mediators differently depending on the stimulus. Differentiated release has also been suggested for stimulation with neuropeptides.[180] Anti-IgE stimulation favoured the release or preformed mediators, whereas activation of Toll-like receptors induced the release of only certain cytokines.[180] Mannitol stimulation of CBMC elicited a small, though significant, release of LTC_4. However, since LTC_4 is 1000 times more potent on airway smooth muscle than histamine it may still be of importance in causing bronchoconstriction in subjects with EIB.[121]

The ratio between the released amount of PGD_2 and LTC_4 was used as an index of a possible stimulus-dependent effect. The favoured release of PGD_2 was most pronounced in response to mannitol challenge. The difference in the ratio of released PGD_2 and LTC_4 after mannitol and anti-λ stimulation may be due to the higher Ca^{2+} dependency of the 5-LO as compared to the COX pathway. During anti-λ stimulation the increase in intracellular Ca^{2+} is achieved by release from both extracellular and intracellular stores as in contrast to non-IgE stimulation where Ca^{2+} is mainly released from intracellular stores. In line with this, the ratio PGD_2: LTC_4 was almost 1:1 after stimulation with the calcium trigger A23187 (**Table II**).

Table II. Ratio of released PGD_2 and LTC_4 after stimulation. Cells were stimulated with anti-λ (2 ug/ml), mannitol (0.7M), the combination of mannitol and anti-λ or with A23187 (5 µM). Values are presented as ratio of released PGD_2 vs LTC_4 in cells from four to eight individual experiments, n = 5-15.

	PGD_2 : LTC_4
Control	14 : 1
Anti-λ	49 : 1
Mannitol	156 : 1
Combination	15 : 1
A23187	1.2 : 1

In summary, this is the first study documenting a significant release of eicosanoids in mast cells in response to mannitol with a predominant release of PGD_2 in CBMC. The results indicate that eicosanoid mediators released from mast cells may be of importance in asthmatic and inflammatory reactions triggered by hyperosmolarity.

BIOSYNTHESIS OF PGD_2 IN CBMC VIA THE COX-1 AND COX-2 PATHWAYS

The aim of **Paper II** was to further investigate the biosynthesises of the dominating prostanoid, PGD_2 in mast cells with focus on how COX-1 or COX-2 may be involved. The studies were performed with the use of selective and unselective COX inhibitors. Indomethacin and diclofenac are unselective COX inhibitors. The compounds FR122047 and Sc-560 are COX-1 selective with Fr122047 being most selective. Rofecoxib and lumiracoxib are COX-2 selective with the latter being most potent.

Mast cells (CBMC) were pretreated with pharmacologic inhibitors and stimulated by IL-1β, anti-IgE or their combination under normoxic (21% O_2) or hypoxic (4% O_2) conditions for different time periods. Interleukin-1β was used as a stimulus since it was previously reported to induce COX-2 expression in monocytes.[181]

The release of PGD_2 was induced at 0.5h after stimulation with anti-IgE alone or in combination with IL-1β. At 24h, IL-1β alone also induced the release of PGD_2. Furthermore, immunoreactive TXB_2 and PGE_2 were detected in CBMC in response to stimulation with IL-1β, anti-IgE or their combination for 24h. The profile of prostanoid release in CBMC was $PGD_2 >> TXB_2 \geq PGE_2$ and this relation were independent of stimuli. The results obtained correspond well with findings in human lung mast cells where PGD_2 was the dominating cyclooxygenase product after anti-IgE stimulation, followed by lower levels of released TXB_2 and PGE_2.[182] Immunoreactive PGD_2 and TXB_2 were also analysed with HPLC (**Fig. 8**). Previously, the leukaemia mast cell line HMC-1 has been found to release TXB_2 and PGE_2 after arachidonic acid and/or ionophore A23187 stimulation.[183]

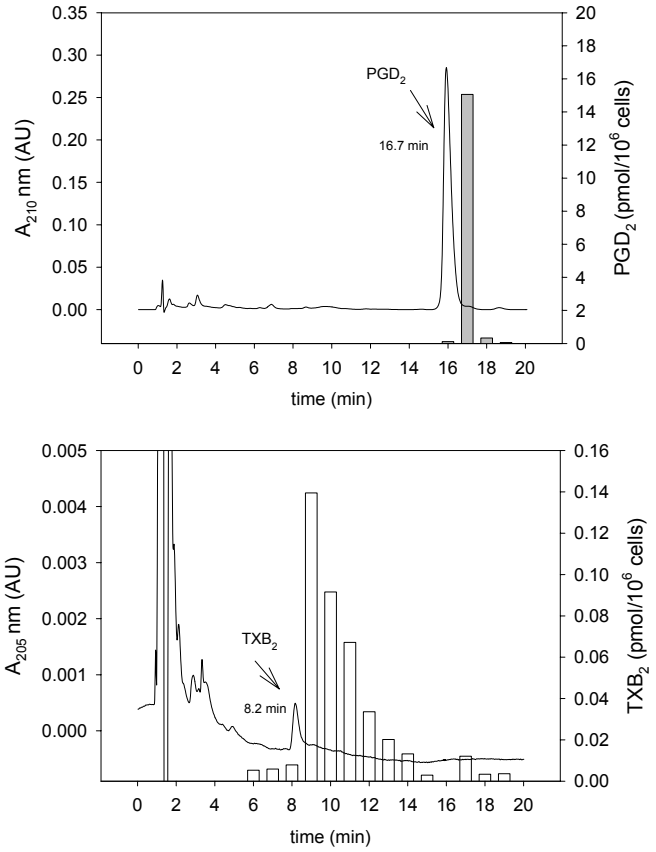

Figure 8. RP-HPLC analyses of synthetic PGD_2 (upper panel, unpublished data) and synthetic TXB_2 (lower panel, **Paper II)** in supernatants from CBMC stimulated with anti-IgE (2 µg/ml) (upper panel) and anti-IgE and /or IL-1β (50ng/ml) (lower panel) for 0.5h. One ml fractions were collected, derivatised and analysed for immunoreactive PGD_2-MOX (upper panel) and TXB_2 (lower panel).

Stimulation with IL-1β was found to induce COX-2 expression at 24h without any effect on PGD synthase or COX-1. Treatment with IL-1β alone for 0.5h did not induce release of PGD_2, whereas previous results with significant release after anti-IgE alone were confirmed. However, after 24h, IL-1β significantly increased the release of PGD_2, although in lower amounts than after anti-IgE stimulation. Additionally, there was a synergistic effect of IL-1β and anti-IgE on PGD_2 release at 24h.

Anti-IgE induced PGD_2 release was inhibited by the selective COX-1 inhibitor FR122047 and the selective COX-2 inhibitor lumiracoxib. However, the COX-1 inhibitor was most effective at 0.5h. The combined stimulation with anti-IgE and IL-1β was inhibited to a higher extent by the selective COX-1 inhibitor as the selective COX-2 inhibitor failed to inhibit PGD_2 release at 0.5h.

In contrast, IL-1β did not stimulate release of TXB_2 at 0.5h. However, at 24h both anti-IgE and IL-1β significantly induced the release of TXB_2, but there was no synergistic effect using the combination. The selective COX-1 and COX-2 inhibitors were equally effective on inhibiting TXB_2 release induced by the combination of IL-1β and anti-IgE at 24h.

Our data, showing that PGD_2 release induced by an early immunological response in CBMC are more COX-1 than COX-2 dependent, is in line with previous results from CBMC cultures.[178, 184] It was reported that the selective COX-2 inhibitor NS-398 and the unselective COX inhibitor indomethacin dampened anti-IgE induced PGD_2 at 0.5h.[178, 184] Also, CBMC stimulated with anti-IgE for 2h was found to induce COX-2 mRNA transcripts [185] which would indicate that late (after 2h) prostanoid formation induced by immunological stimulus may be relatively more COX-2 dependent.

Hypoxia may stimulate an increased production of mediators including prostanoids in many cell types.[186] As asthmatic and allergic responses are associated with local and sometimes also systemic hypoxia, it was of interest to assess whether or not hypoxia affected release of PGD_2 and other products from CBMC. However, our results showed that hypoxia had no effect on IL-1β, anti-IgE or their combined induction of PGD_2, TXB_2 or PGE_2 release in CBMC. Interestingly, this indicates that mast cells, as opposed to other inflammatory cells, remain unaffected by hypoxic environmental changes.

In summary, IL-1β induced the expression of COX-2 and also the release of PGD_2 after 24h in CBMC. The early response of PGD_2 release induced by IL-1β in combination with anti-IgE was more COX-1 dependent, whereas the late response was more COX-2 dependent. CBMC has the capacity to produce immunoreactive TXB_2 in addition to PGD_2 after IL-1β, anti-IgE or combined stimulation. The combination of IL-1β and anti-IgE had synergistic effects on PGD_2 release after 24h of stimulation as compared to each stimulus alone. Hypoxia did not induce any additional release of PGD_2, TXB_2 or PGE_2 after stimulation.

EXPRESSION OF 15-LO-1 IN CBMC

In **Paper III,** the aim was to investigate if CBMC express 15-LO-1 and if so, if interleukin-4 (IL-4) regulates the expression with subsequent effects on mediator release.

For investigation of IL-4 effects on mast cells, CBMC were cultured according to two protocols, with serum ($CBMC^{MNC}$) or without serum ($CBMC^{SC}$). This was followed by treatment with IL-4 for 120h. The results showed that the expression of 15-LO-1 was induced by IL-4 in both $CBMC^{MNC}$ and $CBMC^{SC}$ in a time dependent manner. Furthermore, IL-4 was mandatory for the expression of 15-LO-1.

For investigation of catalytic activity of the expressed 15-LO protein, $CBMC^{MNC}$ were cultured with or without IL-4 for 120h and incubated for 5 min with [1-^{14}C] labelled arachidonic acid in the presence of indomethacin. Indomethacin was added to all incubations to exclude COX derived 15-hydroxylated metabolite formation. It was previously shown in the human mast cell line HMC-1 that the formation of 15-HETE was inhibited by indomethacin, suggesting that the 15-HETE was derived via the COX pathway.[183] In IL-4 treated CBMC, a major radioactive peak eluted at the retention time of authentic 15-ketoeicosatetraenoic acid (15-KETE) and a minor radioactive peak was detected at a retention time corresponding to 15-hydroxyeicosatetraenoic acid (15-HETE). The relation 15-KETE to 15-HETE was 9:1 in CBMC. For confirmation of the *in vivo* significance of the 15-LO expression, we found 15-LO expression co-localised to a subset of mast cells in skin and lung biopsies.

There are different opinions regarding the function of 15-HETE and other 15-LO derived metabolites, they may in fact act as both pro- and anti- inflammatory mediators.[94, 98, 100] 15-HETE was the main arachidonic acid metabolite in human bronchi.[187] It was also found that human lung tissue from asthmatic subjects produced more 15-HETE than specimens from non-asthmatic subjects.[188] However, inhalation of 15-HETE prior to allergen inhalation sustained the airway response in the asthmatic individuals.[95] In contrast, inhalation of 15-HETE prior to histamine provocation did not further induce any bronchoconstriction.[95] These findings support a pathophysiological role for 15-HETE in asthmatic diseases.

Osmotic activation of mast cells may be of importance in the pathophysiology of EIB (**Paper I**). It has been found that nasal lavage fluid from patients with allergic, active or inactive, rhinitis had increased levels of 15-HETE after inhaling 900 mOsm mannitol compared to placebo.[189] In addition, the increase in 15-HETE could be related to a decrease in nasal peak inspiratory flow in patients with an active rhinitis.[189] Further, we found that IL-4 treated CBMC, stimulated with 0.7M mannitol showed a strong tendency of increased release of 15-HETE as compared to isotonic control treatment ($P = 0.05$) **(Paper III)**.

In summary, this report demonstrates the expression of enzymatically active 15-LO-1 in human mast cells after treatment with IL-4. Both $CBMC^{MNC}$ and $CBMC^{SC}$ converted arachidonic acid to 15-KETE in the presence of IL-4. The expression of 15-LO-1 in mast cells might be of importance for the function of mast cells in asthma and other inflammatory disorders. It remains to be elucidated whether a putative pathophysiological role of 15-LO-1 in mast cells is pro-or anti inflammatory.

COMPARISON OF TWO MODELS OF CBMC PREPARATIONS

During the course of the studies, presented in **Paper II and III,** two different methods for preparation of CBMC were established, one with serum added from day 1 ($CBMC^{MNC}$) and one without serum during the first weeks in culture ($CBMC^{SC}$).

After 8-10 weeks in culture with serum, the yield of $CBMC^{MNC}$ cells was low, about 1% of the starting mononuclear cell amount were derived into tryptase positive $CBMC^{MNC}$. In contrast, $CD34^{+}$ selected mononuclear cells cultured in serum-free medium were tryptase positive already after 3-5 weeks ($CBMC^{SC}$). Further, the cell number increases and reaches more than 100% of the starting amount since the cells were dividing.

We wanted to investigate whether there was a difference between CBMC cultured by the two different protocols and thus, mediator release and enzyme expression were investigated in $CBMC^{MNC}$ and $CBMC^{SC}$.

Elevated levels of PGD_2 were released after stimulation by anti-IgE, IL-1β or with the combination of both. $CBMC^{MNC}$ released almost 4-times the amount of PGD_2 compared to $CBMC^{SC}$ after anti-IgE and IL-1β stimulation. However, levels were increased to the same extent in the two different culturing methods. The unselective COX inhibitor indomethacin significantly inhibited the induced PGD_2 release to the same extent in $CBMC^{MNC}$ and $CBMC^{SC}$ (**Fig. 9**).

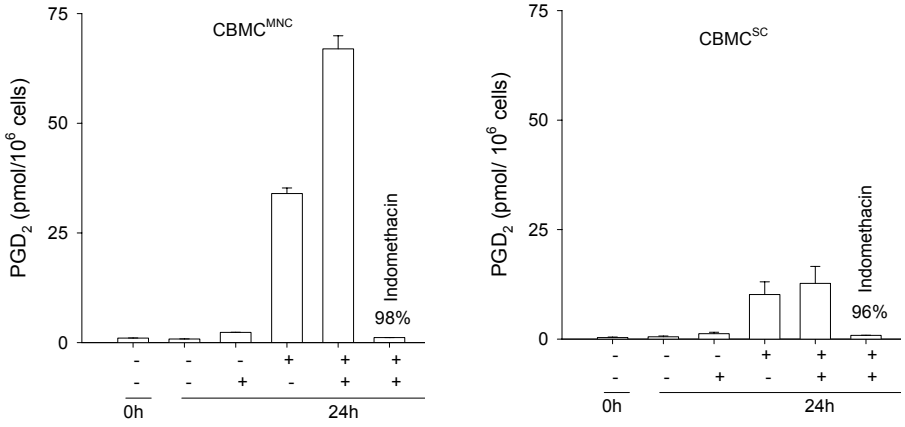

Figure 9. Release of PGD_2 after stimulation of $CBMC^{MNC}$ and $CBMC^{SC}$ with anti-IgE (2 ug/ml), IL-1β (50 ng/ml) or the combination of both. Percent inhibition of the combined stimulation with indomethacin (unselective COX inhibitor).

Further, when comparing the two different cell preparations it was found that the amount of 15-KETE produced was 6-times higher in cells cultured in medium supplemented with serum ($CBMC^{MNC}$) compared to cells cultured in serum free medium ($CBMC^{SC}$) (**Fig. 10, Paper III**).

Together, this would indicate that mast cells cultured without serum have less ability to produce COX and LO derived metabolites. This most probably depends on the lower enzyme expression rather than its activity.

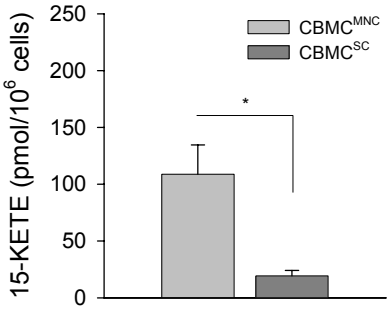

Figure 10. 15-KETE formation in IL-4 treated $CBMC^{MNC}$ and $CBMC^{SC}$ stimulated with arachidonic (**Paper III**). Data are expressed as mean ± SEM from five experiments respectively, n = 5 and $^*P < 0.05$.

Previously, there has been one report investigating the difference in culture conditions with or without serum.[190] The authors reported that cells cultured without serum released less amount of histamine and expressed fewer FcεRI receptors as compared to cells initially cultured without serum but with the addition of 10% FCS after week 8. They also found that cells cultured without serum showed a minor percent of chymase positive cells as compared to cells cultured with serum after week 8.[190] In a previous study, the addition of a serum-associated lipid growth factor lysophosphatic acid (LPA) to serum free CBMC culture system induced mast cell differentiation and proliferation.[191] Thus, there seem to be factors in serum that may effect CBMC maturation and it remains to be investigated whether addition of LPA may affect eicosanoid formation and release in CBMC.

In summary, cells cultured in serum free medium showed less capacity to release mediators as compared to cells cultured with serum from day 1. However, the profile of eicosanoids formed was similar for the two conditions. The great advantage of faster cell proliferation and maturation make the culturing without serum an attractive alternative.

MANNITOL-INDUCED BRONCHOCONSTRICTION AND MAST CELL MEDIATOR RELEASE

In **Paper IV**, the aim was to examine if mannitol-induced bronchoconstriction was related to mast cell mediator release with excretion of the PGD_2 metabolite 9α, 11β-PGF_2. Atopic subjects with asthma and a history of EIB (n = 12) and non-atopic subjects without asthma (n = 9) were subjected to mannitol challenge by inhalation of cumulatively increasing doses. Lung function tests were performed and urine samples were collected before and after mannitol challenge for analysis of urinary LTE_4 and 9α, 11β-PGF_2.

Previously, mannitol as a challenge test has been found to identify patients with EIB.[165, 166] In this study, the asthmatic subjects showed a sustained airway response with a mean fall in FEV_1 of 36% after mannitol inhalation of a mean cumulative dose of 272 mg. In the non-asthmatic subjects, there was no fall in FEV_1, though they inhaled the highest dose of mannitol (635 mg). Increased levels of urinary LTE_4 and the

PGD_2 metabolite 9α, 11β-PGF_2 could be demonstrated after mannitol challenge in both the asthmatic and the non-asthmatic subjects.

In the asthmatic subjects, urinary 9α, 11β-PGF_2 reached a maximum at 30 min following the mannitol challenge, and the levels declined toward baseline levels at 90 min. In contrast, the LTE_4 levels were sustained from 30 to 90 min, and there was no significant decline. There was no significant increase of urinary N^{τ}-methylhistamine after mannitol challenge in the asthmatic subjects.

In the control subjects, time-course for the excretion of 9α,11β-PGF_2 differed somewhat from the asthmatics, with values having decreased back to baseline at 60 min after challenge. However, for LTE_4, the time-course for the urinary excretion of LTE_4 was similar to the asthmatic subjects. In contrast to the asthmatic subjects, there was a small increase in the urinary excretion of N^{t}-methylhistamine after challenge with mannitol in the control subjects $P < 0.05$.

When comparing the urinary excretion between the groups, we found a significant increased release of 9α,11β-PGF_2 in subjects with asthma as compared to control subjects despite the fact that the asthmatic group inhaled less than half the amount of mannitol. This increase was related to bronchoconstriction. There was no difference regarding either the urinary excretion of LTE_4 or N^{t}-methylhistamine between the groups. In a previous study it was shown that exercise significantly increased the urinary excretion of 9α, 11β-PGF_2 in asthmatic subjects with EIB as compared to control subjects with no effect in either urinary LTE_4 or N^{τ}-methylhistamine.[172] Further, the increase in 9α, 11β-PGF_2 was associated with bronchoconstriction.[172] This supports a similar mechanism causing 9α, 11β-PGF_2 release and bronchoconstriction in subjects with EIB after mannitol or exercise challenge tests.

The reason why mediator release also occurred in the non-asthmatic group is most probably due to the high amount of mannitol used for challenge. The reason why only the asthmatic subjects displayed bronchoconstriction is presumably their increased level of bronchial hyper-responsiveness. Increased infiltration of mast cells in the airway smooth muscle of the asthmatic subjects may also contribute to the different responses. These factors are most important in EIB.[192]

In summary, these results demonstrate that the airway narrowing provoked by inhalation of mannitol in asthmatic subjects is associated with increased urinary excretion of eicosanoids. Specifically interesting is the pronounced formation of the bronchoconstrictive PGD_2 in response to mannitol which strongly indicate mast cell activation.

PHARMACOLOGICAL INTERVENTION AND MANNITOL-INDUCED BRONCHOCONSTRICTION

The aim of **Paper V** was to find out if a mast cell stabiliser or a β_2-agonist protected against mannitol-induced bronchoconstriction via inhibition of mast cell activation.

Atopic asthmatic subjects (n = 14) with EIB were treated with placebo, formoterol or sodium cromoglycate (SCG) prior to mannitol challenge in a double blind crossover design. After treatment (15 min) they were subjected to a cumulative mannitol challenge using a dose pre-determined to cause ≥ 25% reduction in FEV_1. Lung function (FEV_1) and urine sample were collected before and after mannitol challenge.

A mean 29% fall in FEV_1 was seen after placebo. Both cromoglycate and formoterol significantly inhibited the response to inhaled mannitol ($P < 0.001$) by providing a 63% and a 95% protection of the maximum fall in FEV_1, respectively. In association with the mannitol-induced bronchoconstriction on the placebo day, there was an increase in the urinary excretion of both 9α,11β-PGF_2 and LTE_4. By contrast, there was no significant increase in the urinary excretion of 9α,11β-PGF_2 in the presence of either SCG or formoterol after mannitol challenge. In fact, the levels of 9α,11β-PGF_2 after mannitol challenge were significantly lower at 90 min as compared to baseline in the presence of SCG and lower at both 60 and 90 min with eformoterol. However, the urinary excretion of LTE_4 in the presence of either eformoterol or SCG was maintained as compared to placebo.

The importance of mast cells in osmotically driven bronchoconstriction found in the previous study (**Paper IV)** was further supported by replicating data on urinary excretion of the PGD_2 metabolite 9α,11β-PGF_2 and by the effect of cromoglycate on the urinary excretion of 9α,11β-PGF_2. The decreased urinary levels of 9α,11β-PGF_2 were also related to protection from bronchoconstriction even though cromoglycate had no bronchodilator effect. Previously, SCG has been found to prevent EIB in children and adults.[193, 194] In the present study, the urinary excretion of LTE_4 remained unchanged, a finding that may suggest a source of leukotrienes other than mast cells. However, CysLTs are likely to be involved in the airway response to mannitol as the time course of recovery of lung function is faster in the presence of the leukotriene antagonist montelukast.[167]

In summary, these results clearly demonstrated inhibition of bronchoconstriction in association with inhibition of the release of a mast cell mediator by sodium cromoglycate and the β_2-agonist formoterol in response to an osmotic stimulus *in vivo.* The results strongly support a role for the mast cell in release of mediators in response to exercise mimetic.

GENERAL DISCUSSION AND FUTURE PERSPECTIVE

The results reported in this thesis demonstrate that mast cells can be activated by mannitol stimulation *in vitro* and *in vivo* causing release of arachidonic acid derived PGD_2, LTC_4, 15-HETE and granulae stored histamine.

MAST CELLS IN DISEASE

The relative number of mast cells has been found to be increased in smooth muscle and BAL fluid of asthmatic subjects.[32, 195, 196] Further, subjects with allergic asthma had a significantly thicker bronchial smooth muscle layer than control subjects or non-allergic asthmatic subjects.[196] Lung tissue from asthmatic subjects was shown to contain a higher percentage of degranulated mast cells as compared to control subjects, and this was related to disease severity.[38] In addition, subjects with asthma had increased number of mast cell colony forming cells in the blood.[197] This constitutes a hypothesis that infiltration of mast cells into the airway smooth muscle and interaction with other cells are important in the development of asthma. In **Paper IV**, an increased urinary excretion of 9α,11β-PGF_2 was seen as an index of mast cell activation in both asthmatic and non-asthmatic subjects. The increase of 9α,11β-PGF_2 was more pronounced in the asthmatic group in spite of a lower dose of inhaled mannitol. The results may be due to an increased number of mast cells in the asthmatic subjects.

Mastocytosis is a disease characterised of mast cell proliferation and accumulation resulting in organ or tissue hyperplasia.[198] Patients with mastocytosis have increased urinary excretion of PGD_2 metabolites.[199] It was recently found that patients with mastocytosis had an increased systemic and urinary TX formation.[200] Further, the formation of TX correlated with excretion of urinary 9α,11β-PGF_2 and N^{τ}-methylhistamine, suggesting that the TX forming cell source might be mast cells and not platelets.[200, 201] For the first time, we found release of TXB_2 in CBMC (**Paper II**). Since CBMC may be regarded as an appropriate model of human mast cells this strongly support the hypothesis that mast cells may be a source of TX.

HYPEROSMOLAR STIMULATION AND MAST CELL INVOLVEMENT IN VIVO AND IN VITRO

Bronchoconstriction caused by mannitol inhalation correlates with other indirect challenge methods mimicking exercise such as, eucapnic hyperventilation (EVH) and hypertonic saline.[164, 165] In **Paper IV** we found that asthmatic subjects with EIB as well as control subjects released elevated amount of urinary 9α, 11β-PGF_2 and LTE_4 in response to mannitol challenge. Further, **Paper I** is the first documentation of significant release of PGD_2 and LTC_4 in mast cells in response to mannitol stimulation *in vitro*. Together these results support mast cell activation in response to hyperosmolar stimulation both *in vivo* and *in vitro*. As by 25 October 2006 mannitol (Aridol®) received marketing approval in Sweden for use in diagnosis and management of asthma. Mannitol is also approved for the same indication in Australia.

Nasal challenge with cold dry air induces the release of histamine, PGD_2 and LTC_4 into nasal lavage (NAL) fluid in subjects with rhinorrhea.[202] There is also evidence of increased levels of histamine and LTs in NAL fluid after nasal challenge by

hyperosmolar mannitol solutions in non-asthmatic subjects.[203] Further, mannitol-induced 15-HETE production has previously been found in NAL fluid in patients with allergic rhinitis.[189] Interestingly, this increase was related to a decreased nasal peak inspiratory flow (nPIF) in patients with active rhinitis.[189] In relation to this, in **Paper III**, 15-HETE was released from IL-4 treated CBMC after mannitol stimulation, indicating that 15-HETE might play a role in osmotically driven responses such as EIB.

In **Paper IV**, there was a small increase of urinary N^{τ}-methylhistamine after mannitol challenge in control subjects but not in subjects with asthma. In the *in vitro* setting, CBMC were found to release high amounts of histamine after mannitol stimulation *in vitro* (**Paper I**). Previously, it was also found that human lung mast cells was activated with histamine release *in vitro* by small changes in osmolality[37] However, different subtypes of mast cells are exposed *in vivo* and *in vitro*. In **Paper I** we used cells cultured in serum which favours the formation of the MC_{TC}-type of mast cells.[108] The enriched lung mast cells however, belong to the MC_T-type of cells known to be less responsive to non-immunological stimulation.[22, 169] The low levels of urinary N^{τ}-methylhistamine after mannitol challenge in subjects with asthma and control subjects *in vivo* (**Paper IV**) was also found with exercise.[172] Whereas allergen challenge previously was found to induce significantly increased levels of urinary N^{τ}-methylhistamine, the relative increase of 9α, 11β-PGF_2 above baseline was much higher in the asthmatic subjects.[88] This implicates that also *in vivo* the type of stimuli used will affect the profile of mediator release.

Hyperosmolar stimulation was reported to activate human lung mast cells *in vitro* with histamine release.[37] However, neither the release of leukotrienes nor prostaglandins were significantly induced.[169] The disagreement between previous results with no effect on the release of PGD_2 or LTC_4 as compared to our results in **Paper I** with significant levels of released PGD_2 and LTC_4 after mannitol stimulation may be due to the different subtypes of mast cells used. However, it may also depend on the cell purity. In **Paper I** mast cells were 99% tryptase positive as compared to the cells prepared from human lung parenchyma with a purity of 1-10% mast cells.[169]

Thus, the subtype of mast cells used, the potency of stimuli and the purity of cells are important and seem to determine the amount of released mediators.

MEDIATOR FUNCTION IN EIB

As previously discussed, when comparing the urinary excretion of 9α, 11β-PGF_2, LTE_4 and histamine in subjects with EIB and control subjects after mannitol challenge (**Paper IV)** there was only a significant difference in the excretion of urinary 9α, 11β-PGF_2 . This was also found in a previous study were patients were subjected to exercise.[172] Together, this further supports the concept that mannitol challenge mimics exercise as a stimulatory event. In our experiments *in vitro*, the amounts of released PGD_2 were 156-times higher than the amounts of LTC_4 after mannitol stimulation (**Paper I**), thus, supporting our *in vivo* data with significantly increased release of the PGD_2 metabolite 9α, 11β-PGF_2 after mannitol challenge (**Paper IV**). Mast cell derived PGD_2 may thus be released in sufficient amounts in response to hyperosmolar stimulation to cause physiological effects. Since 9α, 11β-PGF_2 was the main C20 metabolite found in urine after PGD_2 injection and mast cells are the predominant PGD_2 producing cells, 9α,11β-PGF_2 is a good marker of mast cell activation *in vivo*.[58, 201, 204]

The urinary excretion of 9α, 11β-PGF_2 after mannitol challenge was related to bronchoconstriction in subjects with EIB (**Paper IV**). The potency of both PGD_2 and 9α, 11β-PGF_2 on the airway smooth muscle further support this finding.[113, 117] In our study, the non-asthmatic subjects inhaled almost three times the amount of mannitol compared to the asthmatic subjects and yet no bronchoconstriction was obtained (**Paper IV**). It is known that the epithelium lining the airways can be damaged in subjects with EIB, leading to airway hyperreactivity.[205] Thus, airway sensitivity and the increased infiltration of mast cells indicate that our results obtained from mannitol-induced bronchoconstriction in asthmatic subjects is due to mast cell involvement. Further, the β_2-agonist (formoterol) and disodium cromoglycate (SCG) protected against mannitol-induced bronchoconstriction in asthmatic subjects even though SCG has no direct bronchodilatory effect (**Paper V**). This protection may at least in part be caused by the inhibition of released PGD_2 since urinary excretion of 9α,11β-PGF_2 was attenuated, implicating the importance of mast cell activation in the airway response to exercise (**Paper V**).

The urinary LTE_4 excretion was sustained in subjects with EIB as well as in control subjects after mannitol challenge and there was no difference between the groups (**Paper IV**). In **Paper V**, pre-treatment with either formoterol or SCG had no effect on urinary LTE_4 excretion, possibly suggesting another source than mast cells. Neither was the urinary excretion of N^{τ}-methylhistamine different between the groups (**Paper IV**) Thus, it seems as the analysis of PGD_2 or its metabolite 9α,11β-PGF_2 is a better marker of mast cell activation as compared to histamine since PGD_2 and 9α,11β-PGF_2 were found *in vitro* (**Paper I**, **II**) and *in vivo* (**Paper IV**, **V**), respectively.

It may be speculated that endogenous protective mechanisms may be lost in the asthmatic subjects leading to more sensitive airways. Inhalation of PGE_2 before exercise in asthmatic subjects with EIB protected against a fall in FEV_1 and it reduced the duration of bronchoconstriction.[206] Thus, if the production of PGE_2 or relevant receptor expression is lost, airways may be more easily triggered. Atopic asthmatic subjects treated with PGE_2 before allergen challenge reduced the level of PGD_2 in BAL fluid compared to placebo treatment.[64] In another report, exercise induced the levels of histamine, Cys-LTs and tryptase in sputum samples from subjects with EIB whereas decreased levels of PGE_2 and TXB_2 were obtained.[205] It is known that PGE_2 inhibit mast cell mediator release.[207] This strengthens the hypothesis that protective mediators such as PGE_2 may be missing in subjects with EIB. Epithelial cells are a major source of PGE_2 and loss of epithelium would imply imbalance of the mediator that inhibits mast cell activation and relaxation of smooth muscle.[64] In **Paper II** a small but significant production of PGE_2 was demonstrated by the mast cells. However, it remains to be elucidated in detail how mast cells respond to PGE_2 in healthy as well as asthmatic subjects.

PHARMACOLOGICAL INTERVENTION

The use of H_1 histamine receptor antagonists in subjects with persistent asthma is debated. There are reports of decreased asthma symptoms and also improvement in lung function after treatment with a H_1 receptor antagonist.[102, 208] Treatment with the H_1 receptor antagonist fexofenadine in asthmatic subjects was found to decrease the sensitivity to inhaled mannitol but, there was no airway protection.[167] In another study, treatment with the histamine antagonist loratadine showed no protective effect on exercise-induced bronchoconstriction in subjects with asthma compared to placebo.[209]

Thus, since H_1 antagonists presumably have no effect on the underlying inflammation, the effect on EIB seems to be limited. In another study, asthmatic subjects were pretreated with the histamine receptor antagonist, terfenadine, the cyclooxygenase receptor antagonist, flurbiprofen, a combination of both or with placebo followed by exercise.[210] It was found that the combination of terfenadine and flurbiprofen protected against bronchoconstriction.[210] There was no protection against the fall in FEV_1 with either inhibitor alone. Thus, both histamine and COX derived products seem to be involved in EIB.

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Leukotrienes are likely to be involved in the airway response to mannitol as the time course of recovery of lung function was faster in the presence of the leukotriene receptor ($CysLT_1$) antagonist, montelukast.[167] Montelukast has also been shown to protect against exercise-induced asthma via limiting the decline and shortening the lung function recovery.[211-213] The $CysLT_1$ antagonist zafirlukast and the histamine antagonist loratadine given in combination before exercise significantly inhibited but did not completely block the airway response to exercise.[209] Treatment with zafirlukast alone protected from a FEV_1 fall and resulted in a faster recovery as compared to placebo treatment.[209] Together, these results suggest that leukotrienes are involved in EIB and that CysLTs might play an important role in sustaining the bronchial response to dry air.

As mentioned above, neither a histamine antagonist by itself or in combination with a leukotriene receptor antagonist completely blocks the airway response to mannitol or exercise. This would implicate the importance of other active metabolites such as cyclooxygenase derived products. Such a hypothesis was supported in a study where the cyclooxygenase inhibitor indomethacin protected against exercise-induced bronchoconstriction.[214] In line with this, the combination of a histamine antagonist, a leukotriene agonist and a COX inhibitor completely abolished allergen induced constriction in sensitised guinea pig lungs.[215] In the future, effects of agents blocking the synthesis or the receptor binding of PGD_2 would provide further information regarding the mechanism of action. Further indomethacin was also found to reduce the decreased arterial oxygen saturation after exercise in children.[214]

Cromones (sodium cromoglycate (SCG) and nedocromil sodium) are used as "mast cell stabilisers". Even though these drugs showed no or few side effects, their use has been limited because of large differences in efficacy *in vivo*. In adult asthmatic patients only 20-30% respond to these substances.[216] However, cromones are commonly used for treatment of eye inflammation during pollen season and for treatment of asthma in children.[152] *In vitro*, SCG have been shown to inhibit human lung mast cell derived PGD_2 and histamine release after immunological activation.[150, 217, 218] In **Paper V**, SCG was found to be a potent inhibitor of $9\alpha,11\beta$-PGF_2 excretion in subjects with asthma.

Inhalation of β_2-adrenoreceptor agonists are the most widely used treatment for acute relief of bronchoconstriction.[152] Further, administration of β_2-agonists before exercise has been found to reduce the fall in FEV_1 by 78-80% in the majority of subjects.[219] β_2-agonists dilate bronchial smooth muscle contraction (via binding to G protein-coupled receptors) but it also reduces the responsiveness to a number of different triggers.[220] *In vitro* β_2-agonists inhibit mast cell mediator release.[218, 221] In fact, immunologically induced release of human lung tissue mast cell PGD_2, LTs and histamine was inhibited to a higher extend with a β_2-agonist as compared to SCG.[218, 221] Further, *in vivo*, β_2-agonists prevented allergen induced mast cell derived mediators.[222]

In **Paper V**, we found a reduced urinary excretion of 9α,11β-PGF_2 after administration of formoterol in subjects with asthma. However, the effect of decreased mast cell mediator release after β_2-agonist pre-treatment followed by stimulation is probably independent of bronchodilation and may be explained by inhibition of mast cell release. .[222] When the agonist binds to its receptor it stabilises the cell and prevents the binding of other ligands and thus inhibits cell activation and mediator release that in turn might affect bronchial smooth muscle.[219]

CELLS INVOLVED IN EICOSANOID FORMATION IN ASTHMA AND EIB

Not only mast cells can be triggered with mannitol but also eosinophils form LTC_4 and basophils release histamine after mannitol stimulation *in vitro*.[37, 223] Hence, the released leukotrienes or histamine may not only be derived from mast cells. In **Paper III** mannitol stimulation of CBMC induced the release of 15-HETE and 15-LO was co-localised with tryptase both in lung and skin mast cells. Thus, indicating that mast cells may also contribute to the formation of 15-LO derived products *in vivo*. We can hypothesise that the loss of epithelial cells lining the airways in asthmatic subjects and thus loss in 15-LO derived products may to some extent be reconstituted by infiltrating 15-LO containing mast cells. However, the *in vivo* effects caused by mast cell derived 15-KETE and 15-HETE and their patophysiological role remains to be elucidated.

The findings from **Paper I**, **IV** and **V** support that PGD_2 and its metabolite 9α,11β-PGF_2 might be involved in mannitol-induced bronchoconstriction in subjects suffering from asthma and EIB. Mast cells are the main PGD_2 producing cell and after IgE dependent activation the production was found to be 100-1000 fold lower in eosinophils, macrophages and T-lymphocytes.[59] Eosinophils do not possess the capacity to form PGD_2, they can only metabolize PGD_2 to 9α,11β-PGF_2 *in vitro*.[224] However, the contribution of this metabolic route to the urinary level of 9α,11β-PGF_2 in the present studies are likely to be very minor. There are contradictory results concerning macrophages and their capacity to form PGD_2. Alveolar macrophages derived from BAL fluid from asthmatic and control subjects were found to produce PGD_2.[225, 226] However, no data regarding the cross reactivity of the antibody used in the immunoassay or the cell number used in their experiments were presented. Basophils are also activated by increased osmolarity with release of histamine [169], both anti-IgE and hyperosmolar stimulation significantly induced the release.

Cysteinyl leukotrienes have been found in BAL fluid from asthmatic subjects after allergen challenge.[227] Eosinophils are common in sputum samples of asthmatic subjects, possibly implying that they might be the source of leukotriene production. Mannitol has also been shown to induce leukotriene formation in eosinophils in both asthmatic and control subjects.[223] Thus, eosinophils may be the source of LTE_4 excreted in subjects with EIB challenged with mannitol (**Paper IV** and **V**). Previously, hyperosmolar stimulation alone did not induce any CysLT release either in basophils or lung mast cells *in vitro*.[169] It was found that immunological stimulation significantly induced the release of CysLTs both in basophils and mast cells whereas the combination of immunological stimulation in a hyperosmolar solution significantly dampened anti-IgE induced CysLT released.[169] Eosinophils can release elevated amounts of LTC_4 and it was shown that eosinophils from asthmatic patients had a 3-fold increased activity of their 5-LO enzyme.[228] Thus, in addition to the infiltrating amounts of eosinophils, their enzymatic activities might also be higher as compared to control subjects.[228] While the number of eosinophils is increased in the airways of

asthmatic subjects and eosinophils are a source of cysteinyl leukotrienes [229] the activity of SCG or β_2 agonists on eosinophils in the presence of osmotic stimuli remains to be determined.

Circulating histamine levels are to 98% contained within basophils.[230] Serum histamine levels are not a good marker of mast cell histamine release since mast cells reside in tissue and basophils in blood. However, histamine levels in plasma induced by allergen challenge are suggested to be derived from the lung.[222] There are contradictive results on the source and mechanism of histamine release in response to bronchial challenge. For example, atopic asthmatic subjects challenged with allergen obtained significantly increased serum histamine levels during the early response but, there was no effect on the late FEV_1 response or in urinary excretion of N^{τ}-methylhistamine.[231] Hyperosmolar mannitol stimulation induced histamine release from both basophils and mast cells *in vitro* but basophils were found to respond at lower concentrations.[169] There was no additive effect with the combination of immunological stimulation in a hyperosmolar solution for histamine release in basophils.[169] In contrast, in human lung mast cells there was a synergistic effect on histamine release by immunological stimulation in a hyperosmolar solution.[169] Interestingly, this synergistic effect on histamine release was also found in CBMC in **Paper I**.

STIMULUS DEPENDENT RELEASE AND REGULATION OF MAST CELL MEDIATOR RELEASE

Mast cells are known to release many different mediators in response to immunological stimulation. However, for non-IgE mediated reactions there is often a stimulus dependent release of preformed and *de novo* synthesised mediators. In our work, there was no correlation between the released PGD_2, LTC_4 or histamine after mannitol provocation though we found a correlation for released PGD_2 and LTC_4 after immunological stimulation (**Paper I**). It has also been reported that IL-1 stimulated CBMC released newly synthesised IL-6 but not tryptase.[232]

Differentiated release has also been suggested for neuropeptide stimulation of mast cells, which favoured the release or preformed mediators.[180] In contrast, triggers binding to toll-like receptors favour the release of certain cytokines.[180] Different triggers may also potentiate each others effects regarding mediator release. In **Paper I** we found that immunological stimulation in a hyperosmolar solution had a synergistic effect on histamine release at 30 min in CBMC. Interestingly, allergic asthmatic subjects with EIB have been shown to have an enhanced bronchial reaction during pollen season.[233] This may be caused by the synergism of allergen and the hyperosmolar effect caused by exercise. In fact, such a mechanism was strongly indicated in **Paper I** were a synergistic effect of histamine release was seen with the combined stimulation of mannitol and anti-IgE. In **Paper II**, the combination of immunological stimulation and stimulation of IL-1β was synergistic for PGD_2 release after 24h of stimulation. These results suggest that inflammation may lead to increased mediator release and potentiation of allergic responses *in vivo*.

DEVELOPMENT OF MAST CELLS OF ASTHMATIC PHENOTYPE

Interleukin-4 is proposed to have an important role in inflammatory diseases such as asthma. Elevated levels of IL-4 have been measured in BAL fluid from atopic asthmatic subjects compared to either control subjects or non-atopic asthmatic subjects .[234] Previously it was found that IL-4 treated CBMC enhances the anti-IgE related mediator release of LTC_4 with a 27-fold increase compared to non-treated cells.[175] Further, IL-4 induced the expression of LTC_4 synthase and stimulated the activity of the enzyme[175] and it was also reported that mast cells released more PGD_2 and histamine upon anti-IgE challenge in the presence of IL-4.[175] This latter phenomenon was explained by the IL-4 induced expression of the high affinity IgE receptor (FcεRI).[175] It has also been suggested that CBMC express a closely related $CysLT_1$ receptor that can be induced by IL-4.[235] This receptor changed the sensitivity to LTC_4 which was not found for $CysLT_1$ or $CysLT_2$ receptors.[235] Further, the selective $CysLT_1$ receptor antagonist MK571 inhibited IL-4 induced release of mediators in CBMC.[176] Thus, inflammatory diseased driven by a Th2 cytokine expression such as asthma might directly affect mast cells and cause cell activation.

In vivo, asthmatic subjects produce more CysLTs as compared to normal controls.[236] The enzyme expression in lung biopsy tissue representing the 5-LO and COX pathways were characterised before and during pollen season.[237] It was found that immunostaining for 5-LO, FLAP, LTA_4 hydrolase and LTC_4 synthase increased during pollen season whereas there was no effect on COX-1, COX-2 or PGD_2 synthase expression.[237] It is clear that LTC4 synthase is expressed in mast cells in human bronchial mucosa.[238] Thus, indicating that if it is possible, the skewing of LT generation in mast cells would make them more "asthma like". Immunologically stimulated human lung mast cells produce PGD_2/LTC_4 in a ratio of 1:3.[58] In **Paper I** we found a ratio of 49:1 in CBMC after immunological stimulation. Thus, treatment of CBMC with IL-4 would suggest them to produce more CysLTs generating and also to reflect human lung mast cells.

It has been reported that IL-4 induced 15-LO expression is restricted to monocytes, macrophages, dendritic cells and epithelial cells.[239-242] 15-HETE has been associated with inflammatory cells found in subjects with asthma.[241] Increased levels of 15-HETE have been found in BAL fluid and in sputum samples from asthmatic subjects compared to normal controls.[94, 243] These findings suggest a pathophysiological role for 15-HETE in asthmatic diseases. In **Paper III** we found that IL-4 upregulate the expression of 15-LO in CBMC and that this was related to an increased product formation. Thus, indicating that IL-4 is an important mast cell regulatory cytokine that may be induced in subjects with asthma.

Finally, whether IL-4 has the ability to upregulate LTC_4 synthase, an IL-4 dependent CysLT receptor and 15-LO *in vivo* remains to be elucidated.

CONCLUSIONS

* Osmotic challenge of CBMC with mannitol-induced release of PGD_2, CysLTs and histamine. The results indicate that eicosanoid mediators released from mast cells may be of importance in asthmatic and inflammatory reactions triggered by hyperosmolarity.

* The dominating COX product in mast cells, PGD_2, was found to be formed via both COX-1 and COX-2 pathways in CBMC. Unselective and selective COX inhibitors were equally effective in inhibiting PGD_2 formation at shorter incubations times. Interleukin-1β induced the expression of COX-2 at 24h. At this time-point, there was a relatively greater inhibition of PGD_2 formation by a selective COX-2 inhibitor than during acute challenge (0.5h). The results suggest a predominant COX-2 related PGD_2 formation during late allergic and inflammatory responses.

* 15-lipoxygenase was expressed in CBMC pretreated with IL-4. The major arachidonic acid derived 15-lipoxygenase products were 15-KETE and 15-HETE in a relation 9:1. Further, *in vivo*, mast cells were found to co-express 15-LO-1 and tryptase in human lung and skin tissue samples, indicating a potential functional role of 15-lipoxygenase products from mast cells in asthma and other inflammatory disorders.

* Mannitol-induced bronchoconstriction was associated with an increased urinary excretion of the PGD_2 metabolite and mast cell marker 9α,11β-PGF_2 in subjects with asthma. This is probably due to increased responsiveness to released mediators in these subjects. This supports mast cell activation, which was also seen after mannitol inhalation by non-asthmatic subjects. The healthy subjects did however not bronchoconstrict. The difference is probably due increased responsiveness to liberated mediators in the asthmatic subjects.

* Further support of mast cell involvement in mannitol-induced EIB was the finding that both SCG and formoterol protected against bronchoconstriction in subjects with asthma. Since SCG does not provide any bronchodilatory effect by itself the finding supports mast cell stabilisation. In addition, this protection could be related to decreased urinary 9α,11β-PGF_2 excretion.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Mastceller är framförallt kända för att framkalla symptom vid akuta allergiska reaktioner men idag vet man att de även bidrar till akuta och kroniska inflammatoriska reaktioner. Normalt finns det ett begränsat antal mastceller i vävnader men det har visat sig att individer med astma har ett ökat antal mastceller i luftvägarna. Luftvägarnas retbarhet kan också härledas till detta ökade antal mastceller. Det är därför viktigt att förstå mastcellens aktiveringsprocesser. Mastceller bildas från stamceller i benmärgen och rekryteras till vävnad via blodbanan där de cirkulerar som omogna mastceller. Själva utmognadssprocessen sker först när de når vävnaden. För att studera mastceller krävs det därför endera tillgång på vävnad eller stamceller. I denna avhandling har navelsträngsblod använts som stamcellskälla och mastceller har mognats ut med hjälp av stamcellsfaktor (scf) och interleukin-6 (IL-6).

Det är sedan tidigare känt att immunologisk aktivering av mastceller leder till frisättning av mediatorer som lagrats i granula eller som nysyntetiserats. I denna avhandling har icke-immunologisk aktivering av mastceller via hyperosmolär eller cytokinstimulering undersökts och frisättningen av mediatorer har analyserat. I två delarbeten undersöktes effekter av mannitolinhalering hos astmatiker som en modell för ansträngningsutlöst bronkkonstriktion. Mekanismerna bakom ansträngningsutlöst bronkkonstriktion och mannitolinducerad bronkkonstriktion kan härledas till uttorkning av luftvägarna. Upp emot 80-90% av vuxna obehandlade astmatiker reagerar med bronkkonstriktion till följd av ansträngning.

I **delarbete I** undersöktes mastcellens roll vid hyperosmolär stimulering**.** Mastceller fick mogna ut från navelsträngsblod och stimulerades med mannitol, anti-IgE eller en kombination av båda. Efter stimulering analyserades cellernas frisättning av prostaglandin D_2 (PGD_2), leukotrien C_4 (LTC_4) och histamin. Mannitolstimulering ökade frisättningen av samtliga mediatorer och det var ingen korrelation mellan frisatta metaboliter. Anti-IgE aktiverade också till frisättning av samtliga mediatorer och vi fann en korrelation mellan frisatt PGD_2 och LTC_4. Stimulering med en kombination av anti-IgE i en hyperosmolär lösning hade en synergistisk effekt på frisättningen av histamin. Mannitol aktiverar celler till mediatorfrisättning men på ett annat sätt än anti-IgE.

I **delarbete II** studerades den cyklooxygenas (COX)-relaterade frisättningen av PGD_2, tromboxan B_2 (TXB_2) och PGE_2 efter interleukin-1β (IL-1β) och/eller anti-IgE stimulering av mastceller. Interleukin-1β ökade enzymuttrycket av COX-2 i mastceller. Kombinationen av IL-1β och anti-IgE hade en synergistisk effekt på frisättningen av PGD_2 jämfört med vardera stimuli för sig. Den selektiva COX-1 hämmaren FR122047 minskade frisättningen av PGD_2 inducerad av IL-1β tillsammans med anti-IgE vid 0.5h till en högre grad än den selektiva COX-2 hämmaren lumiracoxib. Förutom PGD_2 frisattes även tromboxan B_2 (TXB_2) och PGE_2 efter stimulering. Hypoxi (4%O_2) påverkade inte mediatorfrisättnigen jämfört med normoxi (21% O_2). Prostaglandin D_2 bildades från både COX-1 och COX-2.

I **delarbete III** studerades om mastcellen kunde uttrycka 15-lipoxygenas (15-LO) och därmed producera 15-LO relaterade produkter. Interleukin-4 ökade enzymuttrycket av 15-LO och efter stimulering med arakidonsyra bildades 15-hydroxyeicosatetraenoic acid (15-HETE) och 15-ketoeicosatetraenoic acid (15-KETE) i förhållande 1:9. En subpopulation av mastceller från lunga och hud uttryckte 15-LO tillsammans med tryptas som är en mastcellsmarkör. Mastceller utmognade från navelsträngsblod men även från lunga och hud kan uttrycka 15-LO. Den fysiologiska funktionen av 15-LO-deriverade produkter återstår att utreda.

I **delarbete IV** mättes lungfunktion och urinmetaboliter från astmatiker med ansträngningsutlöst bronkkonstriktion och friska kontroller som stimulerades via mannitolinhalation. Patienter med astma fick inhalera mannitol tills lungfunktionen (FEV_1) sänktes med 25%. Friska kontroller inhalerade den högsta dosen mannitol (635mg). Lungfunktionen mättes var 5-10e min efter provokationens slut tills 90 min passerat. Urinprover samlades 30, 60 och 90 min efter mannitolprovokationen för mätning av den mastcellsspecifika prostaglandin D_2 (PGD_2) metaboliten 9α,11β-PGF_2, leukotrien E_4 (LTE_4) samt histaminmetaboliten N^{τ}-metylhistamin. Urinutsöndring av 9α,11β-PGF_2 och LTE_4 ökade både hos astmatiker och kontroller efter mannitolprovokation. Vid en jämförelse av den ökade frisättningen mellan grupperna var det bara 9α,11β-PGF_2 som ökade signifikant hos astmatikerna. Kontrollerna reagerade inte med bronkkonstriktion trots att de inhalerade nästan tre-faldigt mer mannitol. Den ökade utsöndringen av 9α,11β-PGF_2 kan härledas till bronkkonstriktion hos astmatiker med ansträngningsutlöst astma. Detta beror sannolikt på den ökade känsligheten i luftvägarna eller att antalet mastceller i luftvägarna kan vara fler.

I **delarbete V** mättes lungfunktion och urinmetaboliter från astmatiker med ansträngningsutlöst bronkkonstriktion som förbehandlats med en mastcellsstabiliserare (kromoglikat, SCG), luftrörsvidgare (β_2-agonist, formoterol) eller placebo innan mannitolinhalation. Vid ett första besök bestämdes den dos av mannitol som sänkte lungfunktionen (FEV_1) med 25%. Vid de övriga tre besöken var varken försökspersonerna eller försökspersonalen informerade om vilken substans som delades ut. Lungfunktionen mättes och urinprover samlades och analyserades på samma sätt som i delarbete IV. Förbehandling med SCG och formoterol skyddade till 63% och 95% från mannitolinducerad sänkning av lungfunktionen jämfört med placebo. Urinutsöndringen av 9α,11β-PGF_2 och LTE_4 ökade efter placebo behandling. Förbehandling med endera SCG eller formoterol minskade utsöndring av 9α,11β-PGF_2 men de hade ingen effekt på LTE_4 utsöndring. Båda drogerna skyddade mot mannitolinducerad bronkkonstriktion och detta var i samband med minskad utsöndring av 9α,11β-PGF_2 vilket stärker mastcellens roll i mannitolinducerad bronkkonstriktion.

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