The National Institute of Environmental Medicine
Division of Physiology,
The Unit for Experimental Asthma and Allergy Research

AN EXPERIMENTAL STUDY OF MEDIATORS IN ALLERGIC BRONCHOCONSTRICTION WITH FOCUS ON EICOSANOIDS

Ewa Selg

Karolinska Institutet

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For Peter and Anders

With love

ABSTRACT

Mast cells have a central role in the inflammatory response in airways of both atopic and non-atopic asthmatics. The mast cell-derived mediators such as histamine, leukotriene C_4 and prostaglandin D_2, released upon activation of the cell, produce acute bronchoconstriction that is characteristic of the early asthmatic response (EAR), and contribute to the development of airway inflammation and hyperresponsiveness following the initial reaction. There is a great need for relevant experimental *in vitro* models to study the airway effects of the mast cell-derived mediators. The overall aim of the experimental studies presented in this thesis was accordingly an evaluation of the relevance of the isolated perfused and ventilated guinea pig lung (IPL) as an experimental model to study the EAR.

The reactivity of the IPL to histamine, cysteinyl-leukotrienes (CysLTs: leukotriene (LT) C_4, D_4 and E_4) and prostanoids, established by intravascular challenge of the normal lungs with these mediators, was closely similar to that of human airways. The cyclooxygenase (COX)-dependent modulation of the responses to bronchoconstrictive agonists in IPL followed two different patterns. The responses to histamine and LTD_4 were amplified by agonist-induced secondary release of bronchoconstrictive COX products. Both COX isoenzymes, COX-1 and COX-2 participated in the LTD_4-induced generation of COX products. In contrast, the response to PGD_2 did not actively promote the agonist-induced release of COX products, but was down-modulated by relaxant COX products presumably tonically formed in airways.

Investigation of the pharmacology of the PGD_2 response in IPL by the use of COX inhibitors gave rise to the hypothesis that diclofenac also was a thromboxane (TP) receptor antagonist. Further investigation of the effects of diclofenac and its structural analogue lumiracoxib on the TP-mediated responses in standard airway and vascular pharmacology *in vitro* models and in human platelets confirmed that these drugs, in addition to being COX unselective and highly COX-2 selective inhibitors, respectively, also were competitive TP receptor antagonists.

The IPL was demonstrated to provide a model where a specific, dose-dependent and reproducible bronchoconstriction was obtained in response to intravascular challenge with antigen (ovalbumin) in lungs actively sensitised to ovalbumin. The mediators of the antigen-induced bronchoconstriction were investigated by pharmacological interventions with an antihistamine, an antileukotriene or COX inhibitors as well as by measurements of the antigen-induced mediator release. It was established that similarly to the situation in human airways, histamine, CysLT and prostanoids mediated the antigen response in IPL. Furthermore, both COX-1 and COX-2 contributed to the immediate antigen-induced generation of prostanoids. The results of the inhibition of two classes of mediators at a time disclosed interactions among the mediators at several levels. The complete inhibition of the antigen response produced by inhibition of all the three mediator classes at a time demonstrated that the concerted actions of histamine, CysLTs and prostanoids could solely explain the immediate antigen-induced bronchoconstriction in this model. In conclusion, the IPL is considered a relevant experimental model for studies of the EAR, and it is proposed to test the concept of inhibition of the three mediator classes in subjects with asthma.

Key words: leukotrienes, prostaglandins, histamine, guinea pig lung, ovalbumin, mast cell, cyclooxygenase, airway smooth muscle, COX inhibitors, NSAIDs

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV):

I. **Sundström E**, Låstbom L, Ryrfeldt Å and Dahlén S-E
Interactions among three classes of mediators explain antigen-induced bronchoconstriction in the isolated perfused and ventilated guinea pig lung.
J Pharmacol Exp Ther (2003) 307:408-418

II. **Selg E**, Buccellati C, Andersson M, Rovati GE, Ezinga M, Sala A, Larsson A-K, Ambrosio E, Låstbom L, Capra V, Dahlén B, Ryrfeldt Å, Folco GC and Dahlén S-E
Antagonism of thromboxane receptors by diclofenac and lumiracoxib.
Br J Pharmacol (2007 Dec;152(8):1185-95

III. **Selg E**, Andersson M, Låstbom L, Ryrfeldt Å and Dahlén S-E
Opposite effects of COX inhibitors on bronchoconstriction induced by leukotriene D_4 and prostaglandin D_2 in isolated perfused and ventilated guinea pig lung.
Submitted manuscript

IV. **Selg E**, Låstbom L, Ryrfeldt Å, Kumlin M and Dahlén S-E
Effects of selective and non-selective COX inhibitors on antigen-induced release of prostanoid mediators and bronchoconstriction in the isolated perfused and ventilated guinea pig lung.
Accepted for publication in Prostaglandins, Leukotrienes and Essential Fatty Acids

CONTENTS

LIST OF ABBREVIATIONS

AchE	Acetylcholinesterase
BAL	Bronchoalveolar lavage
BHR	Bronchial hyperresponsiveness
COX	Cyclooxygenase
CysLT	Cysteinyl-leukotriene
EAR	Early phase asthmatic response
EIA	Enzyme immunoassay
FLAP	5-lipoxygenase-activating protein
HEK293	Human embryonic kidney cell line
IPL	Isolated perfused and ventilated guinea pig lung
IPs	Inositol phosphates
LAR	Late phase asthmatic reaction
5-LO	5-lipoxygenase
LT	Leukotriene
NSAIDs	Non-steroidal anti-inflammatory drugs
OVA	Ovalbumin
PAF	Platelet activating factor
PG	Prostaglandin
TP	Thromboxane receptor
TX	Thromboxane

INTRODUCTION

There is a large body of evidence supporting a pivotal role of mast cells in both atopic and non-atopic asthma. Histamine and lipid mediators derived from arachidonic acid, and termed eicosanoids, such as leukotriene C_4 and prostaglandin D_2, are released in asthmatic airways from mast cells activated by allergen. The mast cell-derived mediators cause acute airway obstruction, characteristic of the early response to allergen in asthmatic airways, but also contribute to the development of the major features of asthma such as chronic airway inflammation and bronchial hyperresponsiveness that develops several hours after the initial reaction. The mediators and their receptors are key targets for therapeutic interventions. In airway pharmacology, it is thus a great need for experimental models for studies of the actions of new or existing pharmacological agents for asthma treatment, and for the detailed exploration of novel mechanisms or drugs before going into humans. Such models should be relevant with respect to central components of the response to allergen in human airways. In the studies presented in this thesis, the mediators of the immediate antigen-induced airway obstruction were studied in the isolated perfused and ventilated guinea pig lung model (IPL) that allows studying the mediator mechanisms with relevance to asthma in intact lungs. These studies were undertaken in order to evaluate the utility of the IPL in asthma pharmacology.

GENERAL BACKGROUND

Asthma

Asthma is the most common cause of significant respiratory morbidity (Bjerg et al., 2007; Hartert and Peebles, 2000; Masoli et al., 2004), with the highest and still rising prevalence in the Western world (Eder et al., 2006). The clinical features of asthma are episodes of airflow obstruction that reflect bronchial smooth muscle contraction, bronchial wall edema and mucous plugging. The airflow obstruction in asthma is variable and at least partially reversible with the administration of bronchodilator. Asthmatic subjects also display bronchial hyperresponsiveness (BHR) that manifests as exaggerated airflow obstruction in response to challenges with a variety of agonists of bronchoconstriction such as methacholine, carbachol and adenosine.

The histopathology of inflammation in asthma also involves varying degrees of epithelial disruption, goblet cell metaplasia, accumulation of CD4+ lymphocytes and eosinophils in the submucosa and epithelium, thickening of the subepithelial collagen layer, mast-cell degranulation as well as hypertrophy and hyperplasia of the structural elements of the airway including the bronchial smooth muscle and vasculature (Baraldo et al., 2007; Chetta et al., 1996; Jeffery et al., 1989; Laitinen et al., 1985; Wardlaw et al., 2000). The chronic inflammation and consequent structural changes in asthma is often called remodelling (Bousquet et al., 2000). Mast cells, eosinophils and lymphocytes are involved in the development of inflammation in asthma. However, a role for other cell types such as fibroblasts (Levi-Schaffer et al., 1999) and airway smooth muscle (Ebina et al., 1993) has also been proposed. Most likely, the chronic airway inflammation in asthma is due to overlapping effects of several effector cells and signal molecules involved.

Mast cells and their role in asthma

Mast cells are hematopoietic immune effector cells that are found in all vascularised tissues. They play an important role in the tissue homeostasis, wound healing and host defence, particularly in bacterial infection. They are especially abundant in the tissues forming interfaces with the external environment, such as skin and gastrointestinal, genitourinary and respiratory tracts (Metcalfe et al., 1997).

Lung mast cells contain predominantly tryptase and are generally classified as ”mucosal”, in contrast to the ”connective tissue” mast cells in the skin and the peritoneal cavity (Enerback, 1966; Weidner and Austen, 1990). Two subpopulations of lung mast cells may be involved in the asthmatic response (Forsythe and Ennis, 1998). One population, termed human lung mast cells, is located close to blood vessels beneath the basement membrane and may be obtained by enzymatic or mechanical disruption of whole lung tissue. Another population of cells may be recovered by bronchial-alveolar lavage (BAL) and is termed the BAL mast cells. These cells are located between the epithelium and the basement membrane. The BAL mast cells may be the first to be exposed to inhaled allergens, and thus mediate the initial stage of the asthmatic response, whereas the human lung mast cells most likely play a role in the subsequent development of chronic disease.

Mast cells are significantly more numerous in airways of asthmatics than in those of non-asthmatics (Carroll et al., 2002; Djukanovic et al., 1990; Laitinen et al., 1993; Pesci et al., 1993). In addition, mast cells, but not T cells or eosinophils, infiltrate the bronchial smooth muscle in subjects with asthma, but not in normal subjects or those with eosinophilic bronchitis, a factor likely to be important in determining the asthmatic phenotype (Brightling et al., 2002).

The assumed dominant signal for mast cell activation in asthma is cross-linkage of immunoglobulin E (IgE) bound to high-affinity receptors (FcεRI) by a specific allergen. Although allergen exposure is strongly linked to asthma once established, allergen avoidance has a minor effect in the established disease, that appears self-perpetuating (Bradding et al., 2006). Moreover, in subjects with atopic asthma, but also in those with non-atopic and occupational asthma, a role of chronic mast cell activation is supported by the observation of mast cells exhibiting features of ongoing degranulation in bronchial mucosa (Broide et al., 1991; Pesci et al., 1993).

The mechanisms of chronic mast cell activation in asthma are complex, as mast cells can be activated by a broad range of FcεRI-independent stimuli, such as neuropeptides (Forsythe et al., 2000; Heaney et al., 1995), suggesting the role of a neurogenic pathway in asthma, adenosine (Forsythe et al., 1999), eosinophil-derived granule proteins (O'Donnell et al., 1983) and changes in osmolality (Eggleston et al., 1990; Gulliksson et al., 2006). Moreover, mast cells can be activated through c-kit receptors by stem cell factor bound on fibroblasts during mast cell-fibroblast interaction (Hogaboam et al., 1998), TNF-α, monomeric IgE, proteases, immunoglobulin free light chains and Toll-like receptor ligands (Bradding et al., 2006). The

chronic ongoing mast cell degranulation appear to occur as piecemeal release of the granulae constituents and has thus a different mechanism than that of the FcεRI-dependent acute degranulation (Bradding et al., 2006).

Early and late phase asthmatic responses

Early phase asthmatic response

Approximately 80-90% of children and 50-60% of adults with asthma (Wright, 2002) have allergen-specific IgE, usually against aeroallergens. When allergic bronchoconstriction in such subjects is studied in the laboratory, the early phase asthmatic response (EAR) develops 15-30 min after inhalation challenge with the specific allergen, and manifests as airflow obstruction due to bronchial smooth muscle contraction and bronchial wall edema (Inman et al., 1995). The EAR is caused by the release of bronchoconstrictor mediators in particular from mast cells activated by allergen challenge. Thus, mast cell-derived mediators, such as histamine, cysteinyl-leukotrienes, prostaglandin D_2 and tryptase are detected in the BAL fluid during the EAR (Casale et al., 1987; Liu et al., 1991; Wenzel et al., 1989). In addition to causing acute airway obstruction, these mediators contribute to development of airway inflammation and hyperresponsiveness that occurs later on after allergen challenge.

Other cells bearing the FcεRI, such as basophils and dendritic cells (Tunon-De-Lara et al., 1996), epithelial cells (Campbell et al., 1998) and monocytes and macrophages (Cheng et al., 2006) have also been proposed to participate in the allergen-induced bronchoconstriction. However, mast cells that predominate quantitatively and release a broad range of bronchoconstrictive mediators, are most likely to be the main cells that initiate the IgE-dependent early phase response.

Late phase asthmatic response

The late phase asthmatic response (LAR) manifests as a second wave of airflow obstruction occurring 6-8 h after the EAR, and is fully developed after 12 h. The LAR follows the EAR in approximately 25-50% of subjects (O'Byrne et al., 1987; Smith et al., 1992). The more sustained airflow obstruction during the LAR has been assumed to be due to swelling of the bronchial wall during ongoing inflammation. The increased number of eosinophils, neutroplils and basophils in the BAL fluid (Liu et al., 1991; Smith et al., 1992) and induced sputum (Gauvreau et al., 2000),

and the increase in BHR due to inflammation and epithelial injury (Gauvreau et al., 2000; Liu et al., 1991; Smith et al., 1992) are detected during the LAR. The increase in BHR can persist several days after the initial reaction (Smith et al., 1992).

The important contribution of mast cells also to the LAR is supported by inhibition of this response, besides inhibition of the EAR, produced by pharmacological antagonists of mast cell-derived mediators (Hamilton et al., 1998; Roquet et al., 1997), cromones (cromoglycate, nedocromil) that interfere with FcεRI-dependent mast cell activation (Aalbers et al., 1991; Bleecker et al., 1996; Kopferschmitt-Kubler et al., 1993; Pelikan and Knottnerus, 1993), and anti-IgE antibodies (D'Amato, 2006; Fahy et al., 1997). Furthermore, the urinary mast cell markers can be detected both during the EAR and the LAR (O'Sullivan et al., 1998). The observations that the number of mast cells correlated with BHR support the possible contribution of mast cells to the chronic inflammation in asthmatic airways as well (Brightling et al., 2002; Wardlaw et al., 1988).

Allergen challenge by inhalation is a very useful clinical and research tool for evaluating allergic airway disease. It is however important to recognise that experimental allergen challenge does not completely mimic the pathophysiology of chronic asthma, as not all subjects with asthma have demonstrable allergy (Wuthrich et al., 1995). Furthermore, the majority of spontaneous asthma exacerbations occur in response to infectious agents rather than allergen exposure (Freymuth et al., 1999; Gern and Busse, 1999). On the other hand, the histopathologic similarity of allergic and non-allergic asthma supports a similar pathophysiologic basis (Humbert et al., 1996; Ying et al., 1997; Ying et al., 2001), with mast cell activation and mediator release as a key component (Broide et al., 1991; Pesci et al., 1993).

Mast cell-derived mediators

The mast cell-derived mediators, released upon activation through the high-affinity receptors for IgE (FcεRI), include pre-formed granule-associated inflammatory mediators such as histamine, neutral proteases, pre-formed cytokines and proteoglycans. These mediators are released within minutes by exocytosis (Metcalfe et al., 1997) and contribute to bronchoconstriction, enhanced vascular permeability and initial recruitment of inflammatory cells.

Histamine is found in the BAL fluid after allergic reactions (Wenzel et al., 1988) and contributes to the bronchospasm, edema and mucus secretion via the action on H_1 receptors

(White, 1990). Moreover, histamine can activate bronchial epithelial cells and eosinophils and modulate the cytokine network (Sirois et al., 2000; White, 1990). Histamine stimulates also the release of neuropeptides (Joos et al., 2000).

Among mast cell proteases, β-tryptase is the predominant protease of the BAL mast cells (Welle, 1997). Tryptase interacts with various cell types via protease-activated receptors (PARs) and can cleave basement membrane proteins, inactivate neuropeptides with bronchodilatory properties such as vasoactive intestinal peptide, and stimulate mucus secretion (Welle, 1997).

Mast cells are unusual in that cytokines are stored in granules. However, activated mast cells release proinflammatory, chemoattractive and immunomodulatory cytokines over a period of several hours. Mast cells are a source of profibrogenic cytokines such as TGF-β and FGF, proinflammatory cytokines such as IL-4, IL-5 and IL-13 that regulate both IgE synthesis and the development of eosinophilic inflammation as well as IL-3, IL-6, IL-8, IL-10, IL-13 and TNF-α (Bradding and Holgate, 1999). There is recent evidence for a pivotal role of TNF-α in pathophysiology of severe asthma (Berry et al., 2006; Howarth et al., 2005). TNF-α activates mast cells (Coward et al., 2002) and inflammatory mediator production from neutrophils and macrophages (Sirois et al., 2000), stimulates expression of adhesion molecules on endothelial cells and subsequent transmigration of inflammatory leucocytes (Klein et al., 1989) and contributes to the development of bronchial fibrosis together with TGF-β (Djukanovic, 2000).

Mast cell activation triggers also rapid (minutes) release of lipid mediators that are the products of endogenous arachidonic acid metabolism such as prostaglandin D_2 and leukotriene C_4. Release of leukotriene B_4 has also been reported in mast cell activation in some models (Malaviya and Abraham, 2000; Okabe et al., 2006; Weller et al., 2005), but it is not clear if it also occurs in response to the immunological challenge in human lung (Kumlin and Dahlén, 1990). These mediators are capable of inducing the bronchospasm, swelling of the airway wall, leucocyte infiltration, hyperplasia of bronchial smooth muscle and mucus secretion (Bradding et al., 2006). Their role is particularly evident during the EAR that is nearly completely prevented by pharmacological interventions that block the effects of these mediators (Roquet et al., 1997). Mediators from degranulating mast cells are thus critical to the pathology of the asthmatic lung *(Figure 1)*.

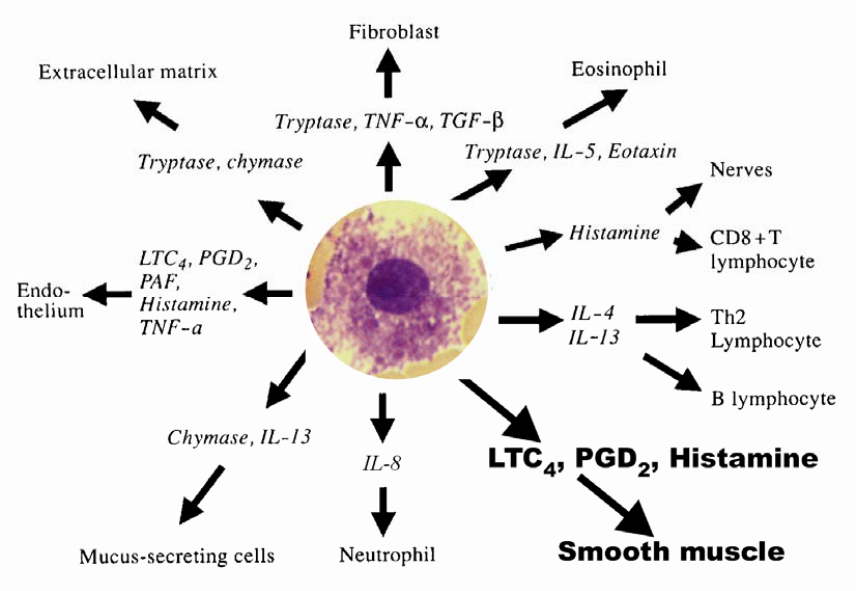

Figure 1. Role of mast cell mediators in the development of chronic allergic asthma. In bold enlargement: the focus of this thesis.

Eicosanoids

Eicosanoids (twenty carbon polyunsaturated fatty acid derivatives) are lipid mediators that are not stored in cells but synthesised *de novo* from arachidonic acid, the major precursor in mammalian cells. The eicosanoid family consists of three major clans, which include the prostanoids (prostaglandins and thromboxanes) that are synthesised via the cyclooxygenase pathway, the leukotrienes and certain mono-, di- and tri-hydroxy acids that are synthesised via lipoxygenase pathways, and the epoxides that are formed by a cytochrome P-450 (epoxygenase) pathway.

Arachidonic acid is stored in an esterified form in membrane glycerophospholipids until mobilised by phospholipases (PLA_2). There are several classes of PLA_2, but the type IV cytosolic PLA_2 ($cPLA_2$) is the key enzyme for eicosanoid production, as cells lacking this enzyme are unable to synthesise eicosanoids (Funk, 2001). Translocation of $cPLA_2$ upon cell activation to the nuclear envelope and endoplasmic reticulum is cell-specific and agonist-dependent (Evans et al., 2001). The arachidonic acid release is usually receptor-mediated and is induced by many types of hormones, autocoids, growth factors, antigens, immune complexes, bacterial peptides, tumor

promoters and other stimuli (Smith, 1989). However, responses to mechanical stresses on cells such as shear forces acting on vascular endothelial cells or mechanical trauma that induces formation of prostanoids, may not be receptor-mediated (Smith, 1989).

5-lipoxygenase pathway of arachidonic acid metabolism

Leukotrienes (LTs) are synthesised from arachidonic acid in the 5-lipoxygenase (5-LO) pathway *(Figure 2)*. The LTs are produced predominantly by inflammatory cells of myeloid origin like mast cells, macrophages and polymorphonuclear leucocytes that possess 5-LO (Samuelsson et al., 1987). The 5-LO is located in the nucleus in some cells and in the cytosol in the others (Peters-Golden and Brock, 2001). Activation of these cells is a signal for translocation of 5-LO together with $cPLA_2$ to the nuclear envelope (Peters-Golden and Brock, 2001). In cooperation with 5-LO activating protein (FLAP), 5-LO transforms arachidonic acid initially to the 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is further converted either to 5-HETE, or to the highly instable epoxide LTA_4 (Rådmark et al., 1980; Smith, 1989).

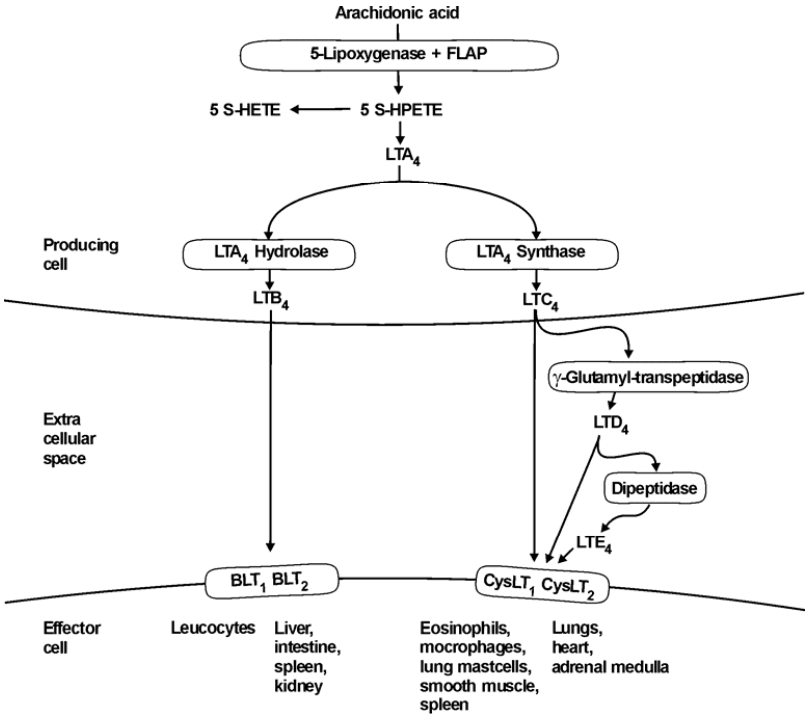

Figure 2. Biosynthesis of leukotrienes and receptors mediating their biological effects.

Once formed, LTA_4 is rapidly transformed to LTB_4 by LTA_4 hydrolase, which is present in almost all cells (Haeggström et al., 2007; Samuelsson and Funk, 1989). Thus, cells lacking 5-LO activity can synthesise LTB_4 in the presence of activated monocytes that provide LTA_4 (Jakobsson et al., 1991).

The LTA_4 can also be conjugated with glutathione by LTC_4 synthase at the nuclear envelope to form LTC_4 (Penrose and Austen, 1999). The LTC_4 is transported out of the cell by transporters from the multidrug resistence-associated protein family (MRP1) (Robbiani et al., 2000), and further converted extracelularly by γ-glutamyl transpeptidase to LTD_4, which in turn is converted by dipeptidase to LTE_4 (Peters-Golden and Henderson, 2007). Together, LTC_4, LTD_4 and LTE_4 are termed cysteinyl-leukotrienes (CysLTs).

Cyclooxygenase pathway of arachidonic acid metabolism

Prostanoids are synthesised from arachidonic acid in the cyclooxygenase pathway *(Figure 3)*. PGG/H synthase referred to as cyclooxygenase (COX) is the key enzyme in the biosynthesis of prostanoids. The released arachidonic acid is presented to COX, which is located in the endoplasmic reticulum and the nuclear envelope (Smith et al., 2000).

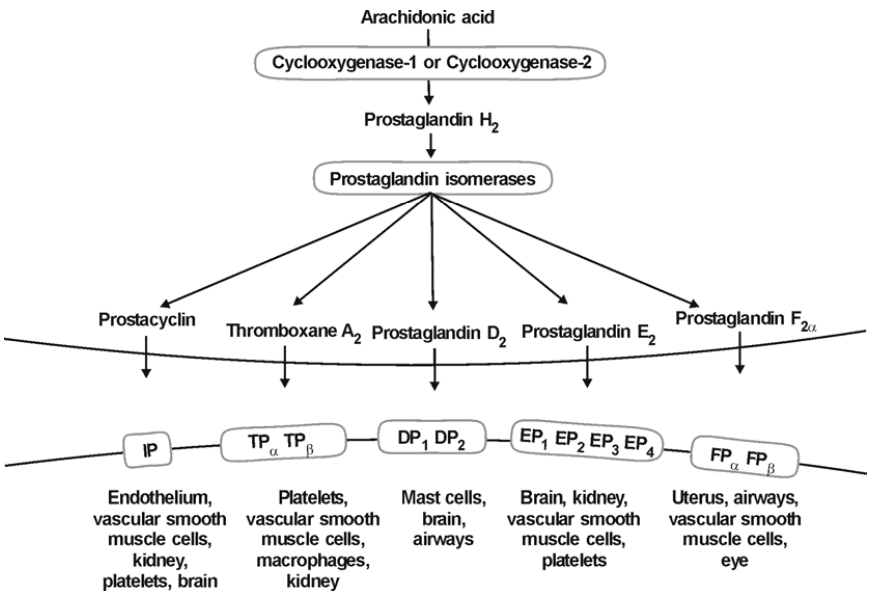

Figure 3. Biosynthesis of prostanoids and receptors mediating their biological effects.

There are two isoforms of COX referred to as COX-1 and COX-2 that catalyse the same reactions, conversion of arachidonic acid to the prostaglandin endoperoxide PGH_2 (Smith et al., 1996). Generally, COX-1 is constitutively expressed and ubiquitous and is responsible for basal production of prostanoids involved in homeostasis, whereas the inducible COX-2 is expressed by monocytes and macrophages at sites of inflammation and plays a key role in mediating the inflammatory process (Smith et al., 1998). The expression of COX-2 is upregulated by a variety of inflammatory stimuli, growth factors and bacterial endotoxin (Smith et al., 2000).

The precise molecular mechanisms explaining the division of labour between the two COX isoenzymes when they are co-localised in the same cell are unknown. The different requirements for arachidonic acid concentration for COX-1 and COX-2 may be one factor in their regulation, allowing the two COX isoenzymes to function within the same cell, as low substrate concentrations favour prostanoid synthesis via COX-2 whereas high concentrations favour COX-1 (Smith et al., 2000).

COX has two catalytic activities, a bis-oxygenase (cyclo-oxygenase) involved in the endoperoxide PGG_2 formation (Hamberg and Samuelsson, 1973), and a hydroperoxidase activity mediating reduction of PGG_2 to PGH_2. The cyclo-oxygenase activity but not the hydroperoxidase activity is specifically inhibited by non-steroidal anti-inflamamtory drugs (Van Der Ouderaa et al., 1980).

Finally, PGH_2 is converted into prostaglandins (PGs) of the D_2, E_2, F_2 and I_2 series as well as thromboxane (TX) A_2 by the specific terminal synthases, with different structures and tissue-specific distribution. Thus, conversion of PGH_2 to TXA_2 and PGI_2 is catalysed by TX synthase (TXAS) (Wang and Kulmacz, 2002) and PGI synthase (PGIS) (Li et al., 2007), respectively, both of which belong to the cytochrome P-450 family, and are localised in the endoplasmic reticulum and the nuclear envelope. PGD synthase (PGDS) that isomerises PGH_2 to PGD_2 occurs in two distinct forms, the lipocalin-type PGDS (L-PGDS), a secretory enzyme that is abundant in the CNS (Urade and Eguchi, 2002), and the hematopoietic PGDS (H-PGDS) , which represents the σ-class of the cytosolic glutathione S-transferase family (Kanaoka and Urade, 2003). The lung-type (Watanabe et al., 1989) and liver-type (Suzuki-Yamamoto et al., 2002) PGF synthase (PGFS-I and II, respectively) that convert PGH_2 to PGF_2 are cytosolic proteins that belong to the aldo-ketoreductase family. Membrane-bound PGFS that is stimulus-inducible has also been described (Nakashima et al., 2003). Several types of proteins such as constitutive cytosolic PGES

(cPGES) and inducible nuclear membrane-bound PGES (mPGES) have PGES activity (Murakami et al., 2002).

The coupling of the two COX isoenzymes and the terminal prostaglandin (PG) synthases is regulated in cell-specific manner. The possible factors determining the actual profile of prostanoid production are a) Co-localisation of a series of enzymes in a particular subcellular compartment (Brock et al., 1999; Ueno et al., 2001), b) Preferential utilisation of particular enzymes depending on their intrinsic kinetic properties and the prevailing levels of substrates and regulatory factors (Caughey et al., 2001; Penglis et al., 2000) and c) Differential expression of particular enzymes (Caughey et al., 2001; Matsumoto et al., 1997; Penglis et al., 2000).

Biological effects of leukotrienes

LTB_4 is a potent stimulus for the activation of leucocytes (Dahlén et al., 1981; Ford-Hutchinson et al., 1980). Biological activities of LTB_4 are mediated by two subtypes of BLT receptors, BLT_1 and BLT_2 that belong to the G-protein-coupled rhodopsin-type receptor superfamily of seven transmembrane domains (GPCRs) (Brink et al., 2003) *(Figure 2)*.

The CysLTs are proinflammatory mediators and they activate contractile and inflammatory processes by interactions with two subtypes of cysteinyl-leukotriene (CysLT) receptors, $CysLT_1$ and $CysLT_2$ that belong to the GPCR family (Brink et al., 2003; Peters-Golden and Henderson, 2007) *(Figure 2)*. Decrease in the intracellular cAMP level, evoked by the $CysLT_1$ receptor activation by CysLTs precedes the smooth muscle contraction (Andersson et al., 1982). Interaction of CysLTs and $CysLT_1$ receptors is predominantly expressed in human bronchi, leucocytes , bronchial smooth muscle cells and interstitial lung macrophages (Peters-Golden and Henderson, 2007). Moreover, CysLTs induce migration and enhance degranulation in eosinophils via $CysLT_1$ receptors (Ohshima et al., 2002). Human mast cells also express $CysLT_1$ receptors (Sjöström et al., 2002). The CysLTs play an important role in development of chronic airway inflammation and airway remodelling in asthma (Holgate et al., 2003). The potent $CysLT_1$ receptor antagonists, such as pranlukast (Onon®), zafirlukast (Accolate®) and montelukast (Singulair®), and the 5-LO inhibitor Zileuton® are approved for the treatment of asthma.

Biological effects of prostanoids

Owing to metabolic instability in the circulation, prostanoids are believed to primarily act as local hormones (autocoids). Thus, they exert their effects in the proximity of the sites of their synthesis, in autocrine or paracrine manner (Smith, 1989). Prostanoids exert their biological effects via interactions with the specific prostanoid receptors that belong to the GPCR family (Coleman et al., 1994) *(Figure 3)*. The prostanoid receptors based on specificity for TXA_2, PGI_2, PGE_2, PGF_2 and PGD_2 are classified as TP, IP, EP, FP and DP receptors, respectively (Coleman et al., 1994), but they can be cross-activated by other prostanoids as well. For example, the TP receptor, in addition to TXA_2, is activated by PGH_2, PGE_2, PGF_2 and PGD_2 (Devillier and Bessard, 1997) and isoprostanes (Kang et al., 1993). On the basis of pharmacological action, prostanoid receptors can be classified into three groups: the relaxant receptors (IP, DP, EP_2, EP_4) that mediate increase in cAMP and induce smooth muscle relaxation, the contractile receptors (TP, FP, EP_1) that mediate Ca^{2+} mobilisation and induce smooth muscle contraction, and the inhibitory receptors (EP_3) that mediates decreases in cAMP and inhibits smooth muscle contraction (Coleman et al., 1994).

Thromboxane is a potent smooth-muscle contracting mediator and is involved in the development of bronchial hyperreactivity, mucus secretion, angiogenesis and smooth-muscle proliferation (Devillier and Bessard, 1997). Two isoforms of TP receptors have been identified and designated as TP_α that is expressed in numerous tissues and TP_β that is a splice variant cloned from endothelium, with limited tissue distribution (Huang et al., 2004). It is unclear if the two isoforms have different physiological functions *in vivo*.

Prostaglandin D_2 activates two receptor subtypes, the DP_1 and the DP_2 receptor. The DP_1-mediated effects of PGD_2 in airways involve a weak relaxation in bronchial and pulmonary vein tissue (Walch et al., 1999), whereas the DP_2 receptor mediates eosinophil chemotaxis and degranulation (Emery et al., 1989).

Prostaglandin $F_{2\alpha}$ causes bronchoconstriction in both healthy and asthmatic airways (Mathé and Hedqvist, 1975; Mathé et al., 1973; Smith et al., 1975) via activation of TP receptors (Devillier and Bessard, 1997). FP receptors are weakly expressed in the lungs (Sugimoto et al., 1994).

The effects of PGI_2 (prostacyclin) involve smooth-muscle relaxation in pulmonary vessels (Szczeklik et al., 1980), inhibition of increased vascular permeability (Schutte et al., 2001), and the inhibitory effect on the mediator release from inflammatory cells (Tran et al., 2001). In

addition to the IP receptor, PGI_2 activates also the EP_2 receptor (Wheeldon and Vardey, 1993). In isolated human bronchi, smooth-muscle relaxation is produced by stimulation of the IP receptor by PGE_1 (Norel et al., 1999).

The effects of PGE_2 are mediated by four subtypes of EP receptor, EP_1, EP_2, EP_3 and EP_4 that all respond to PGE_2 but differ in their actions and responses to various PGE analogues (Coleman et al., 1994). In the lungs, all four EP receptor subtypes have been demonstrated (Narumiya et al., 1999). The EP_1-mediated effect of PGE_2 contributes to the spontaneous airway tone in isolated guinea pig trachea (Ndukwu et al., 1997), and mediates the contractile activity of exogenous PGE_2 in this preparation (Coleman and Kennedy, 1985; Ndukwu et al., 1997), whereas airway smooth muscle relaxation is EP_2 receptor-mediated (Norel et al., 1999; Tilley et al., 2003).

Role of COX-1 and COX-2 in biological responses

In the isolated perfused guinea pig lung, the basal pulmonary prostanoid production as well as the bradykinin-induced release of PGI_2 was predominantly COX-1 dependent (Amann et al., 2001). COX-1 contributed also to some other rapidly (within minutes) occurring inflammatory responses such as the immediate IgE-dependent PGD_2 release from mast cells (Reddy et al., 1999), the arachidonic acid-induced ear edema (Loftin et al., 2002) and the PAF-induced TXA_2 release in rat lung (Uhlig et al., 1996).

In contrast to the simplistic view that COX-2 always is inducible, constitutive COX-2 expression has been described in the lungs, and was localised to tracheal and bronchial epithelial cells (Asano et al., 1996; Ermert et al., 1998b) as well as macrophages and mast cell-like cells in the connective tissue surrounding the blood vessels (Ermert et al., 1998a). COX-2 was constitutively expressed also in isolated guinea pig trachea (Nieri et al., 2006) and appeared to mediate the spontaneous tone in this preparation (Charette et al., 1995). Constitutively expressed COX-2 contributed also to the arachidonic acid-induced (Ermert et al., 1998a) and the PAF-induced (Fabi et al., 2001) vascular response in isolated rat lungs. COX-2 can also be induced in most cell types in the lung by a broad range of proinflammatory stimuli (Belvisi et al., 1997; Bradbury et al., 2002; Mitchell et al., 1994)

Non-steroidal anti-inflammatory drugs

The COX enzyme is an important pharmacological target. Aspirin (acetylsalicylic acid) was the first non-steroidal anti-inflammatory drug (NSAID), introduced 1889 for the treatment of arthritis. Several decades later, new NSAIDs such as indomethacin (1963), diclofenac (1965) and ibuprofen (1969) were introduced *(Figure 4, upper panel)*. A biochemical basis for the antipyretic, anti-inflammatory and analgesic effect of NSAIDs was provided by the discovery that aspirin, but also virtually all other NSAIDs inhibited prostanoid formation and that the anti-inflammatory activity of NSAIDs *in vivo* correlated with their relative COX-inhibitory potency *in vitro* (Vane, 1971). Prior to this discovery, it had incidentally been suggested that some NSAIDs were prostaglandin antagonists (Collier et al., 1963; Collier and Shorley, 1963).

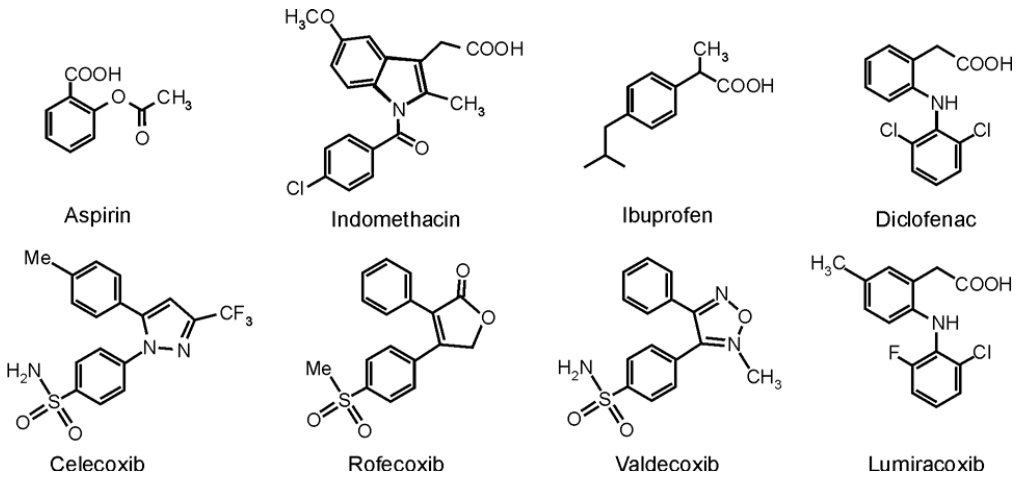

Figure 4. Chemical structures of non-selective NSAIDs *(upper panel)* and COX-2 selective inhibitors (coxibs) *(lower panel)*.

COX inhibition is not the sole but the major mechanism of action of NSAIDs. Aspirin causes irreversible COX inhibition by acetylation of serine residue located 70 amino acids from the *C*-terminus (DeWitt and Smith, 1988). Indomethacin, meclofenamate and flurbiprofen also cause irreversible COX inhibition but without covalent modification of the enzyme (Rome and Lands, 1975). Other common NSAIDs such as ibuprofen, flufenamic acid and sulindac are competitive inhibitors (Rome and Lands, 1975).

After the discovery of a second COX isoform (COX-2) that was inducible by inflammatory cytokines (Fu et al., 1990), the view has developed that the constitutively expressed COX-1 is responsible for production of prostanoids regulating the physiological processes in the gastrointestinal, renal, hemostatic and reproductive system. Accordingly, inhibition of COX-1 was regarded as a main cause of adverse effects associated with the use of NSAIDs such as platelet dysfunction, impaired renal function and gastrointestinal bleedings, the latter effect accounting for a large number of deaths every year due to the wide-spread use of NSAIDs (Wolfe et al., 1999). The inducible COX-2 emerged as the isoform that was primarily responsible for the production of prostanoids in the pathophysiological processes of acute and chronic inflammation. This has led to the development of selective COX-2 inhibitors (coxibs) *(Figure 4, lower panel)*, a new class of NSAIDs with improved gastrointestinal safety (FitzGerald and Patrono, 2001). Initial data were promising, documenting that the coxibs had the same efficacy as the traditional COX unselective NSAIDs, but were associated with less gastrointestinal bleedings (Bombardier et al., 2000; Silverstein et al., 2000).

The simple hypothesis that COX-2 inhibition only affected pro-inflammatory prostaglandins was however soon questioned, as it was demonstrated that constitutively expressed COX-2 may play a role in physiological renal function (Catella-Lawson et al., 1999; McAdam et al., 1999b; Whelton et al., 2000), and that COX-2 plays a role both in the physiological functions of the gastrointestinal tract and in the ulcer healing (Hawkey et al., 1998; Wallace, 2001) *(Figure 5)*.

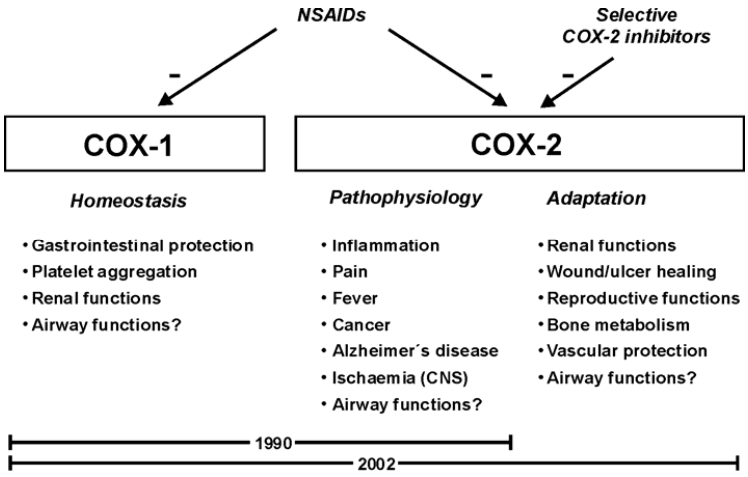

Figure 5. Physiological and pathophysiological roles of the COX isoenzymes.

The finding that the systemic biosynthesis of prostacyclin (PGI_2) in man is predominantly COX-2-dependent (McAdam et al., 1999a) represented a turning point in the risk-benefit assessment of coxibs. This finding suggested that at least in theory the physiological balance between the pro-thrombotic and vasoconstrictory actions of COX-1-derived TXA_2 in platelets and the anti-aggregatory and vasorelaxant actions of COX-2-derived PGI_2 in endothelium would be tilted by COX-2 inhibition in favour of aggregation, implicating cardiovascular hazard.

In fact, with some delay, the adverse cardiovascular effects of coxibs emerged most clearly from several long term studies with primary endpoints other than cardiovascular safety, where an increased number of serious adverse cardiovascular events associated with the use of rofecoxib (Bresalier et al., 2005), celecoxib (Solomon et al., 2005) and valdecoxib (Nussmeier et al., 2005) was observed. Furthermore, long term follow-up of gastrointestinal outcomes associated with the use of some coxibs suggested similar incidence of upper gastrointestinal ulcer complications as those associated with NSAIDs (Juni et al., 2002). Thus, it has been difficult to develop safer NSAIDs by targeting COX-2. This presumably illustrates the complexity of the biologically active products formed along the COX pathway.

AIMS OF THE THESIS

The overall aim of this experimental study was to evaluate the relevance of the isolated perfused and ventilated guinea pig lung model (IPL) in asthma pharmacology.

- The aim of the studies presented in paper I and IV was to characterise the mediators of the immediate antigen-induced bronchoconstriction in IPL.

- The aim of the studies presented in paper IV was also to explore the specific role of the two COX isoenzymes, COX-1 and COX-2 in the immediate antigen-induced generation of prostanoids and ensuing bronchoconstriction in IPL. The role of COX-1 and COX-2 in the antigen-induced generation of prostanoids has not been previously studied in this model, and only to a limited extent in other models.

- The aim of the studies presented in paper I and III was to characterise the intrinsic effects of histamine, cysteinyl-leukotrienes and prostanoids, as well as to establish their mechanisms of action in IPL. A closely associated objective of these studies was to evaluate the relative role of COX products in modulation of the bronchoconstrictive responses to histamine, LTD_4 and PGD_2 in this model. The influence of COX inhibition on the airway response to PGD2 has not been assessed prior to this work.

METHODS

Isolated perfused and ventilated guinea pig lung

General aspects

The isolated perfused and ventilated lung provides the possibility to study the effects of different interventions in an intact organ with physiological cell-to-cell contacts, and to continuously monitor physiological lung-function variables. Experiments in isolated lung cells in culture and isolated fragments of the lung tissue are not always predictive of the results in whole lungs or intact animals (Uhlig, 1998). In the isolated lungs, many important properties are thus preserved. Compared to experiments *in vivo*, this method allows studying the definitive lung effects and avoiding interference from other organs. A limitation of the isolated lung model is deprivation of nervous regulation and lymph drainage, the effects of which are unknown.

Massive postmortem bronchoconstriction occuring 10 min after excision of the lungs is a unique phenomenon of guinea pig lungs, less than 50% of which can be inflated even at high inflation pressure and most gas is trapped during deflation (Lai et al., 1984b). Depletion of pulmonary blood volume due to exsanguination (Lai et al., 1984b) and airway dehydration and hypocarbia due to ventilation with room air (Reynolds and McEvoy, 1988), inducing release of an endogenous constrictor agent (Lai et al., 1984a; Lai et al., 1984b) was proposed as a cause of this phenomenon. The release of tachykinins (substance P and neurokinin A) from afferent nerve fibers was identified as an important mechanism of the postmortem bronchoconstriction in guinea pig lungs (Lai and Cornett, 1987; Martins et al., 1991). Substance P is a potent bronchoconstrictor (Lundberg et al., 1983). Interestingly, in guinea pig, the severe bronchospasm in postmortem lungs was dependent on stimulation of afferent nerve fibers alone, a mechanism apparently unique for the airways of this species (Lai and Cornett, 1987). In contrast, in the airways of other mammals, the afferent fibers do not affect the airway smooth muscle contractions directly but via a reflex arc involving the efferent fibers (Russell and Lai-Fook, 1979). Tachykinins mediated also bronchoconstriction induced by hypocapnia (Reynolds and McEvoy, 1989) and ventilation with cold air (Yoshihara et al., 1998) in guinea pigs *in vivo*. It is thus likely that the postmortem bronchoconstriction in guinea pig lung is predominantly due to the release of tachykinins.

The experimental setup

The isolated perfused and ventilated guinea pig lung (IPL) used in this thesis (Paper I-IV) is displayed in *Figure 6*. Male Dunkin-Hartley guinea pigs weighing 400-600 g were used. Whole lungs were prepared essentially as described in detail by Kröll et al. (Kroll et al., 1986), with following modifications: 1) Positive pressure ventilation was not employed during preparation 2) The buffer with addition of 2% bovine serum albumin, fraction V, was used only during the surgical procedure and the initial part of a stabilisation period 3) In order to prevent the postmortem bronchoconstriction, the IPL was stabilised by addition of salbutamol (33 nM) to the perfusion buffer throughout (Atzori et al., 1992).

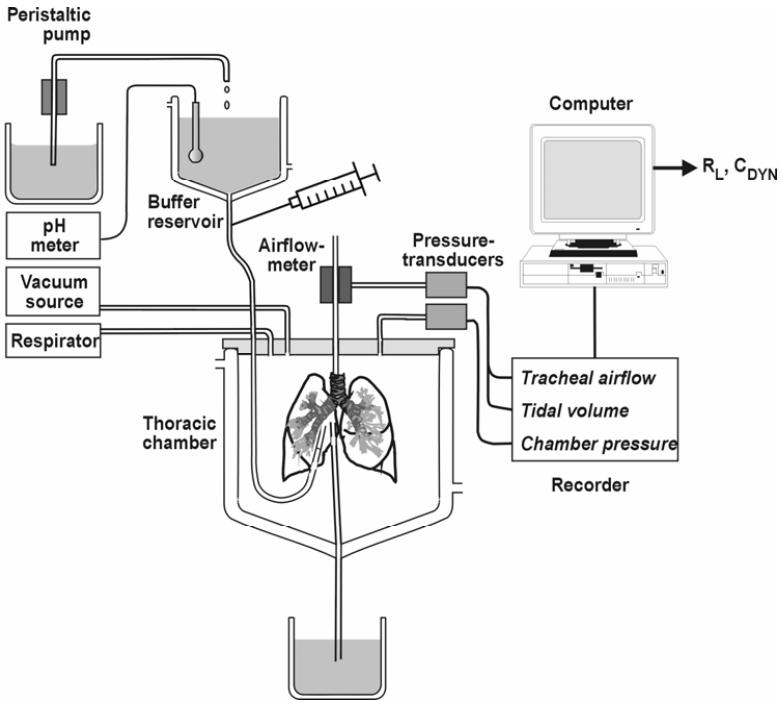

Figure 6. The isolated perfused and ventilated lung setup.

The lungs were perfused via the pulmonary artery with Krebs-Ringer bicarbonate buffer at a constant hydrostatic pressure of about 1.2 kPa (composition of Krebs-Ringer buffer in mM: NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 24.9; KH_2PO_4, 1.2, with addition of

5.5 mM glucose, 12.6 mM HEPES and 33 nM salbutamol). The perfusion of the isolated lungs via the pulmonary artery is commonly used and sufficient for most purposes (Uhlig, 1998). Blood from the pulmonary artery reaches all parts of the lung including large upper airways (Baile et al., 1994), and bronchial artery-pulmonary artery anastomoses are present in guinea pig (McLaughlin, 1983). Constant pressure perfusion permits higher perfusate flow rates than constant flow perfusion, and is less likely to cause hydrostatic edema (Uhlig, 1998).

The lungs were ventilated at 60 breaths min^{-1} by creating an alternating negative pressure (-0.32 to -0.58 kPa) inside the chamber using an animal respirator (model 7025, Ugo Basile, Biological research apparatus, Varese, Italy) and a vacuum source connected to the chamber. Negative pressure ventilation mimics more closely the situation *in vivo* and is less prone to induce barotrauma and edema than positive pressure ventilation (Dritsas et al., 1969). Tracheal airflow was measured with a heated pneumotachograph (Hans Rudolph, Kansas City, MO, USA) connected to the transducer in the EMKA system (EMKA Technologies, Paris, France) that also measured chamber pressure. Lung-function parameters: airway conductance and dynamic compliance were calculated simultaneously and recorded by a computerised data acquisition system with software IOX (EMKA Technologies).

Only lungs with stable baseline values for airway conductance, dynamic compliance and perfusion flow were used. The lungs were allowed to equilibrate for 10 minutes in the medium without albumin and with single pass perfusion before administration of the test compounds, which were injected in a constant volume (0.1 mL) of vehicle into the perfusate inlet catheter. The experimental protocols and other details are described in the individual papers.

The peak effects on airway conductance and dynamic compliance were evaluated as % of baseline values. Since changes in dynamic compliance followed similar pattern as changes in airway conductance throughout all experiments, we show only the values of airway conductance. Bronchoconstriction in preparation is expressed as % decreases in airway conductance related to baseline.

Isolated preparations of guinea pig trachea, guinea pig aorta and rat aorta

The isolated guinea pig trachea preparations (Paper II and III) and guinea pig and rat aorta preparations (Paper II) were used. Male Dunkin Hartley guinea pigs weighing 500-900 g and male Sprague-Dawley rats weighing 180-220 g were used. The tracheal and aortic rings were placed in 5 mL organ baths filled with Tyrode's solution (composition in mM: NaCl, 142.9; KCl,

2.7; $NaHCO_3$, 11.9; glucose, 5.5; $CaCl_2$, 1.8; $MgCl_2$ x $6H_2O$, 0.5; NaH_2PO_4, 0.4). The pH was kept at 7.4 by administration of 6.5% CO_2 in O_2 and the temperature was kept constant at 37°C. The tracheal and aortic rings were mounted on lower and upper organ hooks, connected to the isometric force-displacement transducers (EMKA Technologies). Changes in smooth-muscle tension in the preparations, that is airway and vascular smooth muscle contractions, were recorded and displayed by a computerised data acquisition system with software IOX (EMKA Technologies). Calculations of tension changes were made with help of data analysis software Datanalyst (EMKA Technologies). Contraction responses were generally expressed as per cent of the maximum terminal contractions induced by addition of histamine (1 mM), acetylcholine (1 mM) and KCl (40 mM). The experimental protocols are described in the individual papers.

Animal sensitisation

In the studies of the antigen-induced bronchoconstriction and mediator release (Paper I and IV), lungs from sensitised animals were used. Guinea pigs weighing 200-250 g were sensitised at least four weeks prior to experiments to chicken egg albumin (ovalbumin, OVA) by one i.p. and one s.c. injection of 10 mg OVA dissolved in 0.4 ml 0.9 % NaCl and $Al(OH)_3$ hydrogel 1:1, v:v. This sensitisation protocol has previously been shown to produce reproducible contraction responses to OVA in isolated guinea pig lung parenchyma with no decay in responsiveness over four months after the sensitisation (Wikström-Jonsson and Dahlén, 1994). Guinea pigs respond with production of both IgG_1 and IgE antibodies to active sensitisation according to different protocols (Andersson, 1980; Graziano et al., 1981; Graziano et al., 1984).

Enzyme immunoassay

Measurements of the agonist-induced release of prostanoids in isolated guinea pig lungs and trachea (Paper III) as well as the antigen-induced release of CysLTs and prostanoids in isolated guinea pig lungs (Paper IV) were performed with enzyme immunoassay (EIA).

The EIA analyses of the content of LTC_4, TXB_2 (a stable metabolite of TXA_2), PGD_2-MOX (a stable methoxime derivative of PGD_2), $PGF_{2\alpha}$, PGE_2 and 6-keto $PGF_{1\alpha}$ (a stable metabolite of PGI_2), were performed with polyclonal or monoclonal (for PGE_2) antisera and acetylcholinesterase (AchE)-linked tracers (Cayman Chemical Company). The EIA is based on

the competition between free eicosanoid and the respective AchE-linked eicosanoid for limited specific binding sites on the primary antibody, polyclonal or monoclonal rabbit antiserum. The formed complexes are subsequently bound to the secondary antibody, mouse monoclonal anti-rabbit IgG attached to the solid phase. After incubation and wash, Ellman's reagent containing acetylthiocholine, a substrate for the AchE, is added. The enzyme activity yielding a coloured product is measured spectrophotometrically. The obtained absorbance value is proportional to the amount of the tracer-linked antibody bound to the well, and the amount of eicosanoid in the sample is thus inversely proportional to the absorbance.

Other methods

Platelet aggregation, TP receptor binding and signalling experiments were performed by the Milano group.

Statistical analysis

Data in figures are given as mean ± SD (standard deviation). The obtained experimental data were statistically evaluated by Student's t-test for paired and unpaired observations; $p < 0.05$ was generally considered significant, except of Fig. 5 and Fig. 6 in Paper I, where several comparisons were done and p value was set to 0.02 to minimise the alpha error.

Ethical approval

The experiments in the present thesis were approved by Stockholms Norra Djurforsöksetiska Nämnd (N317/98, N176/01, N127/04) and the ethic committee in Milano, Italy (124/2003-A).

RESULTS AND DISCUSSION

Basal characteristics of responses to bronchoconstrictive agonists

Specific aims

The aim of this part of the thesis was to characterise the intrinsic effects of histamine, cysteinyl-leukotrienes (CysLTs) and prostanoids, as well as to establish their mechanisms of action in the IPL model. The experiments were conducted in lungs from non-sensitised animals.

Characteristics of the responses to histamine and cysteinyl-leukotrienes (Paper I)

Dose-response relations for the effect of the challenge of the IPL with escalating doses of histamine and LTD_4 were parallel *(Figure 7, left panel)* and showed that LTD_4 was on a molar basis about 1500-fold as potent as histamine. This was also documented for D_{50} values (the agonist doses causing 50% reductions in airway conductance) *(Table 1)*. Similar molar ratios for equivalent constrictive effects of histamine and CysLTs were established in isolated guinea pig lung parenchyma as well as in isolated human bronchi (Dahlén et al., 1983a; Dahlen et al., 1982; Dahlén et al., 1980; Hedqvist et al., 1980; Jones et al., 1982). In contrast, mouse and rat airways do not respond with significant bronchoconstriction to challenge with histamine or CysLTs (Hedqvist et al., 1980; Held et al., 1999; Martin et al., 1988; Piper and Samhoun, 1981).

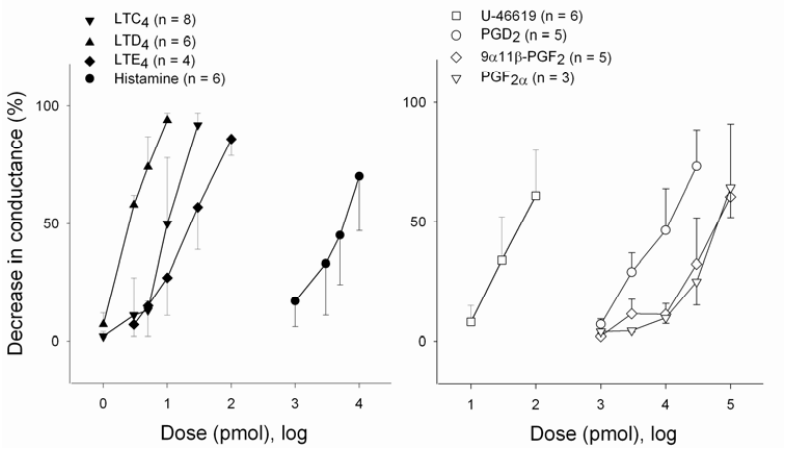

Figure 7. Dose-response relations for the peak decrease in conductance (mean ± SD) obtained by the challenge with escalating doses of cysteinyl-leukotrienes and histamine *(left panel)* and prostanoids *(right panel)* (data from paper I and III).

Agonist	D_{50}			
	mean	sd	n	
Histamine (nmol)	4.7	2.4	5	
LTC_4 (pmol)	10	4	7	
LTD_4 (pmol)	3	0.2	5	
LTE_4 (pmol)	24	15	4	
U-46619 (pmol)	43	15	4	p=0.007 vs. PGD_2
PGD_2 (nmol)	15	8	5	p=0.02 vs. Hi
9α11β-PGF_2 (nmol)	56	38	3	p=0.05 vs. PGD_2
$PGF_{2\alpha}$ (nmol)	60	12	3	p=0.0007 vs. PGD_2

Table 1. Potency of agonists, expressed as the agonist dose causing 50% decrease in airway conductance (D_{50}) (data from paper I and III); n, number of subjects.

As documented for D_{50} values *(Table 1)*, dose-response relations for the responses to LTC_4, LTD_4 and LTE_4 revealed that in IPL, LTD_4 was the most potent bronchoconstrictor, followed by LTC_4 and LTE_4 *(Figure 7, left panel)*. Similarly, LTD_4 has been reported to be more potent bronchoconstrictor than LTC_4 in isolated guinea pig lung parenchyma (Drazen et al., 1980a; Krell et al., 1981; Piper and Samhoun, 1981), whereas other investigators reported that LTC_4 and LTD_4 were equipotent, and significantly more potent than LTE_4 in that model (Sakata and Bäck, 2002; Samhoun and Piper, 1984). In isolated human bronchi, LTC_4 and LTD_4 displayed similar constrictive potencies (Jones et al., 1982; Samhoun and Piper, 1984), whereas LTE_4 was ten times less potent (Samhoun and Piper, 1984).

Effects of receptor antagonists on the response to histamine (Paper I)

The bronchoconstrictive response to histamine in IPL was substantially inhibited by the H_1 receptor antagonist mepyramine (1 μM) (Paper I). This supports the notion that the constrictive effect of histamine in guinea pig airways is mediated by H_1 receptors (Bilcikova et al., 1990; Cortijo et al., 1989; Drazen and Schneider, 1978). The notion that there are inhibitory H_2 receptors in guinea pig airways and that they predominate in the periphery (Bilcikova et al., 1990; Cortijo et al., 1989; Drazen et al., 1980b; Tomioka and Yamada, 1982) was supported by the finding that the response to histamine in IPL was enhanced by the H_2 receptor antagonist metiamide (1 μM) (Paper I). This was the first time demonstration of the role of inhibitory H_2 receptors in the response to histamine in IPL. Relaxant H_2 receptors have previously been described in isolated human bronchi (Black et al., 1972). Human airways have both H_1 and H_2 receptors, but H_1 receptors mediating bronchoconstrictive effect of histamine predominate, whereas H_2 receptor-mediated bronchodilation is relatively weak in man (Braman, 1987; White et al., 1987).

Effects of receptor antagonists on the responses to cysteinyl-leukotrienes (Paper I and previously unpublished data)

The bronchoconstrictive response to LTD_4 in IPL was abolished by the selective $CysLT_1$ receptor antagonist zafirlukast (0.1 μM) (Paper I). In addition, it was found that the response to LTC_4 in IPL also was abolished by this treatment *(Figure 8)*. These findings suggest that the effects of LTD_4 and LTC_4 in this model are predominantly mediated by $CysLT_1$ receptors. As further support, the order of potency of CysLTs in IPL, $LTD_4 > LTC_4 > LTE_4$ *(Figure 7, left panel)*, incidentally fits with agonist potency data for $CysLT_1$ receptors in binding assays (Brink et al., 2003). This justifies the use of LTD_4 as the probe for the CysLTs in this thesis. The predominant part of the response to CysLTs in human airways is mediated by activation of $CysLT_1$ receptors (Buckner et al., 1986; Dahlén, 2000; Yamaguchi et al., 1992).

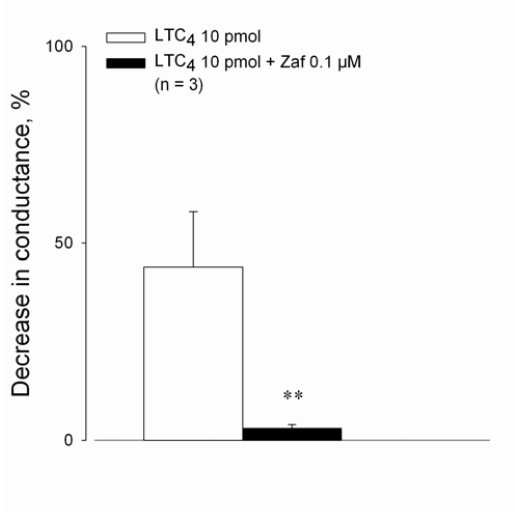

Figure 8. Effect of the selective $CysLT_1$ receptor antagonist zafirlukast (0.1 μM) on the peak decrease in conductance (mean ± SD) following the challenge with LTC_4 (10 pmol); **, $p < 0.01$ (previously unpublished data).

The presence of $CysLT_1$ receptors was also supported in isolated guinea pig lung parenchyma (Wikström Jonsson et al., 1998), and it was established that $CysLT_1$ receptors were activated by either LTD_4 or LTC_4, whereas neither of these agonists activated $CysLT_2$ receptors in this preparation (Sakata and Bäck, 2002). However, in contrast to the situation in IPL, a large part of the response to LTD_4 in guinea pig lung parenchyma was not inhibited by $CysLT_1$ receptor antagonists (Sakata and Bäck, 2002; Wikström Jonsson et al., 1998), and the presence in this preparation of a purported new CysLT receptor, with different properties than those of $CysLT_1$ and $CysLT_2$, was thus proposed (Sakata and Bäck, 2002; Wikström Jonsson et al., 1998). $CysLT_2$

receptors are particularly abundant in guinea pig trachea where they predominantly mediate the constrictive response to LTC_4, whereas LTD_4 activates both $CysLT_1$ and $CysLT_2$ receptors in this preparation (Bäck et al., 2001).

Characteristics of the responses to prostanoids (Paper I and III)

The reactivity of the IPL to TXA_2 was established with the aid of the stable TX mimetic and thromboxane receptor (TP) agonist U-46619 (Abramovitz et al., 2000). This compound was on a molar basis about 350-fold as potent as PGD_2 *(Figure 7, right panel and Table 1)*. Dose-response relations for the effects of challenge of the IPL with PGD_2, its metabolite 9α11β-PGF_2, or the structurally related $PGF_{2\alpha}$ *(Figure 7, right panel)* revealed that PGD_2 was 3-fold less potent than histamine *(Table 1)*. 9α11β-PGF_2 and $PGF_{2\alpha}$ were equipotent, and they were about 4-fold less potent than PGD_2, as documented for D_{50} *(Table 1)*. In contrast, investigation of the constrictive effects of PGD_2, 9α11β-PGF_2 and $PGF_{2\alpha}$ in isolated human bronchi disclosed the greater constrictive potency of 9α11β-PGF_2 (Beasley et al., 1987) and $PGF_{2\alpha}$ (Beasley et al., 1987; Black et al., 1986) than that of PGD_2. However, other investigators reported that PGD_2, 9α11β-PGF_2 and $PGF_{2\alpha}$ were equally potent in this preparation (Seibert et al., 1987). In guinea pig lung parenchyma, PGD_2 and the cyclic ether endoperoxide analogues, 9α11α-PGF_2 and 11α9α-PGF_2, were as effective as, or more effective constrictors than histamine, whereas $PGF_{2\alpha}$ had only a minor constrictive effect, as compared to that of histamine (Schneider and Drazen, 1980). Similarly, comparison of effects of intravenously infused cyclic ether endoperoxide analogues, PGD_2 and $PGF_{2\alpha}$ on pulmonary mechanics in anaesthetised dogs *in vivo*, showed that the cyclic ether analogues and PGD_2 had significantly more potent bronchoconstrictive effects than $PGF_{2\alpha}$ (Wasserman, 1976; Wasserman et al., 1977). Irrespective of the reported small differences with respect to the relative bronchoconstrictor potencies of PGD_2 and its analogues that may depend on the properties of the compounds used in the different studies, these findings confirm the overall similar bronchoconstrictive properties of $PGF_{2\alpha}$ as well as PGD_2 and its metabolites in airways.

It was previously described in isolated guinea pig trachea that PGE_2 exerted mixed relaxant and constrictive effects, depending on muscular tone, whereas PGI_2 had a constrictive effect only (Coleman and Kennedy, 1980). In isolated human bronchi, PGE_2 had highly variable and concentration-dependent effects: low concentrations produced relaxation whereas high concentrations produced contraction in this preparation (Knight et al., 1995b). The contractile effect of PGE_2 in isolated human bronchi was also reported by other investigators (Haye-Legrand

et al., 1986; Mathé et al., 1971), whereas PGI_2 and its analogues, iloprost and ZK 96480 had relaxant effects, but relaxations produced by PGI_2 were variable (Haye-Legrand *et al.*, 1987).

The intrinsic effects of PGE_2 and PGI_2 in the whole guinea pig lungs have not been evaluated prior to the present work. Investigation in IPL of airway effects of exogenous PGE_2 and PGI_2 (the latter by means of the two stable synthetic prostacyclin analogues, ciprostene and 15-keto iloprost) disclosed a weak bronchoconstrictor activity produced only with the highest doses of these prostanoids (Paper III). The observed effects may all relate to actions of higher doses (100-300 nmol) of PGE_2 and the prostacyclin analogues on prostanoid EP_1 receptors. The EP_1 receptors were shown to mediate the constrictive effects of PGE_2 in guinea pig airways (Ndukwu et al., 1997) and the prostacyclin analogues are also agonists on EP_1 receptors (Armstrong et al., 1989; Dong et al., 1986).

It was also investigated if exogenous PGE_2 had a bronchoprotective effect in IPL, that is, could modulate bronchoconstriction induced by histamine. PGE_2 and PGI_2 were previously shown to exert a bronchoprotective effect in other guinea pig airway models, where agonist-induced contractions were relaxed by these prostanoids (Coleman and Kennedy, 1980; Mugridge et al., 1984; Ross et al., 2002; Wasserman et al., 1980). In this thesis, the bronchoprotective effect of PGE_2 was for the first time demonstrated in IPL. The responses evoked by the challenge with escalating doses of histamine in IPL were significantly reduced in the presence of exogenous PGE_2 (100 nM), whereas the control challenge with escalating doses of histamine, performed twice in the same lung preparation, produced super imposable responses (Paper III). The bronchoprotective effect of PGE_2 is most probably due to the action of PGE_2 on the prostanoid EP_2 receptors, as EP_2 receptors are known to mediate smooth-muscle relaxation in airway preparations *in vitro* (Norel et al., 1999; Tilley et al., 2003).

Effects of receptor antagonists on the responses to prostanoids (Paper I, II and III)

As expected, the bronchoconstrictive response to the TX mimetic U-46619 in IPL was abolished by the TP receptor antagonist BAY u3405 (1 μM) (Paper I), and the response to PGD_2 was abolished by BAY u3405 (1 μM) (Paper III) as well as by another selective TP antagonist, SQ 29,548 (1 μM) (Ogletree et al., 1985) (Paper II). This is in line with the previous findings (Beasley et al., 1989; Featherstone et al., 1990; Johnston et al., 1995) that the constrictive effect of PGD_2 in human and guinea pig airways is TP receptor-mediated. The evidence demonstrating a role of the TP receptor in bronchoconstriction caused by PGD_2 in IPL was obtained with the TP

receptor antagonist BAY u3405, which has been reported also to be an antagonist on $CRTH_2$ (DP_2) receptors (Sugimoto et al., 2003). Thus, the question might be raised about the possible contribution also of the DP_2 receptor in mediating PGD_2-induced bronchoconstriction in IPL. However, complete inhibition of PGD_2-induced bronchoconstriction in IPL, obtained with the selective TP receptor antagonist SQ 29,548 (Paper II), similar to that obtained with BAY u3405, allows the conclusion that the TP receptor plays the main role and the DP_2 receptor has no role in mediating PGD_2-induced bronchoconstriction in this model. It has also recently been established in isolated guinea pig trachea that agonists for the DP_2 receptor fail to elicit contractions (unpublished results).

In this thesis, the effects of TP antagonism on bronchoconstriction evoked by 9α11β-PGF_2 and $PGF_{2\alpha}$ in IPL were not investigated. However, the recent findings in isolated guinea pig trachea, where the responses to these prostanoids were significantly inhibited by TP receptor antagonists (unpublished results), in line with the previous findings in other guinea pig airway preparations (Beasley et al., 1989; Featherstone et al., 1990) and in human airways (Coleman and Sheldrick, 1989; Featherstone et al., 1990), provide evidence that the constrictive effects of 9α11β-PGF_2 and $PGF_{2\alpha}$ in guinea pig airways are TP receptor-mediated as well.

Time-courses of the responses to histamine, LTD_4, U-46619 and PGD_2 (Paper I and previously unpublished data)

As it was documented that the bronchoconstrictive responses to LTD_4 in IPL were more long-lived than those to histamine, which were notoriously short-lived, and that the time to the maximal effect for LTD_4 was significantly longer than that for histamine (Paper I), the time-courses of the bronchoconstrictive responses to histamine, LTD_4, U-46619 and PGD_2 were next characterised in this model. Comparing doses producing responses of similar magnitude, it became evident that the time-courses of the responses to histamine and U-46619 were distinctly more short-lived than those of the responses to PGD_2 and LTD_4 *(Figure 9 and Table 2)*. Moreover, comparison between the time-courses of the responses to PGD_2 and U-46619 revealed that although the onset of effect of PGD_2 was similar to that of U-46619, the duration of the bronchoconstrictive effect of PGD_2 (expressed as time to 50% recovery of airway conductance to baseline) was significantly longer for PGD_2 than that for U-46619 *(Table 2)*. In fact, in this particular preparation, the duration of the bronchoconstrictive effect of PGD_2 was longer than that of the classical "slow reacting" substance LTD_4.

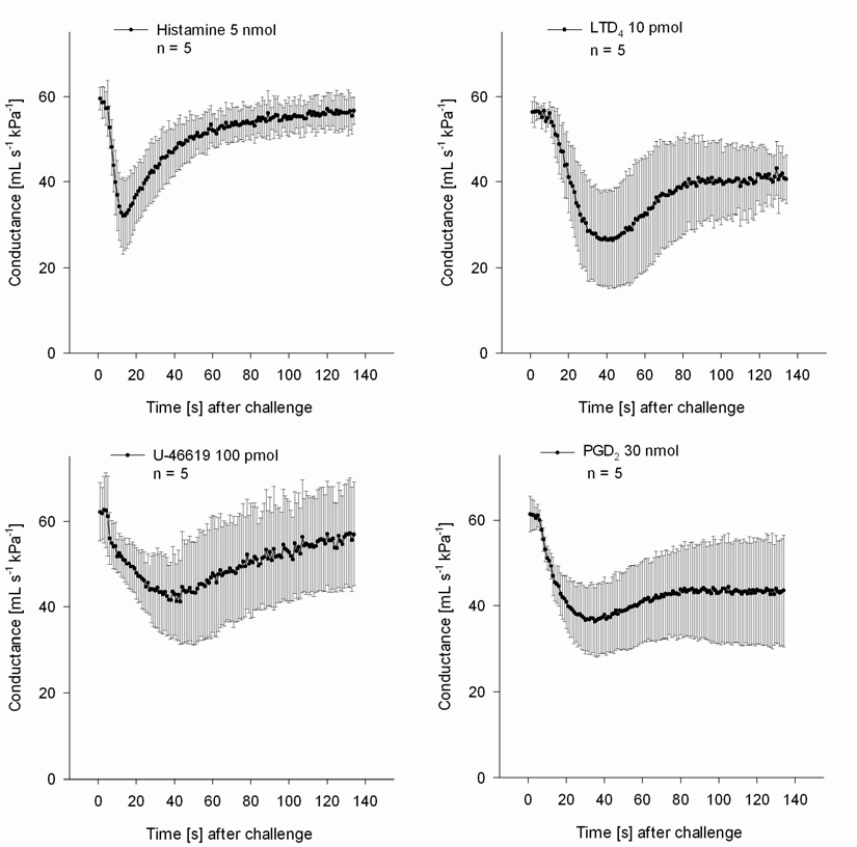

Figure 9. Time-courses of bronchoconstriction induced by histamine, LTD_4, U-46619 and PGD_2 in IPL (previously unpublished data).

It may seem disturbing that the duration of bronchoconstriction induced by PGD_2 in IPL was much longer than that of U-46619, in particular as PGD_2 is rapidly metabolised in the lung (Seibert et al., 1987), whereas U-46619 is a relatively stable TP agonist. However, the different degree of metabolic handling is probably and paradoxically nevertheless the explanation of this difference. The bolus injection of U-46619 only has time to display a first pass pharmacologic effect at the TP receptor whereas PGD_2 also binds to 11-ketoreductase and other enzymes that locally produce metabolites with sustained biologic activity, such as 9α11β-PGF_2. This prostanoid, once formed, may exert the constrictive effect along with PGD_2 and $PGF_{2\alpha}$, as 9α11β-PGF_2 would not be expected to be rapidly inactivated *in situ* by the metabolic enzymes (Seibert et

al., 1987). The immediate PGD_2 metabolite, 9α11β-PGF_2 is also a potent bronchoconstrictor in IPL *(Figure 7, right panel)*. The similar time of onset for the effects of U-46619 and PGD_2 *(Table 2)* supports similar initial effects on the TP receptor. As further support of the role of metabolism into pharmacologically active compounds, it should be recognised that the other compound with long duration of action in the IPL, LTD_4, is metabolised into LTE_4 that also is a potent bronchoconstrictor in IPL *(Figure 7, left panel)*. Taken together, these data do not question that U-46619 mimics PGD_2 in terms of causing bronchoconstriction via activation of TP receptors, but the findings illustrate that the time-course of response to a synthetic compound may differ from that of the natural ligand.

Table 2. Time-courses of bronchoconstriction induced by PGD_2, LTD_4, U-46619 and histamine in IPL (previously unpublished data).

	PGD_2 30 nmol n=5	LTD_4 10 pmol n=5	U-46619 100 pmol n=5	Histamine 5 nmol n=5
Max. decrease (%) in airway conductance (E_{max}), mean ± SD	42 ± 10[ns]	55 ± 22[ns]	37 ± 10[ns]	42 ± 15
Time (s) to E_{max}, mean ± SD	35 ± 2[#]	41 ± 8[#]	37 ± 7[#]	13 ± 2
Time (s) to 50% recovery[a], mean ± SD	53 ± 9[¶¶]	35 ± 23	24 ± 13[¶]	21 ± 7
AUC[b], mean ± SD	2468 ± 767[§]	2406 ± 1138	1586 ± 634	1188 ± 392

[a] From E_{max} to 50% of the conductance value at baseline
[b]Area under the curve: Conductance (mL s^{-1} kPa^{-1}) vs. time 0-134 s after the agonist challenge
[ns] P > 0.05 vs Histamine (Student's two-tailed unpaired t-test)
[#] P < 0.001 vs Histamine (Student's two-tailed unpaired t-test)
[¶] P < 0.05 vs PGD_2; [¶¶] p < 0.01 vs Histamine (Student's two-tailed unpaired t-test)
[§] P < 0.01 vs Histamine (Student's two-tailed unpaired t-test)

Summary: Basal characteristics of responses to bronchoconstrictive agonists

The reactivity of the IPL model to histamine, CysLTs and bronchoconstrictive prostanoids, established by injecting the mediators themselves in lungs from non-sensitised animals, was generally similar to that described in isolated guinea pig and human airway preparations. Furthermore, the bronchoprotective effect of PGE_2 in IPL was for the first time demonstrated in this work.

Analysis of the time-courses of the bronchoconstrictive responses to histamine, LTD_4, U-46619 and PGD_2 in IPL disclosed that the responses to histamine and U-46619 were distinctly more short-lived than those to PGD_2 and LTD_4. In fact, in this particular preparation, the duration of the bronchoconstrictive effect of PGD_2 was longer than that of the classical "slow reacting" substance LTD_4.

The bronchoconstrictive response to histamine in IPL was mediated by H_1 receptors, as shown by significant inhibition of the response by H_1 receptor antagonism. Moreover, the role of inhibitory H_2 receptors was for the first time demonstrated in this model by enhancement of the response to histamine by H_2 receptor antagonism.

The bronchoconstrictive response to LTD_4 as well as that to LTC_4 in IPL was abolished by $CysLT_1$ receptor antagonism, supporting that similar to human airways, the effects of LTD_4 and LTC_4 in this model are predominantly mediated by $CysLT_1$ receptors.

The bronchoconstrictive response to PGD_2 in IPL was abolished by TP receptor antagonism, documenting that the effect of PGD_2 in this preparation was TP receptor-mediated.

Assessment of the influence of the pharmacological interventions on responses to histamine, CysLTs and prostanoids in this part of the thesis allowed also establishing the effectiveness of the pharmacological tools to be used in further studies in this thesis addressing the specific role of these mediators in the immediate antigen-induced bronchoconstriction (Paper I and IV).

Modulation of responses to bronchoconstrictive agonists by cyclooxygenase products

Specific aims

The responses to bronchoconstrictive agonists such as histamine and CysLTs have a cyclooxygenase (COX)-dependent component in guinea pig airways, as suppression of the responses to these agonists by COX inhibitors was described in anaesthetised guinea pigs *in vivo* (Dahlén, 1983; Omini et al., 1981; Rossoni et al., 1980; Weichman et al., 1982), and in isolated peripheral airway preparations from this species (Piper and Samhoun, 1981; 1982; Samhoun and Piper, 1984; Weichman et al., 1982; Wikström et al., 1992). Furthermore, release of COX products induced by histamine or CysLTs was documented in anaesthetised guinea pigs *in vivo* (Omini et al., 1981; Rossoni et al., 1980), in isolated peripheral airway preparations (Piper and Samhoun, 1981; 1982; Samhoun and Piper, 1984; Weichman et al., 1982; Wikström et al., 1992) as well as in isolated perfused lungs from this species (Berti et al., 1980; Berti et al., 1979; Folco et al., 1981; Piper and Samhoun, 1981; 1982; Samhoun and Piper, 1984). However, the contribution of COX products to bronchoconstriction induced by histamine and CysLTs in the IPL has not been assessed prior to this work.

The aim of this part of the thesis was thus to evaluate the relative role of COX products in modulation of the bronchoconstrictive responses to histamine, LTD_4 and PGD_2 in IPL. The influence of COX inhibition on the airway response to PGD_2 has not been addressed previously.

A closely associated objective of this part of the thesis was to further define the airway pharmacology of LTD_4 in this particular model. From *in vitro* studies, it has generally (Piper and Samhoun, 1982; Weichman et al., 1982; Wikström et al., 1992), but not universally (Fitzpatrick and Lawson, 1988), been concluded that TXA_2 mediated the COX-dependent part of the contraction response to leukotrienes. However, data where selective TP receptor antagonists inhibited the response to LTD_4 in isolated guinea pig lung parenchyma (Aizawa et al., 1996) might as well support the alternative explanation that PGD_2 was released in response to LTD_4, and it has not been previously evaluated if selective inhibition of TX biosynthesis attenuates the bronchoconstrictive response to LTD_4. Thus, the effect of TX synthesis inhibition on the response to LTD_4 was investigated in IPL.

Furthermore, as it has previously not been addressed if the COX products that modulate the response to LTD_4 in IPL, or for that matter any other guinea pig airway preparation, are

generated along the COX-1 or COX-2 pathway, the effects of selective COX-1 and COX-2 inhibitors on the response to LTD_4 were investigated.

In order to provide a comprehensive understanding of drug effects and mechanisms involved, for some of the interventions, release of COX products into the lung effluent was also measured.

Evaluation of the relative role of COX products in the response to histamine (Paper I)

Although release of TXA_2 and prostaglandins by histamine has been shown to occur in guinea pigs *in vivo* (Rossoni et al., 1980) and in isolated perfused lungs of this species (Berti et al., 1980; Berti et al., 1979; Piper and Vane, 1969), where it has been suggested to be linked to activation of H_1 receptors by histamine (Berti et al., 1979), the modulatory role of COX products in histamine-induced bronchoconstriction has previously not been examined in IPL. The response to histamine in this preparation was significantly reduced in the presence of the non-selective COX inhibitor diclofenac (10 μM) (Paper I). This suggests that the bronchoconstrictive prostanoids presumably account for part of the bronchoconstriction evoked by histamine in this particular model. Further experiments with selective receptor antagonists and biosynthesis inhibitors as well as by measurements of COX products in the lung effluent will be required to define which COX products that are involved.

Evaluation of the relative role of COX products in the response to LTD_4 (Paper I and III)

The response to LTD_4 in IPL was significantly reduced in the presence of the non-selective COX inhibitor diclofenac (10 μM) (paper I and III), suggesting the COX-dependent component. In the guinea pig, release of the potent bronchoconstrictive TXA_2 (Hamberg et al., 1975) by CysLTs has previously been shown to occur in peripheral airway preparations *in vitro* (Dahlén et al., 1983b; Piper and Samhoun, 1982; Weichman et al., 1982), perfused whole lungs (Folco et al., 1981; Piper and Samhoun, 1981), or in anaesthetised animals *in vivo* (Omini et al., 1981), and it has been linked to $CysLT_1$ receptor activation (Folco et al., 1981; Mong et al., 1986; Wikström et al., 1992). These data suggested that the COX-dependent part of the response to LTD_4 in the IPL could be due to release of TXA_2. The analysis of COX products in the lung effluent after challenge of the IPL with LTD_4 (30 pmol) documented that LTD_4 indeed actively promoted release of TXA_2. In addition, release of PGE_2 and PGI_2 was also detected, although in lesser amounts than for TXA_2

(Paper III). Moreover, both the LTD_4-induced bronchoconstriction, and the TXA_2 release was inhibited by the selective TX synthase inhibitor ozagrel (30 μM) (Paper III). Thus, it can be concluded that the bronchoconstrictive response to LTD_4 in IPL is amplified by the secondarily released constrictive TXA_2. However, significantly more pronounced inhibition of the LTD_4-induced bronchoconstriction by the non-selective COX inhibitor diclofenac than by the TX synthase inhibitor ozagrel (Paper III), suggests that other constrictive prostanoids, such as PGD_2 and $PGF_{2\alpha}$ may amplify the response to LTD_4 as well. This is in line with previous findings that potent TP receptor antagonist show less complete antagonism of leukotriene responses than COX inhibitors (Fitzpatrick and Lawson, 1988). Future studies will be required to define which COX products that are involved.

Investigation of the effect of the selective TX synthase inhibitor ozagrel on the LTD_4-induced release of COX products in IPL disclosed that this treatment, in addition to inhibition of TXA_2 release, substantially enhanced release of PGE_2. Release of PGI_2 also showed a trend towards enhancement under the same conditions (Paper III). This finding suggests shunting of endoperoxides to the PGE_2 and PGI_2 biosynthetic pathways when the TX synthase is inhibited.

A similar finding was previously reported in guinea pig lung where the arachidonic acid-induced release of PGI_2 was substantially enhanced by TX synthase inhibitor dazoxiben (O'Keefe et al., 1985). Thus, suppression of LTD_4-induced bronchoconstriction in IPL by ozagrel may probably be explained as a combination of decreased biosynthesis of TXA_2 and enhanced release of relaxant prostanoids, caused by this intervention.

This part of the thesis attempted also to establish if a single COX isoenzyme was the source of COX products amplifying the bronchoconstrictive response to LTD_4 in IPL. The LTD_4-induced bronchoconstriction was however significantly inhibited by either the highly selective COX-1 inhibitor FR122047 (Ochi et al., 2000) or celecoxib, with presumed COX-2 selectivity (Paper III). Furthermore, in line with the effects of the selective COX-1 and COX-2 inhibitors on the LTD_4-induced bronchoconstriction, the release of TXA_2, but also that of PGE_2 and PGI_2 following the LTD_4 challenge, was substantially depressed by either COX inhibitor (Paper III). Taken together, these findings suggest that both COX isoenzymes, COX-1 and COX-2 initiated production of TXA_2 amplifying the LTD_4-induced bronchoconstriction in IPL. However, significantly more pronounced inhibition of the LTD_4-induced bronchoconstriction obtained with celecoxib, as compared to that obtained with FR122047 (Paper III) suggests that COX-2 plays a more prominent role in the LTD_4-induced generation of constrictive prostanoids.

Similar to guinea pig peripheral airway preparations *in vitro* (Aizawa et al., 1996; Dahlén et al., 1983b; Piper and Samhoun, 1981; Weichman et al., 1982), or anaesthetised animals *in vivo* (Dahlén, 1983; Weichman et al., 1982), it was established that the response to LTD_4 also in IPL had two components. Thus, the actions of low doses of LTD_4 in IPL were significantly reduced in the presence of the non-selective COX inhibitor diclofenac (10 μM) (Paper I and III). However, the effect of diclofenac was surmounted by higher doses of LTD_4 and a parallel shift to the right of the dose-response relations for the LTD_4 challenge was obtained in the presence of this drug. Maximal reduction in lung function was obtained by about 30-fold higher doses, that is, by 300 pmol of LTD_4 in the presence of diclofenac, as compared with 10 pmol of LTD_4 in control. Thus, a 30-fold decrease in sensitivity for LTD_4 in IPL was produced by diclofenac. (Paper I). The diclofenac-resistant component of the response to the highest dose of LTD_4 (300 pmol) was however abolished by the $CysLT_1$ receptor antagonist zafirlukast (0.1 μM) (Paper I). Moreover, the time to maximal effect for LTD_4 in the presence of diclofenac was significantly longer, as compared with that for LTD_4 alone (Paper I). Therefore, it was concluded that in IPL, the effect of LTD_4 in the presence of a COX inhibitor represents the direct action of this mediator on the airway smooth muscle, whereas the effect of LTD_4 in the absence of a COX inhibitor is a combination of the direct and indirect actions, mediated by bronchoconstrictive COX products. It has been reported that no further inhibition of the contractions to CysLTs was achieved by $CysLT_1$ antagonists in the presence of COX inhibitors in isolated guinea pig lung parenchyma, suggesting that the response to CysLTs in this preparation was predominantly due to the actions of bronchoconstrictive COX products (Weichman et al., 1982). However, similar to the findings in the IPL, it has been reported that the residual contractions to CysLTs in isolated guinea pig lung parenchyma pretreated with COX inhibitor were abolished by a leukotriene receptor antagonist (Piper and Samhoun, 1981).

Evaluation of the relative role of COX products in the response to PGD_2 (Paper III)

Possible modulation by COX products of the airway response to PGD_2 has not been investigated prior to this work. The bronchoconstrictive response to a challenge with a single dose of PGD_2 (30 nmol) was repeatable in the same preparation. Investigation of effects of COX inhibitors on bronchoconstriction induced by PGD_2 in IPL disclosed that significant enhancement of the

response to a second PGD_2 challenge was observed in the presence of the non-selective COX inhibitor ibuprofen, the selective COX-1 inhibitor FR122047 as well as the selective COX-2 inhibitor celecoxib (10 μM each) (Paper III), suggesting COX-dependent inhibitory modulation of the bronchoconstrictive response to PGD_2 in this model. It was thus hypothesised that PGD_2 preferentially released bronchoprotective prostanoids such as PGE_2 and PGI_2.

As the PGD_2-induced bronchoconstriction in IPL is mediated by TP receptors (Paper II and III), it was hypothesised that activation of the TP receptor by PGD_2 actively promoted release of relaxant prostanoids. Accordingly, the TP-dependent secondary release of relaxant prostanoids would constitute a modulatory mechanism similar but qualitatively opposite to the $CysLT_1$-dependent release of constrictive prostanoids (Folco et al., 1981; Mong et al., 1986; Wikström et al., 1992). This seemed to be a reasonable explanation of the observed enhancement of the response to PGD_2 by COX inhibitors. Therefore, it was attempted to assess if the PGD_2 challenge in IPL induced release of two potential bronchorelaxant prostanoids, PGE_2 and PGI_2, as well as that of TXA_2. However, initial experiments showed that the lung effluent after injection of bronchoconstrictive doses of PGD_2 contained amounts of this agonist that precluded measurements of COX products because of highly significant cross-reactivity in the enzyme immunoassays. The TX mimetic U-46619 that similarly to PGD_2 evokes bronchoconstriction in IPL by activation of TP receptors (Paper I) was therefore instead selected for this investigation. The compound U-46619 at the used doses did not interfere with the enzyme immunoassays.

However, when IPL was challenged with U-46619 (300 pmol), analysis of COX products in the lung effluent surprisingly disclosed that maximal bronchoconstriction produced by this agonist was not associated with increased release of relaxant prostanoids, such as PGE_2 and PGI_2, above the low baseline levels detected in the lung effluent. In addition, there was no release of TXA_2 in response to TP receptor activation by U-46619 (Paper III). Thus, despite the pharmacological effects of COX inhibitors on the response to PGD_2, these findings indicated that TP receptor activation by U-46619 did not stimulate the secondary release of relaxant prostanoids in IPL. In addition, the finding that the selective TX synthase inhibitor ozagrel (30 μM) had no effect on the bronchoconstriction evoked by PGD_2 in IPL (Paper II) supports that the actions of PGD_2 in this model did not involve release of secondarily formed TXA_2.

These findings gave rise to the hypothesis that the response to a bronchoconstrictive agonist that does not actively stimulate secondary release of relaxant prostanoids, may nevertheless be down-modulated by endogenous relaxant prostanoids, tonically produced in the tissue. Among

the relaxant prostanoids, both PGE_2 and PGI_2 are likely to down-modulate responses to bronchoconstrictive agonists by virtue of functional antagonism, and the bronchoprotective effect of exogenous PGE_2 in IPL was demonstrated in this work (Paper III). Accordingly, the enhancement of PGD_2 responses in the IPL by COX inhibitors might be due to removal of a functional antagonism exerted by the tonical biosynthesis of bronchorelaxant PGE_2 and/or PGI_2 in the airway smooth muscle.

As the high flow rate in the IPL precluded quantitative studies of how COX inhibitors affected the low basal levels of the prostanoids that were detected, we turned to the isolated guinea pig trachea (GPT) kept in a tissue bath under non-flow conditions. In the isolated airway smooth muscle under non-flow conditions, it was in fact established that neither PGD_2 nor U-46619 (10 μM each) actively promoted release of PGE_2, but that there was a continuous release of PGE_2 and PGI_2 from the airway smooth muscle. Moreover, as in the IPL under high flow conditions, the contraction response to PGD_2 in GPT was enhanced by COX inhibitors, such as indomethacin and flurbiprofen (10 μM each) (Paper III).

Obviously, this hypothesis needs to be further tested using other strategies, but these findings question the dogma that agonist-induced secondary release of COX products is a prerequisite for modulatory effects of COX products on the agonist response to occur.

Summary: Modulation of responses to bronchoconstrictive agonists by COX products

The bronchoconstrictive prostanoids presumably accounted for part of the response to histamine in IPL, as suggested by significant reduction of the histamine-induced bronchoconstriction by non-selective COX inhibition.

The secondarily released constrictive TXA_2 accounted for part of the bronchoconstrictive response to LTD_4 in IPL. First, the LTD_4-induced bronchoconstriction was significantly reduced by non-selective COX inhibition. Second, activation of the $CysLT_1$ receptor by challenge of the IPL with LTD_4 actively promoted release of TXA_2 into the lung effluent. Third, both the LTD_4-induced bronchoconstriction and the TXA_2 release were inhibited by TX synthase inhibition. However, the possible contribution of other constrictive prostanoids, such as PGD_2 and $PGF_{2\alpha}$ to the response to LTD_4 in IPL might also be supported by the current data.

Inhibition of the LTD_4-induced bronchoconstriction and the release of TXA_2 by selective COX-1 inhibition as well as selective COX-2 inhibition provided preliminary support for the

contribution of both the COX-1 and the COX-2 pathway in generation of prostanoids amplifying the response to LTD_4.

The response to LTD_4 in IPL had two components. The effect of LTD_4 in the presence of a COX inhibitor represented the direct $CysLT_1$ receptor-mediated action of this agonist on the airway smooth muscle, whereas the effect of LTD_4 in the absence of a COX inhibitor was a combination of the direct and indirect action, mediated by bronchoconstrictive COX products.

The bronchoconstrictive response to PGD_2 in IPL was enhanced by COX inhibition, but activation of the TP receptor by challenge with U-46619 did not actively promote release of relaxant prostanoids. It was hypothesised that the response to a bronchoconstrictive agonist that does not actively stimulate secondary release of relaxant prostanoids may nevertheless be down-modulated by endogenous relaxant prostanoids, tonically produced in the tissue. There was a continuous and solely time-dependent release of PGE_2 and PGI_2 in isolated guinea pig trachea challenged with PGD_2 and U-46619.

Taken together, the findings in this part of the thesis support that there are two different mechanisms by which COX products modulate responses to bronchoconstrictive agonists in IPL *(Figure 10)*.

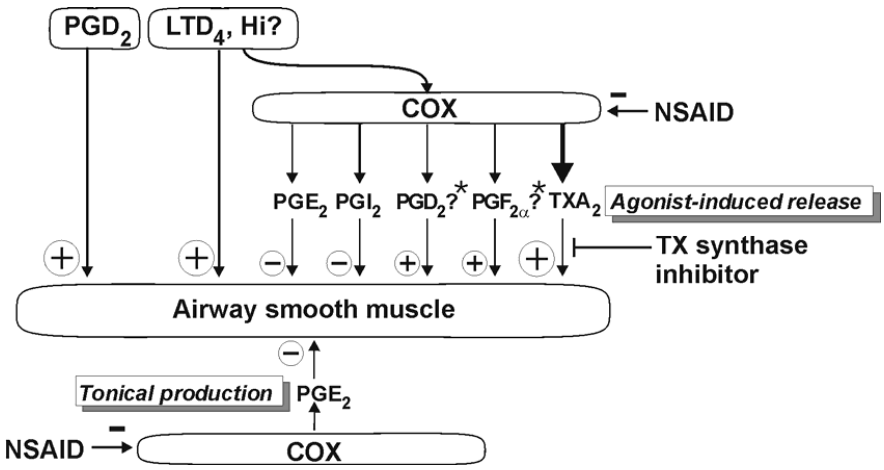

Figure 10. Hypothetical scheme of modulation of responses to bronchoconstrictive agonists in IPL by COX products. + designates enhancement and – designates inhibition; * Release of PGD_2 and $PGF_{2\alpha}$ by LTD_4 not established.

First, the findings with histamine and LTD_4 indicate that responses to these agonists are modulated by a common COX-dependent mechanism, namely the agonist-induced release of bronchoconstrictive COX products, possibly predominantly TXA_2, amplifying the responses. Although it was shown that LTD_4 released both the bronchoconstrictor TXA_2 and relaxant COX products such as PGE_2 and PGI_2, the overall effect of secondarily released COX products on the LTD_4 response was that of amplification, presumably because the greater amounts of the potent bronchoconstrictive TXA_2 dominated over the effects of relaxant COX products. Second, the findings with PGD_2 suggest that the biological activities of COX products presumably tonically formed in the airways determine the modulation of responses to bronchoconstrictive agonists that do not actively stimulate secondary release of COX products. In the guinea pig airways, it appears that the bronchoprotective action of endogenous relaxant prostanoids down-modulates responses to an agonist such as PGD_2 that does not cause receptor-activated release of COX products.

Antagonism of thromboxane receptor by diclofenac and lumiracoxib

Specific aims

Investigation of effects of COX inhibitors on bronchoconstriction induced by PGD_2 in IPL from non-sensitised animals (Paper III) disclosed that the non-selective COX inhibitor diclofenac (10 μM) had a profile of activity that differed from those of other COX inhibitors *(Figure 11)*.

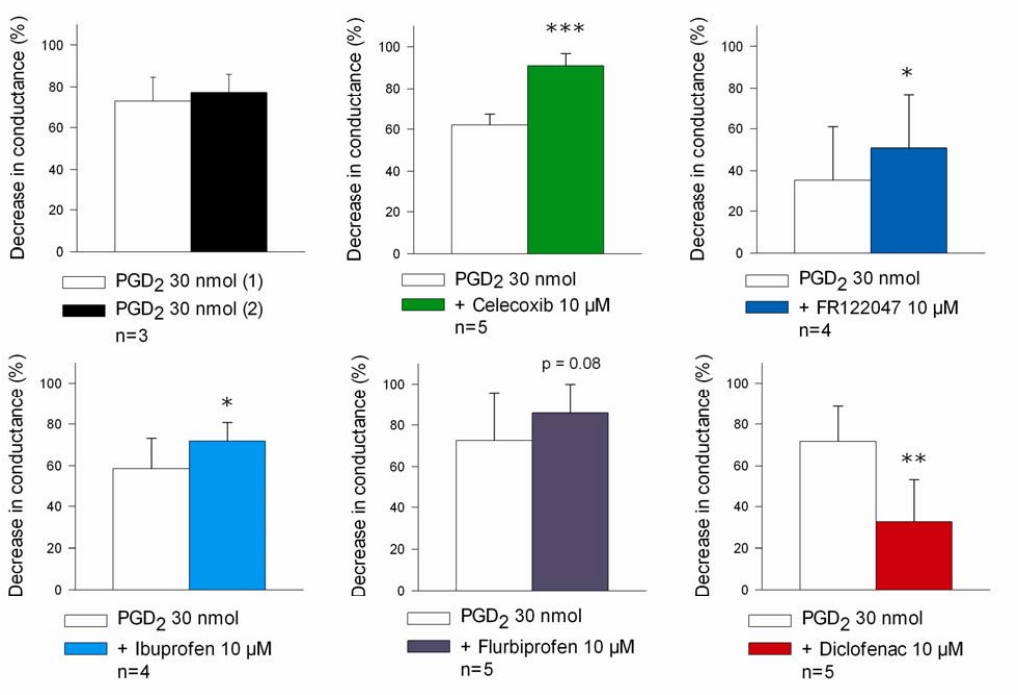

Figure 11. Effects of COX inhibitors on the peak decrease in conductance (mean ± SD) following the challenge with PGD_2 (30 nmol) in IPL; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (data from paper III).

Furthermore, it was observed that diclofenac (10 and 100 μM) inhibited the response to PGD_2 in a concentration-dependent manner (Paper II). In contrast, another non-selective COX inhibitor flurbiprofen (10 and 100 μM), had no significant effect on PGD_2-induced bronchoconstriction in IPL (Paper II). The response to PGD_2 in this model is mediated by activation of TP receptors, as demonstrated by total blockade of the response to PGD_2 by the selective TP receptor antagonist SQ 29,548 (Paper II). In contrast, the selective TX synthase inhibitor ozagrel (30 μM) had no

effect on the bronchoconstriction evoked by PGD_2 in IPL (Paper II). The latter observation excluded the possibility that the response to PGD_2 involved release of secondarily formed TXA_2, a mechanism that at least theoretically might have contributed to the TP activation by PGD_2.

Taken together, the findings in the IPL gave rise to the hypothesis that diclofenac might exert TP receptor antagonism. In this part of the thesis, effects of diclofenac and its structural analogue, lumiracoxib on functional responses due to activation of the TP receptor were further investigated in standard airway and vascular pharmacology *in vitro* models, isolated preparations of guinea pig trachea and guinea pig and rat aorta, respectively, as well as in human washed platelets. Furthermore, receptor binding and effects of diclofenac and lumiracoxib on activation of the TP receptor were studied in HEK293 cells transiently expressing human TP_α receptors.

Characterisation of the TP antagonistic property of diclofenac and lumiracoxib in isolated airway and vascular preparations of guinea pig and rat (Paper II)

In order to optimise the detection of a pharmacologic action of diclofenac and lumiracoxib distinct from COX inhibition, experiments in the guinea pig trachea were performed in a protocol where the endogenous COX was inhibited by pretreatment with flurbiprofen (10 μM). Under these conditions, diclofenac (1-100 μM) concentration-dependently inhibited the airway smooth-muscle contractions evoked by PGD_2, whereas flurbiprofen (1-100 μM) failed to inhibit the response to PGD_2, in line with the findings in the IPL. Nor did diclofenac (1-100 μM) inhibit airway smooth-muscle contractions induced by another agonist, LTD_4 (1pM-1 μM) (Paper II). Together, these findings supported that the inhibitory effect of diclofenac was selective for a constrictive agonist acting on TP receptors. In the same protocol, it was further examined in guinea pig trachea whether the inhibitory and COX-independent effect of diclofenac could be confirmed when airway smooth-muscle contractions were evoked by the selective TP receptor agonist U-46619. This was indeed the case; diclofenac concentration-dependently inhibited the airway smooth-muscle contractions evoked by U-46619 (Paper II).

On the basis of our findings with diclofenac, it was hypothesised that the potent selective COX-2 inhibitor lumiracoxib (Esser et al., 2005), which is a structural analogue of diclofenac, might share the TP receptor antagonism observed for diclofenac. Thus, the actions of lumiracoxib were examined in the guinea pig trachea protocol. Lumiracoxib (10-100 μM) was indeed found to inhibit the airway smooth-muscle contractions induced by U-46619 in a concentration-dependent fashion. In contrast, two other COX-2 inhibitors, celecoxib and rofecoxib (10 and 100

μM each) failed to inhibit airway smooth muscle contractions evoked by U-46619 (Paper II). At this stage, it was concluded that both diclofenac and lumiracoxib, in addition to being COX inhibitors, had the action to inhibit TP receptor dependent contractions of airway smooth muscle. In order to assess the relative contribution of the two different modes of action at different drug concentrations, we next studied the influence of lumiracoxib on U-46619-induced contractions in naïve guinea pig trachea preparations, that is, without the flurbiprofen pretreatment that had been used in the experiments that provided evidence for TP antagonism. Also in the naïve preparation, lumiracoxib (10-100 μM) concentration-dependently inhibited the response to U-46619. In the low concentration range (0.1-1 μM) lumiracoxib, however, only enhanced the contraction response to U-46619 (Paper II).

The actions of diclofenac and lumiracoxib to inhibit TP receptor dependent contractions were also documented in preparations of vascular smooth muscle (Paper II). Thus, in the guinea pig aorta, in a protocol where the preparation was pretreated with indomethacin (10 μM), diclofenac or lumiracoxib concentration-dependently inhibited the response to U-46619. In the aorta from another species, the rat, diclofenac (60 μM) also caused significant inhibition of vascular smooth-muscle contractions evoked by U-46619. Moreover, the antagonistic effect of diclofenac appeared readily reversible as the responsiveness to U-46619 in the rat aorta returned towards control level 15 min after drug removal by changing of media.

Characterisation of the TP antagonistic property of diclofenac amd lumiracoxib in human washed platelets (Paper II)

The TP antagonistic effects of diclofenac and lumiracoxib were confirmed in human washed platelets. Blood was collected in 1mM acetylsalicylic acid. This pretreatment made the platelets unresponsive to the challenge with arachidonic acid (1-3 μM), but they were fully responsive to the calcium ionophore A-23187 (3 μM) or thrombin (0.2 U ml^{-1}). When platelets were challenged with U-46619, a concentration-dependent platelet aggregation occurred, with an EC_{50} value around 60 nM (Paper II). This response was thus truly independent of endogenous TXA_2 formation.

Pretreatment with increasing concentrations (20-100 μM) of diclofenac or lumiracoxib inhibited the U-46619-induced platelet aggregation. Both drugs caused a rightward shift of the concentration-response curve for U-46619 (Paper II). In contrast, neither the selective COX-2 inhibitor, celecoxib, nor the non-selective COX inhibitor flurbiprofen inhibited the platelet

aggregation evoked by U-46619. In addition, pretreatment with lumiracoxib (60 μM) left thrombin- or A-23187-induced aggregation of human platelets unaffected (Paper II).

Although the antagonism of the U-46619-induced platelet aggregation exerted by diclofenac and lumiracoxib was classically surmountable, the strict pharmacologic analysis disclosed a complex interaction in some of the test systems (Schild plot slopes sometimes showed values being different from 1) (paper II), indicating that the effect of diclofenac and lumiracoxib cannot be defined as a pure competitive receptor antagonism. This can presumably be explained by the dual activity of these drugs.

Characterisation of whole cell binding and effects on TP receptor activation of diclofenac and lumiracoxib in HEK293 cells transiently expressing human TP_α receptor (Paper II)

The TP antagonistic effects of diclofenac and lumiracoxib were confirmed in radioligand binding studies in HEK293 cells labeled with [^{3}H]-SQ 29,548. Mixed type curves of [^{3}H]-SQ 29,548 and heterologous competition curves of diclofenac or lumiracoxib were monophasic, fitting a single-site model (Paper II). The data indicated typical binding parameters for the interaction of SQ 29,548 with the TP_α receptor, as previously reported (Capra et al., 2004). In agreement with the results obtained in airway and vascular preparations, as well as in the human platelets, both diclofenac and lumiracoxib were able to compete for the labeled antagonist, albeit with lower affinity than SQ 29,548. No detectable binding in mixed-type curve of [^{3}H]-SQ 29,548 was observed when cells were transfected with the empty vector (Paper II).

Signaling of TP_α receptor was also investigated by measuring the capacity of diclofenac and lumiracoxib to inhibit agonist-induced total inositol phosphates (IPs) production. As expected, HEK293 cells expressing human TP_α receptor responded to agonist stimulation (1 μM U-46619) with approximately a 3-fold increase of the total IPs production. Pretreatment with diclofenac or lumiracoxib inhibited U-46619-induced IPs production whereas ATP-stimulated IPs production was unaffected (Paper II), supporting the proposal that the effect of diclofenac and lumiracoxib was linked selectively to antagonism of the TP receptor.

Summary: Antagonism of thromboxane receptors by diclofenac and lumiracoxib

Diclofenac concentration-dependently and selectively inhibited the airway and vascular smooth-muscle contractions induced by TP receptor agonists such as PGD_2 and U-46619 in isolated preparations of guinea pig trachea and guinea pig and rat aorta, as well as the U-46619-induced aggregation of human washed platelets.

Lumiracoxib shared this activity profile, whereas a number of standard NSAIDs and other selective COX-2 inhibitors did not.

The receptor binding studies and investigation of effects of diclofenac and lumiracoxib on activation of the TP receptor in HEK293 cells transiently expressing human TP_α receptors confirmed the competitive antagonism of the TP receptor by these drugs.

It was thus discovered that the clinically used NSAIDs, diclofenac and lumiracoxib, in addition to being COX unselective and highly COX-2 selective inhibitors, respectively, displayed a previously unknown pharmacologic activity, namely TP receptor antagonism.

The TP antagonism may be an advantage with respect to the cardiovascular hazard of anti-inflammatory drugs different from aspirin, as the TP receptor mediates cardiovascular effects of TXA_2 and isoprostanes that are unopposed when the predominantly COX-2 dependent biosynthesis of PGI_2 (McAdam et al., 1999b) is inhibited by these drugs. The TP antagonism may also be an advantage with respect to anti-inflammatory and analgesic effects of NSAIDs, as the TP-mediated actions of TXA_2 and isoprostanes contribute to inflammation in different clinical settings. However, the TP antagonism has the greatest potential to improve the anti-inflammatory effect of coxibs, as these drugs do not affect biosynthesis of TXA_2 and PGD_2 along the COX-1 pathway.

The TP antagonistic potency of lumiracoxib and diclofenac, documented in this thesis, was however exerted at somewhat higher concentrations than those required for COX inhibition. It is therefore uncertain if the TP antagonism has clinical relevance with respect to the cardiovascular effect profile of these particular drugs, and the TP antagonistic property may not significantly improve the anti-inflammatory effect of diclofenac, as this drug primarily should inhibit the enzymatic formation of COX products along both the COX-1 and COX-2 pathway.

The implication of these findings is that diclofenac and lumiracoxib could represent prototypes of novel chemical entities with dual activity as selective COX-2 inhibitors and potent TP antagonists. Such compounds may give raise to new generation of coxibs that may have,

besides a better gastrointestinal tolerability than NSAIDs, also increased anti-inflammatory efficacy and, potentially, decreased adverse cardiovascular effects.

Mediators of the immediate antigen-induced bronchoconstriction

Specific aims

In this part of the thesis, a comprehensive study of the mediators involved in the immediate antigen (OVA)-induced bronchoconstriction was performed in the IPL from animals previously actively sensitised to OVA, in order to evaluate the relevance of this model in asthma pharmacology. The perfused guinea pig lung has been used extensively in pharmacology to discover release of biologically active principles, and in particular eicosanoids (Kellaway and Trethewie, 1940; Piper and Vane, 1969; Svensson et al., 1975). A literature review disclosed however that the mechanisms involved in the actions of antigen in the perfused guinea pig lung itself, has not received the same attention. The only previous investigation of mediators involved in the response to antigen in perfused guinea pig lung, used challenges with single bolus doses of antigen and provided evidence for a major role of histamine and weak indications of participation of leukotrienes in the response (Selig et al., 1993). In the same study, the COX inhibitor indomethacin paradoxically potentiated the antigen-induced bronchoconstriction, but inhibited release of TXA_2.

The effects of pharmacological intervention with three mediator classes, histamine, leukotrienes and COX products on antigen-induced bronchoconstriction were thus investigated in IPL by the use of specific antagonists and inhibitors. In addition to characterising the effects of interventions with each pathway alone, the effects of combining antagonists/inhibitors of two or all three classes of mediators were investigated in IPL. This has previously not been done although it is documented that interactions occur between mediators in asthmatic airways (Curzen et al., 1987; O'Sullivan et al., 1998; Roquet et al., 1997). In this investigation, challenge with cumulatively increasing doses of antigen was used in order to increase the repeatability of the antigen-induced bronchoconstriction and in addition increase the resolution of the model by allowing studies of shifts in the dose-response relations. The antigen-induced release of histamine, CysLTs and COX products into the lung effluent was also measured, in order to determine mediators involved and to provide a comprehensive understanding of drug effects.

The aim of this part of the thesis was also to explore the specific role of the two COX isoenzymes, COX-1 and COX-2 in the immediate airway response to antigen in IPL. The role of COX-1 and COX-2 in the antigen-induced generation of prostanoids has not been previously studied in this model. This is of interest with respect to the effects of NSAIDs and coxibs

(selective COX-2 inhibitors) in asthmatic airways. Therefore, the effects of selective inhibition of COX-1 and COX-2 on the antigen-induced bronchoconstriction and release of prostanoids were investigated in IPL.

Basal characterisatics of the response to antigen (Paper I and previously unpublished data)

Intravascular challenges with escalating doses of antigen (OVA) of lungs from animals previously actively sensitised to OVA produced a specific and dose-dependent bronchoconstriction (Paper I). This protocol for sensitisation and antigen challenge provides the guinea pig IPL model with a reproducible response to antigen *(Figure 12)*. The differences in the response to OVA between groups of animals sensitised to OVA at different occasions were similar to those observed within a group of animals sensitised to OVA at the same occasion, as documented for D_{50} (the OVA dose that causes 50% decrease in airway conductance) *(Table 3)*.

The onset and duration of the response to OVA in IPL was significantly slower, as compared with that documented for equiactive doses of histamine, U-46619 or LTD_4, producing about 50% decrease in lung conductance (Paper I).

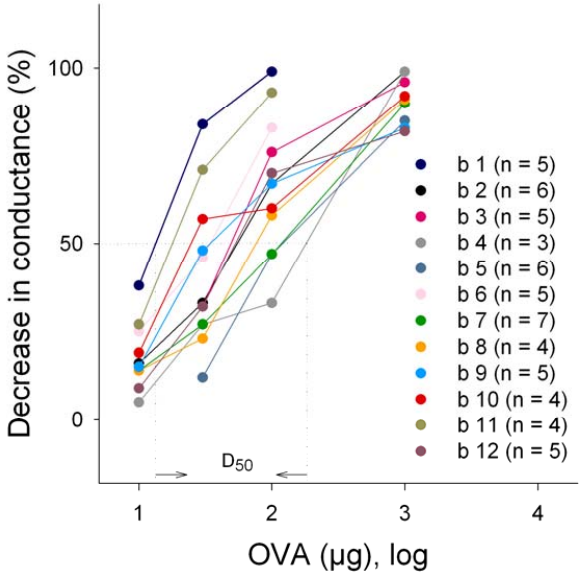

Figure 12. Dose-response relations for the peak decrease in conductance (mean ± SD) following the challenge with escalating doses of antigen (OVA) in the different groups of lungs from guinea pigs, sensitised to OVA at different occasions (previously unpublished data).

batch	OVA D_{50} (µg)			
	median	min	max	n
1	17	10	23	5
10	22	10	538	4
11	23	10	30	4
3	26	17	140	5
6	30	12	65	5
9	45	17	306	5
12	61	30	502	5
2	62	30	321	6
8	87	56	361	4
5	96	59	513	6
7	99	80	289	7
4	458[a)]	25	488	3

[a)] Shifted by 27-fold, as compared with batch 1

Table 3. Doses of antigen (OVA) producing 50% decrease in airway conductance (D_{50}) in the different groups of lungs from guinea pigs, sensitised to antigen at different occasions (previously unpublished data); n, number of subjects.

Contributions of histamine, cysteinyl-leukotrienes and COX products to the immediate antigen-induced bronchoconstriction (Paper I and IV)

Pretreatment with mepyramine (1 µM) caused a significant shift to the right of the dose-response relationship for the bronchoconstrictive response to the challenge with escalating doses of OVA (Paper I). The antigen-induced bronchoconstriction in IPL thus had a histamine component, and the H_1 receptor was mediating the response. Furthermore, pretreatment with the combination of mepyramine and metiamide (1 µM each) did not show a significantly different effect on the response to OVA, as compared to that of mepyramine alone (Paper I). The finding that combined H_1 and H_2 antagonism had the same effect on the antigen-induced bronchoconstriction in IPL as H_1 antagonism alone was in line with previous observations in anaesthetised animals (Advenier et al., 1979).

Significant shift to the right of the dose-response relationship for the response to OVA in IPL was also observed after pretreatment with zafirlukast (0.1 µM) (Paper I), supporting that the $CysLT_1$ receptor-mediated effect of cysteinyl-leukotrienes contributed to the antigen-induced bronchoconstriction in this model as well.

Pretreatment with the non-selective COX inhibitor diclofenac (10 µM) caused significant shift to the right of the dose-response relationship for the response to OVA (Paper I and IV). The clear-cut inhibitory effect on the OVA-induced bronchoconstriction was also produced by another non-selective COX inhibitor, flurbiprofen (10 µM) (Paper IV). A distinct inhibitory effect of the non-selective COX inhibitors on the response to OVA in IPL indicated thus that the third class of mediators was apparently bronchoconstrictive COX products.

Histamine, LTC_4, TXA_2 (measured as immunoreactive TXB_2) and PGD_2 were indeed released in response to challenges of the IPL with three different doses of OVA (100, 1 000 or 10 000 μg) (Paper IV). However, the amounts of histamine, LTC_4, TXA_2 and PGD_2, detected in the lung effluent after the challenge with 100 and 1 000 μg of OVA, were not significantly different, whereas 10 000 μg of OVA produced a substantially greater release of all mediators (Paper IV). Furthermore, as indicated by the relative ratios between the total amount of LTC_4 and that of histamine, TXA_2 and PGD_2, released in response to the challenges with the three different OVA doses (Paper IV), there was a trend that relatively greater amounts of histamine and TXA_2, as compared to those of LTC_4 and PGD_2, were released by the highest dose of OVA (10 000 μg), in line with previous findings (Turner and Dollery, 1988). It is possible that, in addition to mast cells, also airway smooth muscle contributed to the release of COX products detected in the lung effluent.

The extended analysis of COX products released in response to the challenge with the most effective OVA dose (10 000 μg) revealed that in addition to TXA_2 and PGD_2, also $PGF_{2\alpha}$ was released, and release of potentially inhibitory and bronchorelaxant COX products, such as PGI_2 (measured as immunoreactive 6-keto $PGF_{1\alpha}$) and PGE_2, was also detected (Paper IV). Furthermore, it was established that release of all COX products, TXA_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and PGE_2, was abolished in the presence of the non-selective COX inhibitor flurbiprofen (10 μM) (Paper IV). TXA_2 was the most abundant prostanoid released by the antigen challenge in IPL, followed by PGI_2, whereas PGD_2, $PGF_{2\alpha}$ and PGE_2 were released in much lower amounts than those for TXA_2 and PGI_2 (Paper IV). Similarly, in isolated human bronchi challenged with antigen, TXA_2 and PGI_2 were the predominant COX products identified, but PGD_2, $PGF_{2\alpha}$ and PGE_2 were also released (Dahlén et al., 1983a). Other investigators also reported the antigen-induced release of TXA_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and PGE_2 in isolated human bronchi, but PGI_2 and PGE_2 were quantitatively predominant and the preparation had the relatively less capacity to generate TXA_2, PGD_2, $PGF_{2\alpha}$, (Schulman et al., 1982). In antigen-challenged isolated human lung parenchyma, PGD_2 and PGI_2 were quantitatively predominant, whereas TXA_2, $PGF_{2\alpha}$ and PGE_2 were released in similar quantities that were 3-7-fold lower than those for PGD_2 and PGI_2 (Schulman et al., 1981).

Role of COX-1 and COX-2 in the immediate antigen-induced generation of COX products (Paper IV)

In contrast to the significant inhibition of the OVA-induced bronchoconstriction in IPL by the non-selective COX inhibitors (Paper I and IV), neither the selective COX-1 inhibitor FR122047 nor the selective COX-2 inhibitor celecoxib (10 μM each) had significant inhibitory effects on the response to OVA (Paper IV). Moreover, investigation of the respective role of COX-1 and COX-2 as a source for the OVA-induced release of COX products disclosed that the release of PGD_2 was abolished by FR122047 as well as by celecoxib (10 μM each), but neither drug had significant effects on the release of the other COX products (Paper IV) *(Figure 13)*.

	Control	COX-1 inhibition	COX-2 inhibition	COX-1/COX-2 inhibition
PGH_2	+ + +	+	+	–
PGD_2	+	–	–	–
PGE_2	+	+	+	–
$PGF_{2\alpha}$	+	+	+	–
PGI_2	+ +	+ +	+ +	–
TXA_2	+ + +	+ + +	+ + +	–

Figure 13. Semiquantitative (number of plus) attempt to explain the effects of COX inhibition on the immediate antigen-induced generation of prostanoids in IPL. In control, both COX isoenzymes provide the substrate (PGH_2) to the terminal PG synthases. Treatment with either the selective COX-1 inhibitor or the selective COX-2 inhibitor results in diminished formation of PGH_2. Due to dependency on higher PGH_2 level for H-PGDS (Pinzar et al., 2000; Inoue et al., 2003), the selective inhibitors will inhibit PGD_2, but not other prostanoids.

These findings were interpreted as preliminary support for the contribution of both COX-1 and COX-2 to the immediate antigen-induced generation of prostanoids in IPL. These data are thus at variance with those obtained in cellular *in vitro* models, where the immediate generation of prostanoids utilised COX-1, whereas the delayed prostanoid biosynthesis, induced by pro-inflammatory stimuli, utilised COX-2 (Brock et al., 1999; Kawata et al., 1995; Naraba et al., 1998; Reddy et al., 1999). One possible implication of these findings may accordingly be that PGH_2 is used as a substrate by most terminal prostaglandin synthases irrespective of whether generated in reaction initiated by COX-1 or COX-2 *(Figure 13)*, at variance with the proposed selective functional linkage between the two COX isoenzymes and the different terminal prostaglandin synthases (Ueno et al., 2001).

The finding that only release of PGD_2 was abolished by selective COX-1 and COX-2 inhibition (Paper IV) was surprising. However, the substrate affinity of the hematopoietic prostaglandin D synthase (H-PGDS) (Inoue et al., 2003; Pinzar et al., 2000) was one order of magnitude lower, as compared to that of the thromboxane synthase (Hecker and Ullrich, 1989), the cytosolic lung-type prostaglandin F synthase (Suzuki et al., 1999), the cytosolic prostaglandin E synthase (Tanioka et al., 2000) and the prostacyclin synthase (Yeh et al., 2005). It is thus possible that in IPL, the amount of PGH_2 that was available under conditions when one of the two COX isoenzymes was inhibited was not sufficient for generation of PGD_2 by H-PGDS *(Figure 13)*.

Role of specific COX products in the immediate antigen-induced bronchoconstriction (Paper I, III and IV)

It was established that TXA_2 was the most abundant prostanoid released by the antigen challenge in IPL (Paper IV), and the stable TXA_2 mimetic U-46619 was on a molar basis 100-fold more potent bronchoconstrictor than histamine in this model (Paper I) *(Table 1)*. It is thus likely that the COX-dependent component in the antigen-induced bronchoconstriction in IPL was predominantly mediated by TXA_2. This is further supported by the finding that the selective COX-1 or COX-2 inhibitor that abolished PGD_2 release had no effect on the antigen-induced bronchoconstriction (Paper IV). This shows that the action of TXA_2, together with that of $PGF_{2\alpha}$, sufficiently contributed to the airway obstruction. Significant contribution of $PGF_{2\alpha}$ to the antigen-induced bronchoconstriction in this model is however not very likely. It was thus disclosed that much lower amounts of $PGF_{2\alpha}$ were released by the antigen challenge, as compared to those of TXA_2 (Paper IV), and it was previously shown that the bronchoconstrictor potency of $PGF_{2\alpha}$ on a molar basis was 10-fold lower, as compared to that of histamine in this model (Paper III) *(Table 1)*. In addition, as $PGF_{2\alpha}$ is able to exert the bronchoconstrictive effect in airways also by activation of the prostanoid FP receptor (Narumiya et al., 1999), theoretically it is possible that the FP receptor-mediated effect of this prostanoid contribute to the antigen-induced airway obstruction as well. Evaluation of the role of this component is however not possible because to date sufficiently selective FP receptor antagonists are lacking.

The finding that neither selective COX-1 nor selective COX-2 inhibitor reduced the airway obstruction despite suppression of PGD_2 release (Paper IV), suggests also a minor role of this prostanoid in the antigen-induced bronchoconstriction in this model. This is further supported by the finding that PGD_2 was released by the antigen challenge in much lower

amounts, as compared to those of TXA_2 (Paper IV) and that the bronchoconstrictor potency of PGD_2 was on a molar basis 3-fold lower than that of histamine in this model (Paper III) *(Table 1)*. The specific role of PGD_2 remains to be established by selective inhibition of biosynthesis of this prostanoid.

Taken together, these findings nevertheless suggest the main role of TXA_2 and the minor role of $PGF_{2\alpha}$ and PGD_2 as mediators of the COX-dependent component of the antigen-induced bronchoconstriction in IPL.

The findings in this thesis suggest also a predominant role of the constrictive prostanoids and a minor role of the relaxant ones in the immediate antigen-induced bronchoconstriction in IPL, as the observed overall effects of COX inhibitors on the airway obstruction in this model were apparently dependent only on the effects of these drugs on release of the constrictive prostanoids. The non-selective COX inhibition that abolished release of the constrictive TXA_2, PGD_2 and $PGF_{2\alpha}$, was thus associated with significant reduction of the airway obstruction, in spite of the abolished release also of the bronchoprotective PGE_2 and PGI_2 by this treatment (Paper IV). The selective COX-1 and COX-2 inhibition, either of which abolished the release of PGD_2, but did not significantly affect that of PGI_2 and PGE_2 was not associated with reduction of the airway obstruction (Paper IV). The bronchoprotective actions of PGE_2 and probably also PGI_2 were thus not sufficient to counteract the bronchoconstrictive effects of TXA_2 and $PGF_{2\alpha}$.

Interactions among histamine, cysteinyl-leukotrienes and COX products (Paper I, IV and previously unpublished data)

The combination of zafirlukast (0.1 μM) with mepyramine (1 μM) caused even more pronounced and highly significant inhibition of the OVA-induced bronchoconstriction in IPL, as compared with the effects of either drug alone (Paper I). In the presence of this combination of drugs, the preparation tolerated considerably higher doses of OVA and there was substantial reduction of the response to the highest dose of OVA. This finding shows that histamine and cysteinyl-leukotrienes additively contributed to the immediate antigen-induced bronchoconstriction in this model. In line with this finding, the combination of mepyramine (1 μM), zafirlukast (0.1 μM) and diclofenac (10 μM), in order to target all three mediator classes, produced a remarkable inhibition of the OVA-induced bronchoconstriction in IPL, where the preparation tolerated four log orders of increase in the OVA dose without any response to the antigen (Paper I). This provides evidence that when the effects of histamine and cysteinyl-leukotrienes are blocked in the

presence of a potent COX inhibitor, there are no other important mediators of the antigen-induced bronchoconstriction to consider in this model.

Similarly to the findings in the IPL, histamine, CysLTs and COX products contributed additively to antigen-induced contractions in preparations of guinea pig peripheral airways *in vitro* (Ressmeyer et al., 2006) as well as in isolated trachea from this species (Lindström et al., 1992; Martin and Broadley, 2002), whereas in isolated guinea pig lung parenchyma, the response to antigen was mediated by the combined actions of histamine and CysLTs, but COX products played no role in this particular model (Wikström-Jonsson and Dahlén, 1994). Also in isolated human bronchi, a model of human central airways, the IgE-dependent contractions were mediated predominantly by the combined actions of histamine and CysLTs (Björck and Dahlén, 1993; Dahlén et al., 1983a), whereas the significance of COX products was not supported (Björck and Dahlén, 1993; Dahlén et al., 1983a; Norel et al., 1991). However, in preparations of human peripheral airways *in vitro*, the antigen-induced airway contractions were mediated by a synergistic effect of TP receptor agonists and $CysLT_1$ receptor agonists, whereas histamine played no role (Wohlsen et al., 2003). In contrast, in rat airways, histamine, CysLTs and prostanoids play no role in the antigen-induced contractions, and rat airways do not respond with significant contraction to these mediators, whereas serotonin is the major mast cell mediator (Wohlsen et al., 2001).

Compared to control, significant shift to the right of the dose-response relationship for the challenge with OVA was also achieved by the combination of zafirlukast (0.1 μM) and diclofenac (10 μM), inhibiting cysteinyl-leukotrienes and COX products. The effect of this combination of drugs was however not greater than the effects of either drug alone (Paper I). The expected additive effect was thus not observed. Similarly, in preparations of guinea pig peripheral airways *in vitro*, the effect of a combination of an antileukotriene with a TP antagonist on the antigen-induced contractions was less pronouced than that observed when either of these drugs was combined with an antihistamine (Ressmeyer et al., 2006).

However, COX products, such as PGE_2 (Hedqvist et al., 1989; Peters et al., 1982; Raud et al., 1988; 1989) and PGI_2 (Engineer et al., 1978a) may also regulate release of histamine and leukotrienes by feedback inhibition on mast cells. The release of histamine and leukotrienes is thus augmented by COX inhibitors, due to removal of such an inhibitory mechanism (Adams and Lichtenstein, 1985; Engineer et al., 1978b). In fact, the previous observations that the antigen response in the perfused guinea pig lung (Selig et al., 1993) as well as trachea from this

species (Rydberg and Andersson, 1994) was enhanced by indomethacin, may be in line with this proposal. As further support, in isolated guinea pig lung parenchyma, indomethacin enhanced the contraction response to antigen, whereas exogenous PGE_2 shifted the dose-response relations for the antigen challenge to the right and reversed the enhancement caused by indomethacin (Larsson et al., 2005).

It is thus possible that in IPL, removal of PGE_2 inhibition on mast cells by diclofenac may be relatively more prominent effect of this drug under conditions when zafirlukast has blocked both the direct $CysLT_1$-mediated bronchoconstrictive effect of CysLTs, and its component that is due to the secondary release of bronchoconstrictive COX products in the tissue. It is therefore conceivable that under these conditions, decreased release of bronchoconstrictive COX products from mast cells by diclofenac may have little overall effect on the antigen response. Thus, the failure to achieve additive inhibition of the response to OVA in IPL by combining diclofenac and zafirlukast might be explained by enhanced release of histamine and leukotrienes from mast cells by diclofenac. This speculation receives some support from the unexpectedly large inhibition of the OVA response that was observed when the antihistamine mepyramine (1 μM) was added to preparations given diclofenac (10 μM) (Paper I). Further experiments involving direct measurements of histamine, but also leukotriene B_4 (LTB_4) are needed. The influence of LTB_4 on the IPL has never been assessed, but it is generally supposed to be a pro-inflammatory mediator (Samuelsson, 1982).

Pretreatment with the TP receptor antagonist BAY u3405 (1 μM) given alone had no effect on the OVA-induced bronchoconstriction in IPL (Paper I), in contrast to the significant inhibitory effects achieved by the non-selective COX inhibitors given as a single treatment (Paper I and IV). In this context, it should be recognised that non-selective COX inhibitors inhibit both the direct antigen-induced release of COX products from mast cells and the secondary agonist-induced release of constrictive COX products in the tissue amplifying the responses to histamine and CysLTs, whereas BAY u3405 inhibits only the TP-mediated effects of the COX products released. However, the combination of BAY u3405 (1 μM), mepyramine (1 μM) and zafirlukast (0.1 μM) caused a potent inhibition of the response to OVA, and the effect of this combination of drugs was more pronounced, as compared to that achieved by the combination of mepyramine (1 μM) and zafirlukast (0.1 μM) (Paper I). Thus, there was a significant additive effect when BAY u3405 was given to preparations already treated with these antagonists. This finding unravelled

the component of the response to antigen that was solely due to the TP-mediated effects of COX products such as TXA_2, PGD_2 and $PGF_{2\alpha}$ released from mast cells, in the situation when it could be expected that the secondary receptor-activated release of COX products by histamine and CysLTs in the tissue was blocked by mepyramine and zafirlukast, respectively.

Another intriguing observation was that pretreatment with the 5-lipoxygenase (5-LO) inhibitor BAY x1005 (30 μM), a potent inhibitor of leukotriene biosynthesis, had no effect on the antigen-induced bronchoconstriction when given alone (Paper I). This was at variance with the effect of the selective $CysLT_1$ receptor antagonist that given alone inhibited the antigen response in IPL. Also in studies of the EAR, conflicting observations have been made with respect to the effect of 5-LO inhibition, sometimes profound inhibition (Dahlén et al., 1997) and sometimes marginal effect (Hui et al., 1991). However, the combination of BAY x1005 (30μM) with antihistamines (mepyramine and metiamide, 1μM each) caused a major inhibition of the response to OVA in IPL (Paper I). In fact, the effect of this combination of drugs was much more pronounced, as compared to that produced by the antihistamines alone. These findings gave rise to the hypothesis that lack of effect of 5-LO inhibition on the antigen response in IPL was due to increased release of histamine and prostanoids by this treatment.

Investigation of effect of 5-LO inhibition with BAY x1005 (30 μM) on the antigen-induced mediator release in IPL documented that this treatment abolished the release of LTC_4 *(Figure 14)*. Interestingly however, BAY x1005 (30 μM) significantly enhanced the release of PGD_2 and tended to enhance ($p = 0.1$) that of histamine, whereas this treatment had no effect on the release of TXA_2 *(Figure 14)*. Thus, lack of effect of BAY x1005 on the antigen-induced bronchoconstriction in IPL can be explained by the augmented airway effects of histamine and PGD_2 that masked the inhibitory effect of BAY x1005 on the response to antigen due to abolished biosynthesis of the bronchoconstrictive CysLTs by this drug. However, a consequence of an overall 5-LO inhibition caused by BAY x1005 may be also suppression of the formation of lipoxygenase interaction products such as lipoxins. The latter compounds are known to have many important anti-inflammatory effects in other models of inflammation (Brink et al., 2003), and their removal could therefore be expected to produce an exaggerated mast-cell response, similarly to that caused by inhibition of PGE_2 formation due to COX inhibition. Irrespective of the final explanation for the lack of effect of BAY x1005 alone, the combination of BAY x1005 with the antihistamines mepyramine and metiamide again provided striking inhibition of the

antigen response. This lends strong support to the concept that CysLTs and histamine mediate a major part of the immediate antigen-induced bronchoconstriction in the IPL.

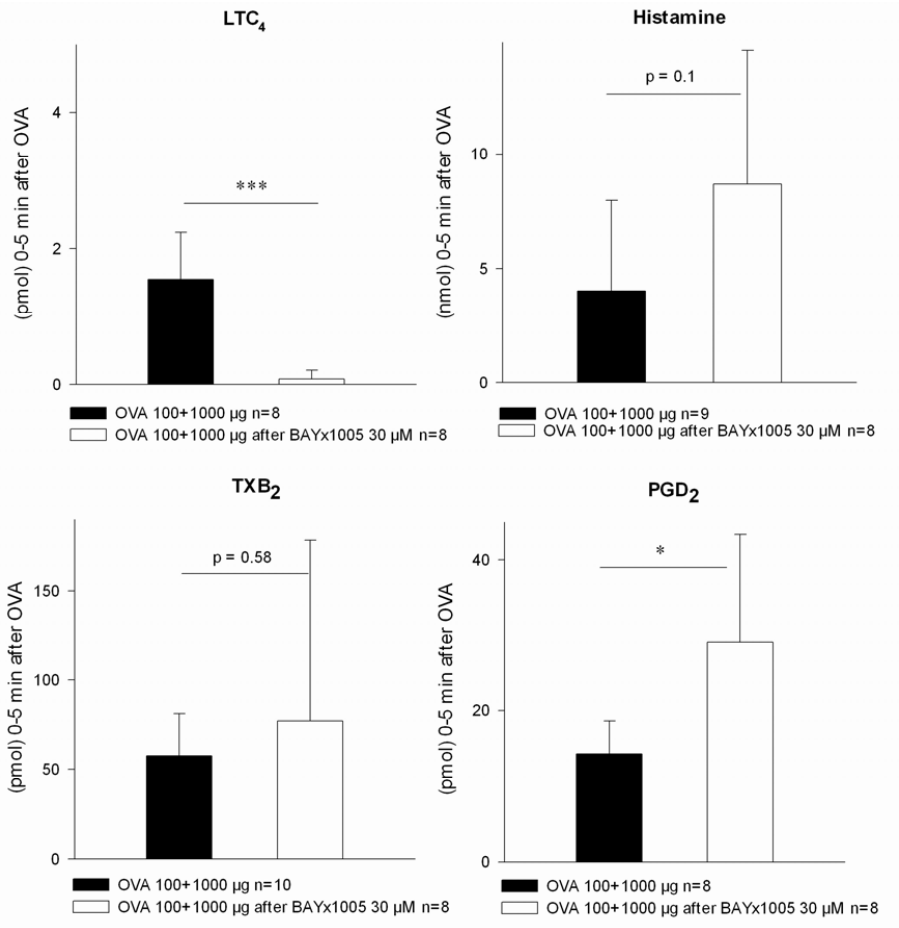

Figure 14. The total amounts (mean ± SD) of LTC_4, histamine**,** TXA_2 (measured as TXB_2) and PGD_2 released during 5 min after the challenge of the lungs from actively sensitised guinea pigs with a single dose of antigen (OVA) in control or after pretreatment with the 5-LO inhibitor BAYx1005 (30 µM). The total amounts of mediators were calculated as area under the curve (AUC): amount in the lung effluent (detected by enzyme immunoassay) vs. time (0-5 minutes after OVA challenge) and the baseline values 5 min before the challenge were subtracted from the AUC. As the amounts of mediators detected in the initial experiments after the control challenge with 100 and 1 000 µg of OVA did not differ significantly (see Figure 1 and Figure 2 Paper IV)), the data obtained with these two OVA doses were pooled.*, $p < 0.05$; ***, $p < 0.001$ (previously unpublished data).

Summary: Mediators of the immediate antigen-induced bronchoconstriction

It was documented that the guinea pig IPL provided a model where a dose-dependent, reproducible and specific response was obtained with intravascular challenge with antigen (OVA) in lungs from animals previously actively sensitised to OVA.

The inhibition of single classes of mediators in IPL produced straightforward results. Three different selective interventions with an antihistamine, an antileukotriene and a COX inhibitor produced about the same 1-1.5 log dose shifts in the dose-response relations for the challenge with escalating doses of OVA, indicating that the antigen-induced bronchoconstriction in this model had a histamine, CysLT and prostanoid component. Moreover, release of histamine, LTC_4 and COX products, such as TXA_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and PGE_2, in response to the OVA challenge was established.

The results of selective inhibition of COX-1 and COX-2 suggested that both COX-1 and COX-2 pathways were involved in the immediate antigen-induced generation of the specific prostanoid end products.

The results of the inhibition of two or three classes of mediators at a time indicated that there were several levels of interactions among the mediators and that these complex interactions may be overlooked when intervention with single classes of antagonists or inhibitors are administered.

Taken together, these findings demonstrate that the antigen-induced bronchoconstriction in IPL is mediated by the concerted actions of histamine, cysteinyl-leukotrienes and several COX products. The pharmacology of the antigen response in this particular preparation is thus closely similar to that of antigen-induced contractions in preparations of human small airways and guinea pig peripheral airways *in vitro*.

Methodological aspects

Effect of salbutamol on the response to histamine (previously unpublished data)

Postmortem bronchoconstriction is a well-known phenomenon in guinea pig lungs but not in perfused lungs of other species (Lai et al., 1984a; Lai et al., 1984b). As previously established, addition of a very low dose of the β_2 receptor agonist salbutamol (33 nM) to the perfusion medium helps to combat this effect and make the isolated perfused and ventilated guinea pig lung preparation viable and responsive to bronchoconstrictive agents for several hours (Atzori et al., 1992) (see also Methods). As it was reported that the presence of this low dose of salbutamol shifted the dose-response relations for bronchoconstrictors slightly to the right (Atzori et al., 1992), it was of great interest to establish the influence of salbutamol on the bronchoconstrictive responses to agonists in this thesis.

The rightward shift of the dose-response relations for the effects of the challenge with histamine in IPL followed increase of the salbutamol concentrations (10-100 nM) *(Figure 15)*. As documented for D_{50} (the agonist dose causing 50% reduction in airway conductance), 3-fold increase in salbutamol concentrations (10-100 nM) resulted in 2-fold increase in histamine D_{50} values *(Table 4)*. Neither the further rightward shift of the histamine dose-response curve nor increase of D_{50} was produced by the highest salbutamol concentration (300 nM), as compared with the effect of 100 nM salbutamol. Moreover, near-maximal bronchoconstriction could still be obtained in the presence of the highest salbutamol concentration (300 nM) *(Figure 15 and Table 4)*.

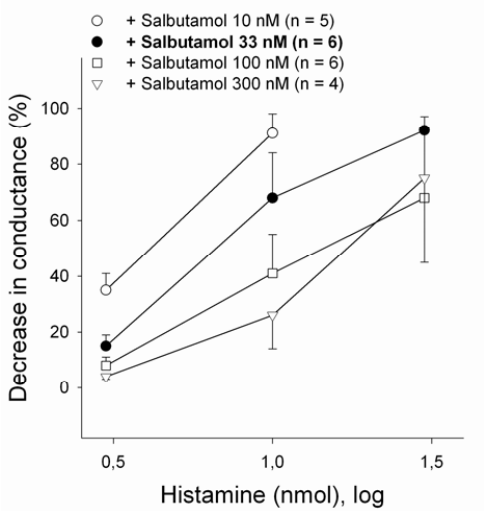

Figure 15. Dose-response relations for the peak decrease in conductance (mean ± SD) following the challenge of the IPL with escalating doses of histamine in the presence of salbutamol at different concentrations (previously unpublished data).

Treatment	Histamine D_{50} (nmol)		
	mean	sd	n
10 nM salbutamol	5	1	5
33 nM salbutamol	8[a)]	1	6
100 nM salbutamol	16[b)]	8	6
300 nM salbutamol	16	2	3

Table 4. Doses of histamine producing 50% decrease in airway conductance (D_{50}) in the presence of salbutamol at different concentrations; n, number of subjects.

[a)] p=0.003 (Student's unpaired two-tailed test) vs. 10 nM
[b)] p=0.03 (Student's unpaired two-tailed test) vs. 33 nM

These findings show clearly that the preparation remains responsive to histamine in the presence of the stablilising concentration of salbutamol (33 nM), applied in all experiments in this thesis. Moreover, in the presence of salbutamol, there was also a trend for enhanced response to a second injection of the TP receptor agonist U-46619 in the guinea pig IPL model (Paper I). This is similar to the finding in the study in the murine IPL model (Held et al., 1999), and suggests that the basic properties of the guinea pig IPL system are intact also in the presence of this dose of salbutamol. Furthermore, as documented extensively in this thesis, the preparation was responsive to other agonists of bronchoconstriction, and maximal bronchoconstrictive effects could be obtained. The findings strongly support the conclusion that the protective effect of salbutamol in IPL predominantly relates to an action other than bronchodilation. There is evidence supporting the role of tachykinins in the development of the severe bronchospasm in the postmortem guinea pig lungs (see Methods page 18). In contrast to other species, the airway smooth muscle in guinea pig has a pronounced non-adrenergic non-cholinergic innervation (Andersson and Grundstrom, 1987) and substance P induces vascular permeability changes, in addition to causing smooth muscle contractions (Lundberg et al., 1984; Lundberg et al., 1983). It was shown in guinea pigs *in vivo* that β_2-adrenoceptor agonists had the antiexudative effect, that is, inhibited the bradykinin- and histamine-induced (Advenier et al., 1992; Erjefält and Persson, 1991b) and capsaicin-induced (Erjefält and Persson, 1991b) airway microvascular leakage and plasma exudation. As the antiexudative effect of β_2-adrenoceptor agonist was independent on the reduction of the mucosal blood flow, and was exerted on both the neural and non-neural exudative response, the direct effect on the permeability-regulating microvascular endothelial cells was proposed (Erjefält and Persson, 1991b). The mechanisms of the protective effect of salbutamol on the postmortem bronchoconstriction in IPL may thus to a large extent depend on the antiexudative effect, possibly in combination with inhibition of the release of tachykinins from airway sensory nerves (Ten Berge et al., 1995; Verleden et al., 1993).

Previous findings in guinea pigs *in vivo* (Erjefält and Persson, 1991a) suggest that the antiexudative effect of salbutamol may also inhibit the antigen-induced plasma leakage in IPL. Another effect of salbutamol with potential relevance in the antigen-challenged IPL is inhibition of the immunologic mediator release from mast cells (Butchers et al., 1991; Church and Hiroi, 1987). However, the concentration range for this effect of β_2-adrenoceptor agonists is the same as that which relaxes the airway smooth muscle (Butchers et al., 1991), and it was shown that salbutamol (10-100 nM) inhibited the IgE-dependent histamine release by 0-20% in the cultured human mast cells (Chong et al., 2003) Thus, a role of the mast cell-stabilising effect of salbutamol in IPL is not very likely.

Comparison of the response to LTD_4 in non-sensitised and sensitised lungs (Paper I)

As it may be speculated that the sensitised state by itself changes airway reactivity to the mediators of bronchoconstriction, much as asthmatics suffer from bronchial hyper-responsiveness to histamine (Hargreave et al., 1981) and LTD_4 (Smith et al., 1985), this thesis involved also an assessment whether or not the active sensitisation changed airway reactivity to LTD_4. This assessment was also considered important, as marked changes in effector cell reactivity could have implications for the dose level of antagonists to be used. It has in fact not been possible to find other publications that have made this direct comparison of mediator reactivity between sensitised and non-sensitised animals.

However, it was documented that the sensitivity to LTD_4 in the IPL was unaltered by the OVA sensitisation. Lungs from non-sensitised but weight-matched littermates had dose-response relations for LTD_4 that were super imposable on those from the sensitised animals (Paper I). The finding indicates that it is not the passive state of being sensitised with an allergen *per se* that changes airway reactivity. Airway hyper-responsiveness in allergic individuals is rather the result of repeated allergen stimulation that occurs naturally by exposure to specific allergen (Ihre and Zetterström, 1993). The animals in this study were not exposed to OVA in the interval between sensitisation and the challenge with OVA in our experiments.

General discussion

Several levels of interactions among mediators of the immediate antigen-induced bronchoconstriction

In the IPL, interactions among the mediators of the antigen-induced bronchoconstriction occur at several levels *(Figure 16)*. First, histamine, cysteinyl-leukotrienes and COX products, released presumably from mast cells as a primary effect of the antigen-antibody reaction, cause bronchoconstriction directly by activation of their respective specific receptors on the airway smooth muscle. In addition, there may be direct interactions between released histamine and cysteinyl-leukotrienes on the airway smooth muscle level, as suggested by some studies (Jacques et al., 1991; Lee et al., 1984). Interactions among mediators can also occur at the receptor level. Although different GPCRs function through distinct signal transduction pathways, a "cross-talk" occurs between pathways and agonist-GPCR interactions can influence the cellular response to another agonist acting on different GPCR (Franco et al., 2007; Kelley-Hickie and Kinsella, 2004; Rozengurt, 2007; Walsh and Kinsella, 2000). The phenomenon of synergism is one example of "cross-talk" between pathways. In fact, synergistic interactions between the TP receptor agonists and the $CysLT_1$ receptor agonists in the anaphylactic contractions were described in human peripheral airway preparations *in vitro* (Wohlsen et al., 2003).

Second, the release of COX products is promoted in IPL by LTD_4, and presumably also by histamine, due to activation of their specific receptors by these agonists. As documented for LTD_4, the bronchoconstrictive COX products predominate quantitatively. The effect of the secondarily released COX products on the responses to histamine and cysteinyl-leukotrienes in IPL is thus that of amplification. The interactions between histamine or LTD_4 and COX products implicate that these mediators may synergise on downstream mechanisms at the effector cell level.

Finally, prostanoids (Feng et al., 2005; Raud et al., 1988), histamine (Bourne et al., 1974; Lichtenstein and Gillespie, 1975) and CysLTs (Sjöström et al., 2002) may also exert feedback control on the release of bronchoconstrictive mediators from the inflammatory cells.

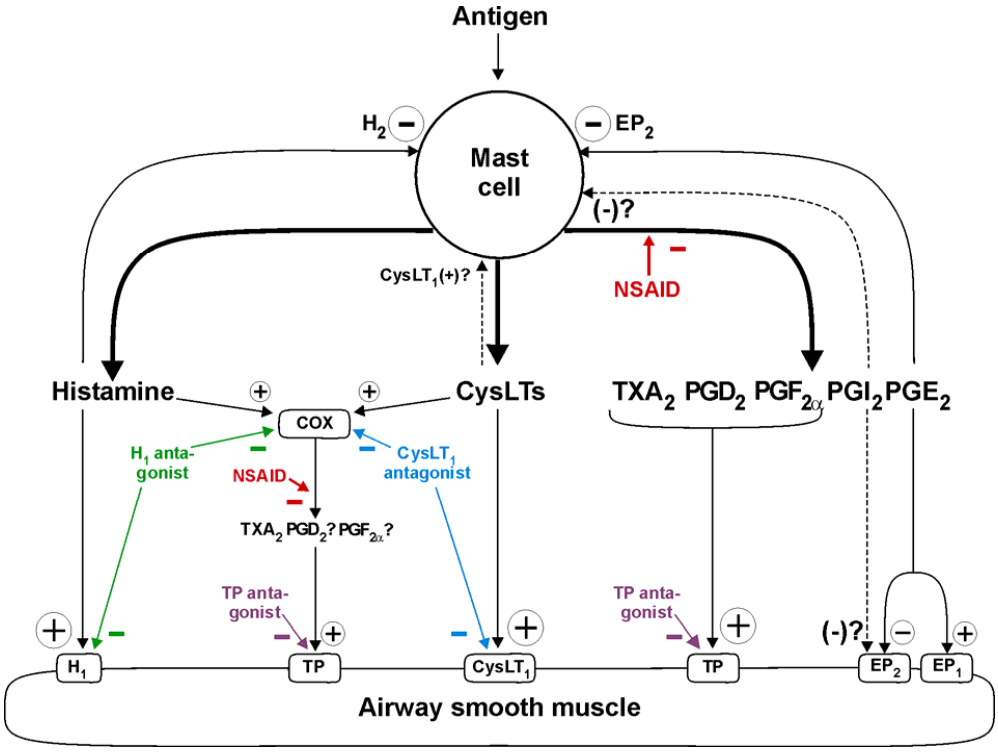

Figure 16. Levels of interactions among mediators of the immediate antigen-induced bronchoconstriction in the IPL.

In this context, it should be appreciated that antagonism of histamine and cysteinyl-leuktorienes by an antihistamine and by a $CysLT_1$ antagonist, respectively, will therefore have a dual action on the response to antigen in IPL. Thus, the antagonists will remove the effect caused by the endogenously released mediator itself, but, also, by blocking the receptor-triggered release of COX products, the antagonist will remove a synergistic mechanism *(Figure 16)*. It should also be recognised that the overall effect of the non-selective COX inhibitors on the response to antigen in this model relates to at least two different actions. Namely, (a) inhibition of the formation of bronchoconstrictive COX products released primarily in response to the antigen-antibody reaction on mast cells, and (b) inhibition of the formation of bronchoconstrictive COX products released secondarily in response to the actions of histamine and LTD_4 in the tissue *(Figure 16)*.

Considered together, the evaluation of drug candidates and experimental interventions in this model needs to take all these levels of actions and interactions into account, and to define the relative importance of each mechanism for a particular intervention.

Role of the COX-dependent modulation of the airway responses to bronchoconstrictive agonists

In the IPL, amplification by agonist-induced release of constrictive COX products appears to predominate for histamine and CysLTs, as the bronchoconstrictive responses to these agonists have been found sensitive to COX inhibitors. Investigations of effects of COX inhibitors on the responses to bronchoconstrictive agonists in human airways *in vitro* and *in vivo* produced conflicting results. In isolated human bronchi, the airway smooth-muscle contractions induced by histamine (Brink et al., 1980; Cerrina et al., 1989; Haye-Legrand et al., 1986), CysLTs (Bourdillat et al., 1987; Jones et al., 1982; Samhoun and Piper, 1984) and LTB_4 (Samhoun and Piper, 1984), were not affected by COX inhibitors, suggesting that COX products had no role in modulation of the contraction responses to these agonists, in spite of both the basal and histamine-induced release of PGE_2 and $PGF_{2\alpha}$, detected in this preparation, and reduction of the histamine-induced release of PGE_2 and $PGF_{2\alpha}$ by COX inhibition (Cerrina et al., 1989; Haye-Legrand et al., 1986). In contrast, other investigators reported significant inhibition of the histamine-induced contractions by TP antagonism in isolated human bronchi, where stimulation with histamine also induced release of $PGF_{2\alpha}$ (Knight et al., 1997). However, in human peripheral airway preparations *in vitro*, contractions induced by CysLTs were not affected by TP antagonism (Wohlsen et al., 2003), supporting the notion that CysLTs do not exert their effect via the secondary release of bronchoconstrictive COX products in human airways (Gorenne et al., 1996).

In human airways, the responses to inhalation challenges with directly acting agonists of bronchoconstriction activating specific receptors on the airway smooth muscle, such as histamine (Hamid et al., 1990; Phillips and Holgate, 1989), LTD_4 (Smith et al., 1987) and methacholine (Fish et al., 1981) were not affected by COX inhibitors. In contrast, other investigators reported suppression of the response to inhaled histamine, but not of that to methacholine by COX inhibition (Curzen et al., 1987). However, inhalation challenge with histamine was not associated with increased urinary concentration of LTE_4 and 11-dehydro-TXB_2, and that with LTD_4 did not result in increased urinary concentration of 11-dehydro-TXB_2 (Kumlin et al., 1992) suggesting

that the secondary release of eicosanoids was not associated with bronchoconstriction evoked by histamine and LTD_4 in human airways.

Here, the objection may be raised that the identification of the distinct amplification by COX products of the responses to histamine and CysLTs makes the IPL model dissimilar to the human airways. However, in view of the conflicting results of investigations of the effects of COX inhibition on the responses to directly acting bronchoconstrictive agonists in human airways *in vitro* and *in vivo,* the role of COX products in modulation of the responses to bronchoconstrictive agonists in human airways remains to be definitively established.

In the IPL, responses to agonists that do not actively stimulate the receptor-triggered release of COX products such as PGD_2 appear to be down-modulated presumably by inhibitory prostaglandins such as PGE_2 and PGI_2, tonically generated in the tissue. In human airways, the inhibitory prostaglandins do not seem to be continuously present. This view is supported by lack of effect of COX inhibition on the initial bronchoconstriction evoked by the challenge with methacholine in normal subjects (Stevens et al 1990) as well as histamine (Manning et al., 1987) and LTD_4 (Manning et al., 1993) in asthmatic subjects. These agonists of bronchoconstriction act directly by stimulation of specific receptors on the airway smooth muscle.

Likewise, COX inhibition had no effect on the initial response to exercise challenge in asthmatic airways (Manning et al., 1993; O'Byrne and Jones, 1986). In contrast to directly acting bronchoconstrictive agonists, the exercise challenge as well as inhalation challenges with hypo- and hyperosmotic or irritant stimuli evokes bronchoconstriction indirectly, that is, by stimulation of mast cells or activation of neural pathways that leads to release of mediators and bronchoconstriction.

Thus, basal production of PGE_2 apparently does not have any important bronchoprotective role in the normal human airways and in the majority of asthmatics, and the bronchoconstrictive stimulus needs to be present before the inhibitory prostaglandins are released. This view is supported by the development of tachyphylaxis to directly acting agonists in normal as well as in asthmatic airways. Tachyphylaxis manifests as diminished bronchoconstrictor response when the challenge with a directly acting agonist is repeated within a few hours after the initial challenge. In asthmatic airways, tachyphylaxis to histamine (Hamielec et al., 1988; Manning et al., 1987; Manning and O'Byrne, 1988) was prevented by pretreatment with COX inhibitors (Manning et al., 1987), and that to LTD_4 was also prevented by COX inhibition (Manning et al., 1993). In contrast to the findings with histamine and LTD_4, tachyphylaxis to inhaled cholinergic agonists,

such as methacholine and acetylcholine, was not observed in asthmatic airways (Manning et al., 1987; Manning and O'Byrne, 1988). However, in normal airways, tachyphylaxis to inhaled methacholine (Beckett et al., 1992; Stevens et al., 1990) was prevented by COX inhibitors (Stevens et al., 1990), as well as that to inhaled LTD_4 (Kern et al., 1986). These findings suggest that the initial challenge of normal as well as asthmatic airways with a directly acting agonist stimulates endogenous PGE_2 release that contributes to the development of tachyphylaxis. As further support, in isolated human bronchi, increased endogenous production of PGE_2 and PGI_2 by epithelium was observed after histamine exposure and was associated with histamine tachyphylaxis (Knight et al., 1995a; Knight et al., 1992), which was abolished by the NSAID pretreatment in tissue donors (Knight *et al.*, 1995a). Furthermore, the contribution of PGE_2 but not PGI_2 to the development of histamine tachyphylaxis in this preparation was supported by the finding that addition of PGE_2 but not PGI_2 reduced the maximal effect of histamine in a subsequent challenge (Knight *et al.*, 1995b). Tachyphylaxis to inhaled histamine that occurred at the smooth muscle level and was dependent on endogenous prostaglandin generation was also described in dogs *in vivo* (Shore and Martin, 1985). However, inhibition of prostaglandin synthesis failed to reverse histamine tachyphylaxis either *in vivo* or *in vitro* in the dog in another investigation (Antol et al., 1988), suggesting that mechanisms other than endogenous PGE_2 release, such as receptor down-regulation or uncoupling may contribute as well.

Stimulation of the endogenous PGE_2 release is also thought to be behind the development of tachyphylaxis to repeated challenge that occurs for many indirectly acting challenges in asthmatic airways. For this phenomenon, the term refractoriness is often used. Refractoriness manifests as diminished bronchoconstrictor response when a challenge with the same stimulus is repeated within a few hours after the initial challenge. Refractoriness after challenges with exercise (Belcher et al., 1987a; Ben-Dov et al., 1983; Edmunds et al., 1978; Hamielec et al., 1988; Manning et al., 1993) was prevented by COX inhibition (Margolskee et al., 1988; O'Byrne and Jones, 1986), as well as after hypo-osmolar stimuli (Mattoli et al., 1987a; Mattoli et al., 1987b), and challenge with sodium metabisulphite (Pavord et al., 1994b). Furthermore, the development of cross-refractoriness, that is, reduced bronchoconstrictor response to a subsequent indirect challenge with a different stimulus than that used for the initial challenge, suggests that stimulation of endogenous PGE_2 release serves as a common protective mechanism in asthmatic airways. Cross-refractoriness was observed between indirect challenges that cause bronchoconstriction by a similar mechanism, such as exercise and hypertonic saline (Belcher et

al., 1987b) as well as those causing bronchoconstriction through different mechanisms, such as exercise and sodium metabisulphite (Pavord et al., 1994a).

It is likely that the modulatory role of endogenous PGE_2 in indirectly acting challenges depends on inhibition of the inflammatory and bronchoconstrictor pathways rather than functional antagonism due to the direct bronchodilatory effect on the smooth muscle. This is supported by the findings that PGE_2 suppressed cholinergic contractions in isolated human bronchi (Ito *et al.*, 1990) and canine trachea (Ito and Tajima, 1981) by inhibiting release of acetylcholine from cholinergic nerve endings. Moreover, it was demonstrated that PGE_2 had inhibitory effects *in vitro* on different cells important in release of contractile mediators (Hedqvist et al., 1989; Peters et al., 1982; Raud et al., 1988; 1989) and development of airway inflammation in asthma (Alam et al., 1993; Chouaib et al., 1987; Pene et al., 1988). However, other mechanisms such as depletion of neural mediator stores can contribute to the development of refractoriness, in addition to endogenous prostaglandin generation.

In summary, the development of tachyphylaxis in response to directly acting bronchoconstrictive agonists in both normal and asthmatic airways and refractoriness in response to indirectly acting stimuli in asthmatic airways, discloses the presence of an inhibitory mechanism that attempts to limit bronchoconstriction, and there is evidence that generation of endogenous PGE_2 is a common factor behind these mechanisms. Loss of protective factors may be important in the pathogenesis of asthma and airway hyperresponsiveness.

Clinical relevance

In guinea pig, IgG_1 is the major anaphylactic antibody (Ovary et al., 1963), whereas IgE is the main homocytotropic antibody mediating mediator release during anaphylaxis in humans (Kay and Austen, 1971). However, IgE besides IgG can sensitise mast cells in guinea pig airways and mediate antigen-induced smooth muscle contractions (Graziano et al., 1984; Ro et al., 1991). Thus, airways of actively sensitised guinea pigs can be used as a model for IgE-mediated anaphylaxis in the lung, but it is difficult to determine the contribution of IgE and IgG_1 antibodies to specific antigen responses. Moreover, the FcεRI and FcγR (FcγRII and FcγRIII), binding IgE and IgG, respectively, are structurally and functionally related (Alber et al., 1992) and cross-linking of these receptors utilises common signalling pathways for cellular activation (Metcalfe et al., 1997; Rose et al., 1997).

In the IPL, a pivotal role of histamine, CysLTs and COX products as mediators of the immediate antigen-induced bronchoconstriction was documented by substantial inhibition of the response to antigen obtained with an antihistamine, an antileukotriene and a COX inhibitor, respectively. Inhibition of the H_1 receptor-mediated effects of histamine has been shown to attenuate also the early asthmatic reaction (EAR) (Curzen et al., 1987; Rafferty et al., 1987; Roquet et al., 1997), indicating a role of histamine as mediator of the response to allergen in asthmatic airways. Treatment with drugs that inhibit the $CysLT_1$ receptor-mediated effects (Dahlén et al., 1991; O'Shaughnessy et al., 1993; Roquet et al., 1997; Taylor et al., 1991) or the biosynthesis of CysLTs (Dahlén et al., 1997; Friedman et al., 1993; Hamilton et al., 1997; Hui et al., 1991) attenuated the EAR to a greater extent than antihistamine drugs. The CysLTs, LTC_4, LTD_4 and LTE_4, are thus the predominant mediators of the response to allergen in asthmatic airways. However, histamine and CysLTs together mediate about 75% of the allergen-induced bronchoconstriction in asthmatics, as shown by the effect of the combined antileukotriene and antihistamine treatment on the EAR (Roquet et al., 1997). This indicates that other mediators contribute to the allergen-induced bronchoconstriction in asthmatic subjects as well. Three main candidates are COX-derived prostanoids, TXA_2, PGD_2 and $PGF_{2\alpha}$ where release following the allergen-induced bronchoconstriction was detected in man (Gréen et al., 1974; Kumlin et al., 1992; Manning et al., 1991; Murray et al., 1986; Shephard et al., 1985; Sladek et al., 1991; Wenzel et al., 1989).

Investigation of the effect of COX inhibition on the EAR has been used as one method to evaluate the contribution of the COX products to the allergen responses in human airways. However, the use of different COX inhibitors has led to conflicting results. Thus, COX inhibition produced the mean overall inhibition of the EAR by 26 % in one study (Curzen et al., 1987), whereas no effect of this pharmacological intervention on the EAR was observed in other studies (Fish et al., 1981; Kirby et al., 1989; Shephard et al., 1985). These conflicting results may be due to suppression by COX inhibition of different pulmonary COX products, both those with bronchoconstrictive actions, such as TXA_2, PGD_2 and $PGF_{2\alpha}$, and those with bronchorelaxant and inhibitory actions, such as PGE_2 and PGI_2. This is at variance with the IPL model where the bronchoconstrictive COX products had the predominant role and the relaxant ones had a minor role in the immediate antigen-induced bronchoconstriction.

The contribution of bronchoconstrictive prostanoids to the immediate allergen-induced bronchoconstriction in human airways has also been addressed more specifically by selective antagonism of the TP receptor that mediates the effects of TXA_2, PGD_2 and $PGF_{2\alpha}$ (Featherstone et al., 1990). The mean overall inhibition of the EAR produced by this pharmacological intervention was 24% (Beasley et al., 1989), almost identical with that obtained with by COX inhibition (Curzen et al., 1987), supporting involvement of the bronchoconstrictive prostanoids. Furthermore, the role of TXA_2 was supported by a mean overall inhibition of the EAR by 22 % produced by selective inhibition of the thromboxane synthase (Manning et al., 1991). However, the intersubject variability was characteristic for the effects of the TP receptor antagonism (Beasley et al., 1989) and the thromboxane synthase inhibition (Manning et al., 1991). Taken together, the effects on the EAR of these three pharmacological interventions with the COX pathway support that bronchoconstrictive prostanoids contribute in a varying degree to the immediate allergen-induced bronchoconstriction in asthmatic airways. The effect of the combination of antihistamines and antileukotrienes with COX inhibitors has not yet been evaluated in man, but the findings in this thesis suggest that the combined inhibition of the three mediator classes has a potential to effectively inhibit the EAR and clinical asthma. This proposal receives some support from the clinical observations that inhibition of two mediator classes at a time had more profound effects than antagonism of single mediators on both the EAR and the LAR (Roquet et al., 1997) as well as on asthma control during a 2-week treatment period (Reicin et al., 2000).

CONCLUSIONS

- Basal characteristics of the bronchoconstrictive responses to exogenous histamine, cysteinyl-leukotrienes and prostanoids in IPL, were generally similar to those in human airways.

- The bronchoconstrictive responses induced by LTD_4 and PGD_2 in IPL were modulated differently by COX products. Whereas bronchoconstriction induced by LTD_4 was amplified by secondarily released constrictive COX products, and most likely TXA_2, that induced by PGD_2 was down-modulated presumably by bronchoprotective PGE_2 and PGI_2 tonically produced in the airways. Furthermore, it appears that both the COX-1 and the COX-2 pathway were involved in the LTD_4-induced generation of COX products.

- The clinically used NSAIDs, diclofenac and lumiracoxib, in addition to being COX unselective and highly COX-2 selective inhibitors, respectively, are competitive TP receptor antagonists, and could thus represent prototypes of new chemical entities with dual activity as selective COX-2 inhibitors and potent TP antagonists. Such compounds may give rise to coxibs with potentially decreased adverse cardiovascular effects.

- The IPL was found to provide a model where a specific, dose-dependent and reproducible bronchoconstriction was obtained in response to antigen (ovalbumin) challenge in the sensitised lungs.

- The immediate antigen-induced bronchoconstriction in IPL was mediated by the concerted actions of histamine, cysteinyl-leukotrienes and several prostanoids. The pharmacology of the antigen response in this model is thus closely similar to that in asthmatic airways.

- It appears that there is no division of labour between COX-1 and COX-2 in the prostanoid-mediated component of antigen-induced broncoconstriction in IPL.

- The IPL is considered a relevant experimental model for the studies of the EAR. Furthermore, interactions among the mediators of the immediate antigen-induced bronchoconstriction in IPL occur at several levels (mast cell mediator release, smooth-muscle activation, effector cell signalling and adaptive responses). The evaluation of experimental interventions and drug candidates in this model thus needs to take all these levels of interactions into account, and to evaluate the relative importance of each mechanism for a particular intervention.

- The remarkable inhibition of the immediate antigen-induced bronchoconstriction in IPL, produced by the combined inhibition of the three mediator classes suggests that this pharmacological intervention has a potential to effectively inhibit the EAR and be useful in the treatment of clinical asthma.

POPULÄRVETENSKAPLIG SAMMANFATTNING (Swedish)

Förekomsten av astma ökar över hela världen och i ett flertal länder har 5-10 % av befolkningen astma. Astmasjukdomen är ofta relaterad till immunologisk sensibilsering, som innebär att vid exponering för olika allergen bildas specifika antikroppar (IgE).

I lungorna finns mastceller som har förmåga att binda antikroppar (IgE) på sin yta. Förnyad exponering för allergen leder till en reaktion mellan allergen och cellbundet IgE. Denna reaktion är en signal till mastcellen att frisätta en rad mediatorer. En del av dessa mediatorer, som histamin, finns lagrade i cellen, medan andra mediatorer, leukotriener och prostaglandiner, bildas efter aktivering av mastcellen. Leukotriener och prostaglandiner är fettsyror och kallas för eicosanoider.

Effekterna av histamin, leukotriener och prostaglandiner förmedlas via interaktion med specifika receptorer. Stimulering av receptorer på glatt muskulatur, slemproducerande körtlar och blodkärl i luftvägar orsakar sammandragning, ökad slemproduktion, slemhinneskador i bronkerna och svullnad. Tillsammans leder detta till luftvägsobstruktion inom minuter efter exponering för allergen.

Bronkkonstriktion beroende av sammandragning av glatt muskulatur är den dominerande effekten av mastcellmediatorerna i den snabballergiska reaktionen. Mastcellmediatorerna spelar också en viktig roll i utveckling av kronisk inflammation, som kännetecknar luftvägar hos astmapatienter.

Hos människan är vissa leukotriener de mest potenta och dominerande bronkkonstriktoriska mediatorerna, men histamin står också för en viss komponent i den snabballergiska reaktionen, liksom en eller fler bronkkonstriktoriska prostaglandiner. Det är dock inte klarlagt vilken relativ betydelse olika mastcellmediatorer har för det bronkkonstriktoriska svaret. Mekanismerna för hur olika mediatorer samverkar och påverkas av astmaläkemedel är också ofullständigt kända.

Syftet med studierna i denna avhandling var därför att systematiskt utreda den relativa betydelsen av olika mastcellsmediatorer vid allergisk bronkkonstriktion i den experimentella modellen, isolerade perfunderade och ventilerade lungor från marsvin (IPL). Detta djurslag valdes därför att luftvägspreparat från marsvin uppvisar ett reaktionsmönster vid allergiska reaktioner som påminner om liknande förlopp hos människor. Undersökningarnas huvudmål var att precisera den roll olika eicosanoider spelar vid allergenutlöst bronkkonstriktion i IPL-modellen.

Studierna i denna avhandling visar att den snabballergiska reaktionen i IPL-modellen motverkas effektivt när preparationen behandlas med astmaläkemedel som hämmar effekter av histamin, leukotriener, eller bronkkonstriktoriska prostaglandinerna. Detta styrker rollen av dessa enskilda mediatorer i allergenutlöst bronkkonstriktion. Studierna visar också att allergenutlöst bronkkonstriktion i IPL-modellen motverkas än mer effektivt med kombinationer av flera olika astmaläkemedel, jämfört med effekterna av de enskilda läkemedlen. Dessa resultat avslöjar att mediatorerna samverkar med varandra och kan ha synergistiska effekter i luftvägarna. Därför kan kombinationer bestående av flera läkemedel visa sig värdefulla i behandling av astma.

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