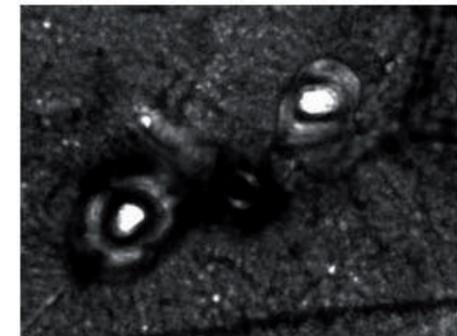
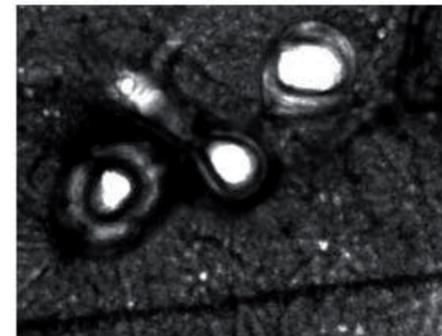


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## Nitric Oxide and Eicosanoids: Significance and Interactions During Antigen-Induced Responses in Peripheral Lung Tissue



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Anna-Karin Larsson

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

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SIGNIFICANCE AND INTERACTIONS DURING ANTIGEN-  
INDUCED RESPONSES IN PERIPHERAL LUNG TISSUE**

Anna-Karin Larsson



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*To my dear family*



## ABSTRACT

Asthma means difficulty in breathing and is described as a chronic, inflammatory disorder that produces narrowing of the lower respiratory tract. The allergen-induced asthmatic bronchoconstriction is primarily caused by an IgE-mediated release of the mast cell mediators, histamine and eicosanoids (leukotrienes and prostanoids). Asthmatics have elevated levels of nitric oxide (NO) in the exhaled air, that has been proposed as a sign of airway inflammation. In the airways, mast cells represent a major source of NO. This formed NO may act in an autocrine fashion to suppress mast cell function, including release of histamine and leukotriene synthesis, and thereby be a regulator of allergen-induced responses. Nevertheless, the function of NO in the peripheral lung is not clear.

The aim of this thesis was therefore to establish the role of nitric oxide and eicosanoids during early allergic airway responses in the peripheral lung. Antigen-induced contractions to the allergen ovalbumin were studied in the lung parenchyma obtained from actively sensitised guinea pigs, an *in vitro* model for mast cell driven antigen-induced contractions. The peripheral lung is a complex tissue with airway smooth muscle, bronchioles, vessels and connective tissue. To further understand and characterise the contractile responses obtained to allergen or agonists in lung parenchymal tissue, studies in guinea pig precision cut lung slices (GP PCLS) were established and performed. Another aim of this thesis was also to compare species differences during the early allergic airway response in the PCLS.

Inhibition of nitric oxide synthase (NOS) enhanced the contractions to cumulative doses of ovalbumin, whereas addition of the different NO donors SNP and NCX 2057 attenuated the antigen-induced contractions. The action of NO was however not relaxation of airway smooth muscle, since NO potently dilated precontracted vascular preparations and weakly relaxed precontracted tracheal rings, while there was no effect on precontracted GPLP. Instead, NO act as inhibitor of allergen-induced mediator release in the peripheral lung. Inhibition of endogenous NO increased the release of leukotrienes, whereas SNP and NCX 2057 distinctly inhibited the release of histamine or leukotrienes during antigen challenge. In conclusion, the findings support that endogenous NO has a protective role in the peripheral lung as a beneficial immunomodulator of the early allergic airway response. The findings also indicate that different NO donors may have selective and protective anti-inflammatory effects in the peripheral lung tissue.

The GP PCLS was established and represents now a new *in vitro* model to simultaneously measure airway and vascular responses under cell culture conditions. The study showed that the pharmacology of the guinea pig PCLS most closely resembled that of the corresponding human tissues. In guinea pigs and humans, leukotrienes and prostanoids were primary mediators of the antigen-induced bronchoconstriction. In contrast, the contractile response to antigen in rat PCLS was mainly mediated by serotonin and modulated by locally formed prostanoids, in particular COX-2 derived PGE<sub>2</sub>, acting at EP<sub>1</sub> receptors. Thus, mechanisms by which eicosanoids contribute to the early allergic airway response differ among species.

**Key words:** nitric oxide, leukotrienes, prostaglandins, histamine, ovalbumin, contractions, distal lung, rat, human, guinea pig, precision-cut lung slices, mast cell

## LIST OF PUBLICATIONS

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

- I. **Larsson A-K**, Bäck M, Hjoberg J and Dahlén S-E.  
Inhibition of nitric-oxide synthase enhances antigen-induced contractions and increases release of cysteinyl-leukotrienes in guinea pig lung parenchyma: Nitric oxide as a protective factor.  
*J Pharm Exp Ther.* 2005; 315:458–465
- II. **Larsson A-K**, Bäck M, Lundberg J and Dahlén S-E.  
Distinct inhibition of mediator release by two different NO donors reduce antigen-induced contractions in the peripheral lung  
*Manuscript*
- III. **Larsson A-K**, Fumagalli F, DiGennaro A, Andersson M, Lundberg J, Edenius C, Govoni M, Monopoli A, Sala A, Dahlén S-E and Folco GC.  
A new class of Nitric Oxide-releasing derivatives of Cetirizine; pharmacological profile in vascular and airway smooth muscle preparations.  
*Br J Pharmacol, E-pub: 12 March 2007*
- IV. Ressmeyer A-R, **Larsson A-K**, Vollmer E, Dahlén S-E, Uhlig S and Martin C.  
Characterisation of guinea pig precision-cut lung slices: Comparison with human tissues.  
*Eur Respir J* 2006; 28: 603–611
- V. **Larsson A-K**, Dahlén S-E, Ressmeyer A-R, Uhlig S and Martin C.  
Modulation of antigen- and serotonin-induced airway contractions in rat precision cut lung slices by prostaglandin E<sub>2</sub>.  
*Manuscript*

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## LIST OF ABBREVIATIONS

COX	Cyclooxygenase
CysLT	Cysteinyl leukotriene
EIA	Enzyme immuno assay
FLAP	5-lipoxygenase activating factor
GPCR	G-protein coupled receptor
GPLP	Guinea pig lung parenchyma
GPTR	Guinea pig tracheal ring
5-HT	5-hydroxytryptamine or serotonin
5-LO	5-lipoxygenase
L-NAME	N <sup>o</sup> -Nitro-L-arginine methyl ester
L-NOARG	N <sup>o</sup> -nitro-L-arginine
LT	Leukotriene
NO	Nitric oxide
NONOate	Diethylamine NONOate
NOS	Nitric oxide synthase
NCX 2057	3-(4-hydroxy-3 methoxyphenyl)-2-propenoic acid 4-(nitroxy) butyl
NCX 2058	3-(4-hydroxy-3 methoxyphenyl)-2-propenoic acid 4-Bromo butyl ester
OVA	Ovalbumin
PCLS	Precision-cut lung slices
PGD <sub>2</sub>	Prostaglandin D <sub>2</sub>
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>
SEM	Standard error of the mean
SNP	Sodium nitroprusside
TXA <sub>2</sub>	Thromboxane A <sub>2</sub>

## INTRODUCTION

Asthma means difficulty in breathing and is described as a chronic, inflammatory disorder that produces narrowing of the lower respiratory tract (1). Attacks are brought about by bronchoconstriction, i.e. spasms of the smooth muscle in the walls of the smaller bronchi and bronchioles, causing the airways to close partially or completely (2, 3). One prominent trigger of bronchoconstriction is exposure to allergen in atopic subjects with asthma. The allergen-induced asthmatic bronchoconstriction may consist of two phases, an early and a late phase. The early reaction in humans is primarily caused by an IgE-mediated release of the mast cell mediators histamine, and the eicosanoids cysteinyl leukotrienes (CysLT) and prostanoids (4-6). This thesis will focus on experimental studies on this early phase.

Asthmatics have elevated levels of nitric oxide (NO) in the exhaled air (7), that has been proposed as a sign of airway inflammation. The increased amounts of NO have been suggested to derive from the lower respiratory tract (8, 9) and exhaled NO is decreased after treatment with inhaled corticosteroids (10). Exhaled NO is reduced shortly after bronchoconstriction to direct and indirect stimuli in asthma (11). In the airways, mast cells represent a major source of NO (12, 13). It has been suggested that NO may act in an autocrine fashion to suppress mast cell functions, including release of histamine (14) and leukotriene synthesis (15). Those effects of NO on mast cell function may be a regulator of allergen-induced responses. Nevertheless, the function of NO in the airways is not clear.

Whereas recent evidence indicates that the inflammation in asthma extends into peripheral airways (2, 16), most studies of mediator mechanisms have been done in models of central and proximal airways. The aim of this thesis was therefore to focus on the peripheral airways, and in particular to establish the impact and interactions of NO and eicosanoids during the early allergic airway response in the peripheral lung. Antigen-induced contractions to the allergen ovalbumin were studied in the lung parenchyma obtained from actively sensitised guinea pigs (GPLP), an *in vitro* model for mast cell driven antigen-induced responses (17). Recent studies of antigen-induced airway constriction in the guinea pig lung indicates that the mediators of the response in this particular species are the same as in allergen-induced airway obstruction in asthmatic subjects (18), namely histamine, CysLTs and several prostanoids. The peripheral lung is a complex tissue with airway smooth muscle,

bronchioles, vessels and connective tissue. To further understand and characterise the contractile responses obtained by challenge with allergen or agonists in the lung parenchymal tissue, a new *in vitro* model, the guinea pig precision cut lung slices (GP PCLS), were established. This is a model of the peripheral lung where contractions of airways and vessels can be studied at the same time. Another aim of this thesis was also to compare species differences in PCLS of rat, human and guinea pig during the early allergic airway response, since both guinea pigs and rodents are widely used in pulmonary pharmacology.

## **BACKGROUND**

### **Asthma and allergy**

Asthma, allergic rhinitis and atopic eczema are causes of chronic ill health and carry a major social and economic burden (19). Allergic rhinitis is the most common immunologic disorder and is characterised by an IgE-mediated inflammation induced by allergen exposure. Allergic rhinitis constitutes a main risk factor for asthma onset (20). Approximately 300 million people world wide have asthma and the prevalence has constantly increased in the past decade, however in some countries the prevalence appear to have reached a plateau. In the developed countries about 10 % of the population are asthmatics (3, 19, 21).

The symptoms of asthma typically include recurrent attacks of breathlessness, chest tightness, wheezing and/or coughing (1). Attacks are brought about by bronchoconstriction, i.e. spasms of the smooth muscle in the walls of the smaller bronchi and bronchioles, causing the airways to close partially or completely (2, 3). This impedes the ventilation, i.e. the transport of oxygen into the lung and the elimination of carbon dioxide out from the lung, with consequent depreciations of gas exchange and blood oxygenation. Severe asthma attacks may therefore even lead to death by asphyxia (22).

Asthma is morphologically characterised by epithelial shedding, airway smooth muscle hypertrophy and hyperplasia, overproduction of mucus and infiltration of inflammatory cells into the airway (23). The pathophysiology of asthma has traditionally been attributed to an inflammatory process that occurs predominantly in the large airways (22). Pathological and physiological evidence however indicate that the inflammatory process extends beyond the central airways to the peripheral airways and the lung parenchyma. Notably, the inflammation in the distal lung has been described to be more severe than in the central airways (2, 22, 24). Such pathologic findings may be extremely important since the total volume and combined surface area of the distal airways are much greater than of the central airways (25).

### *Mediators of allergen-induced airway obstruction in asthmatics*

The allergen-induced asthmatic bronchoconstriction may when triggered by conventional allergen bronchoprovocation consist of two phases, an early and a late phase. In the early phase (the first hour after the exposure to allergen) there is a fall in

lung function that is believed to mainly be due to smooth muscle spasm. This thesis will focus on the early phase. The early asthmatic reaction is primarily caused by an IgE-mediated release of the mast cell mediators, histamine, CysLTs and prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) (4-6). In the late phase (2-24 hours after challenge), bronchospasm continues but the airway obstruction is now amplified by a prominent inflammatory reaction, where vasodilation, mucus secretion, oedema, recruitment and activation of inflammatory cells in the lung, including eosinophils, neutrophils and lymphocytes (1).

#### *Diagnosis and treatment*

The variable airflow limitation in asthma is reversible either spontaneously or by treatment with bronchodilating drugs. This differs from the non-reversible airflow obstruction that is seen in chronic obstructive pulmonary diseases (COPD). Drugs used to treat asthma include bronchodilators and anti-inflammatory agents. Examples of bronchodilators are  $\beta_2$ -adrenoceptors agonists such as salbutamol and terbutaline, but muscarinic-receptor antagonists such as ipratropium bromide may also be used (23). The mainstay anti-inflammatory treatment is represented by inhaled or oral glucocorticosteroids (26). Inhaled corticosteroids are mainly deposited in the central airways (27), making it unclear if corticosteroids effectively treat the inflammation in the distal lung and implying that the small airways are untreated in asthma (22). More recently, CysLT<sub>1</sub> antagonists (LTRA) such as montelukast (Singulair®), zafirlukast (Accolate®) and pranlukast (Onon®) have been introduced as preventive treatment with anti-inflammatory properties (23, 28, 29).

#### **Airway physiology; proximal and distal airways**

The respiratory system is divided into two portions; the upper airways consisting of the nose, pharynx, larynx and the lower airways consisting of trachea, bronchi and intrapulmonary airways. The lower airways are further distinguished into proximal airways referring to the trachea and the main bronchus, whereas the distal airways or lung consist of the bronchioles and alveolar sacs (alveoli) (30) (*Fig 1*). The border between proximal and distal airways is located at the eight generation of the bronchial tree and small airways are referred to have a diameter of less than 2 mm in human lung (2, 22). The lung is further divided into different lobules that consist of elastic

connective tissue containing a lymphatic vessel, an arteriole, a venule and a branch of the terminal bronchioles (30).

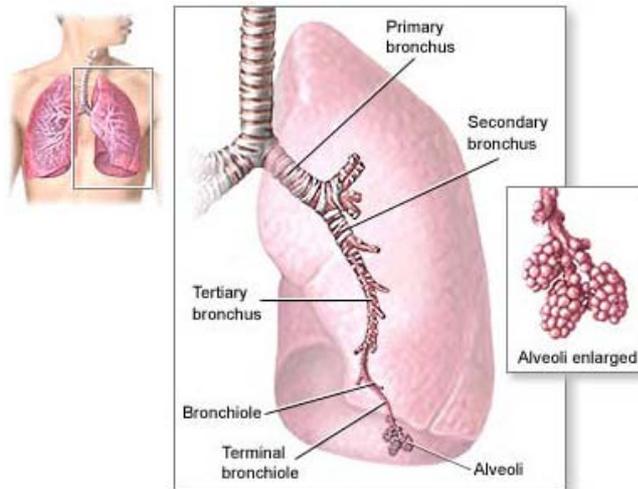


Fig 1. The lung and the bronchial tree.

### **Airway smooth muscle**

The lung parenchyma consists of smooth muscle tissue, small blood vessels and bronchioles (31). Airway smooth muscle (ASM) was earlier thought to be a passive target for mediators or drugs causing contraction or relaxation. Nowadays ASM is known also to be an active tissue. In addition to contracting or relaxing in response to mediators or drugs, ASM may display proliferation, differentiation and induce synthesis of mediators, cytokines, chemokines and growth factors (32-34). Airway smooth muscle mass is increased in asthmatics and is one target for glucocorticoids (35).

#### *Physiology of ASM in the lung*

As the proportion of cartilage decreases, the amount of smooth muscle increases in more peripheral bronchi. Airway smooth muscle encircles the lumen of bronchi in spiral bands. Both the autonomic nervous system and various biologically active molecules mediate contraction of the smooth muscle. The parasympathetic nervous system and mediators of allergic reactions cause constriction of distal bronchioles. During an asthma attack the smooth muscle of bronchioles contracts and the diameter

of the airways are reduced. Because there is no supporting cartilage in the peripheral airways, the muscle spasms may even close off the airways (2, 22).

A smooth muscle fibre is spindle-shaped and contains a single, centrally located nucleus. Smooth muscle can both shorten and stretch to a greater extent than skeletal and cardiac muscle. When smooth muscle filaments are stretched, they initially contract, developing increased tension. This phenomenon is called the stress-relaxation response and it allows the muscle to undergo immense changes in length while still retaining the ability to contract effectively. The muscle contraction is initiated by an increase in the concentration of  $\text{Ca}^{2+}$  in smooth muscle sarcoplasm. It takes longer time for  $\text{Ca}^{2+}$  to reach the filaments in the centre of the fibre and trigger the contractile response than in skeletal muscles. This gives a typical slow onset and a prolonged contraction. The contractions and relaxations are regulated by different mechanisms. The regulator protein calmodulin binds to  $\text{Ca}^{2+}$  in the cytosol and thereby activates the enzyme myosin light chain kinase. This enzyme phosphorylates, through ATP, the myosin head, which then binds to actin and contractions occur. Myosin light chain kinase works rather slow which contribute to the slow smooth muscle contractions (36).

#### *ASM and allergen-induced bronchoconstriction*

In 1910 William Schultz found that isolated intestines from guinea pigs actively sensitised to horse serum contracted in response to challenge with dilute horse serum. Sir Henry H. Dale demonstrated at the same time the specificity and sensitivity of the antigen-induced response in the guinea pig uterus *in vitro*, which included not only the description of the contractile activity of histamine but also the maintained anaphylactic contraction in blood and serum-free tissues. Antigen-induced contraction of isolated smooth muscle has since been named the Schultz-Dale reaction, and is used extensively to study the mechanisms of anaphylactic contractions in a number of tissues, including the airway smooth muscles (37, 38).

#### **Mediators of the allergen-induced bronchoconstriction**

##### *Mast cell activation and release of mediators*

Mast cells are resident in all normal tissues and involved in tissue homeostasis and host defense (39). The mast cell is considered to be a key cell involved in the pathophysiology of asthma. Mast cells are divided into two subpopulations; mast cells

that contain tryptase and chymase in secretory glands or mast cells that only contain tryptase (40). There is an increased infiltration of mast cells in airway smooth muscle in asthmatics, and the number of mast cells correlates with the airway hyperresponsiveness (41). The largest proportion of mast cell is confirmed to be found in the ASM of asthmatics (42), implying a potential interaction between mast cells, ASM, airway obstruction and airway remodelling. The mast cell is activated by both IgE-dependent and IgE-independent mechanisms (1, 43). Upon stimulation, mast cells secrete the autocooid mediators histamine, PGD<sub>2</sub>, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), thromboxane and leukotrienes (44) that induce bronchoconstriction, mucus secretion and mucosal oedema. Mast cells also synthesise and secrete proinflammatory cytokines, such as IL-4, IL-5, IL-10 and IL-13, which regulate IgE-synthesis and eosinophilic inflammation (1, 39). Other compounds that are released upon mast cell stimulation are the proinflammatory cytokines TGF- $\beta$  and TNF- $\alpha$ , different proteases such as tryptase and proteoglycans such as heparine (13). NO is generated by mast cells and implicated as a regulator of mast cell phenotype, activation and function (13, 45, 46).

### *Histamine*

The release of histamine from activated mast cells and basophils contributes significantly to the symptoms of allergic rhinitis, conjunctivitis, urticaria and other allergic reactions. Histamine is an important mediator of airway inflammation in asthma, particularly in the development of the early allergic response. Although histamine has been shown to contribute significantly to the bronchoconstrictor response to allergen, leukotrienes are likely to play a more prominent role in these responses in asthma (47). Histamine is preformed in granules of the mast cell and released immediately when the mast cell is triggered, resulting in a fast contraction of the airway smooth muscle. However, histamine has multiple effects on airways which are mediated by at least four histamine receptors (48, 49). Most of the biologic effects of histamine in allergic reactions are mediated by the binding of histamine to H<sub>1</sub> receptors, which induces contraction of intestinal and bronchial smooth muscles, increases permeability of venules, microvascular leak and activation of sensory nerves. Cetirizine is a selective H<sub>1</sub>-antagonist. The affinity of cetirizine to bind to H<sub>1</sub> receptors is more than 500 times higher compared to H<sub>2</sub> and H<sub>3</sub> receptors (50).

Biologic effects are also mediated via the interaction of histamine with H<sub>2</sub> receptors, which cause vasodilation and stimulate mucus secretion from exocrine glands. H<sub>3</sub>-receptors modulate cholinergic neurotransmission, the release of neuropeptides from sensory nerves and allergen-induced bronchoconstriction (48). H<sub>4</sub>-receptors may be involved in allergic lung inflammation by modulation of allergic responses via its influence on T cell activation (51) and mediate signalling and chemotaxis of mast cells (52). H<sub>1</sub> blocking antihistamines have a central role in the treatment of allergic rhinitis (53), and data from allergen bronchoprovocations of asthmatic subjects suggest that anti-histamines may have beneficial effects as part of combination therapy in the treatment of asthma (4, 54).

#### *Serotonin*

The neurotransmitter serotonin or 5-hydroxytryptamine (5-HT) is implicated in enhancing inflammatory reactions of the lung and been suggested to induce mast cell adhesion and migration (55). 5-HT is released by murine and rat mast cells upon stimulation. 5-HT causes contractions of the airways via the 5-HT<sub>2A</sub> receptor. The induced bronchoconstriction is blocked by the 5-HT<sub>2A</sub> antagonist ketanserin (56-58). The immediate allergic response in rat depends largely on serotonin (59). This response can occur in nearly all airway generations, but is most pronounced in the smallest airways, the terminal bronchioles (58).

#### *Arachidonic acid metabolites: eicosanoids*

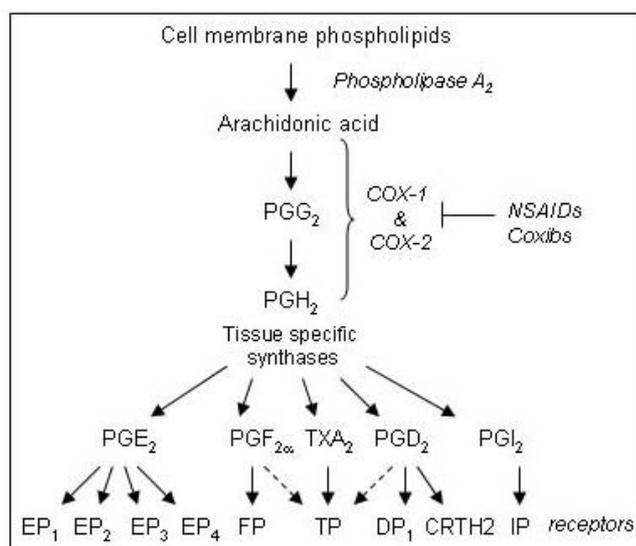
The main source of eicosanoids is arachidonic acid, a 20-carbon polyunsaturated fatty acid that is liberated from phospholipid cell membranes by phospholipase A<sub>2</sub> (PLA<sub>2</sub>) in response to various stimuli (60). The free arachidonic acid is metabolised by several pathways. The cyclooxygenase (COX) pathway generates prostaglandins (PG) and thromboxanes (TX) (61) and the 5-lipoxygenase (5-LO) pathway generates leukotrienes (LT) (62). Leukotrienes, thromboxanes, prostaglandins and lipoxins (LX) are the main classes of the eicosanoids. They are implicated in the control of many physiological processes and are key mediators and modulators of inflammatory reactions (63-65). The eicosanoids are not performed but generated *de novo* from phospholipids. It therefore takes a slightly longer time for the biological effects of these mediators to appear but their effects are more pronounced and longer lasting than histamine (66).

### Prostanoids

In the mid-1930s Ulf von Euler discovered that lipid extracts of human semen contained active compounds that, on injections into animals, stimulated smooth muscle contraction or relaxation and affected the blood pressure. Because of their presumed origin in the prostate gland, he named the compounds prostaglandins (67). Later it was realised that these compounds are widely distributed in animal tissues and members of a large family of compounds formed from the polyunsaturated fatty acid arachidonic acid.

### Prostanoid generation by cyclooxygenase (COX) enzymes

Prostanoids are generated from arachidonic acid *via* the rate-limiting COX reaction (61). COX enzymes, also called prostaglandin G/H synthase catalyze in two steps the conversion of arachidonic acid to the prostaglandin endoperoxides PGG<sub>2</sub> and PGH<sub>2</sub> (68, 69). PGH<sub>2</sub> is then metabolised by tissue specific isomerases or synthases to the prostanoids PGI<sub>2</sub>, TXA<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2α</sub> and PGD<sub>2</sub> (64, 69) (*fig 2*). These prostanoids exit the cell via a carrier-mediated process and activate specific G-protein coupled receptors (GPCR) in target cells (60).



**Fig 2.** Biosynthesis of prostanoids via the COX pathway and tissue specific synthases. Specific GPCR receptors and COX-inhibitors (NSAIDs and coxibs) are included.

The COX enzyme exists in two isoforms termed COX-1 (70) and COX-2 (71). The isoforms are transcribed from distinct genes. COX-1 is situated on chromosome 9 whereas COX-2 is located on chromosome 1. The protein structure of the two isoforms is similar with the exception of the N terminal signal peptide region and a C terminal 18-amino acid insertion in the COX-2 peptide structure (72). This distinction results in broader substrate specificity of COX-2 (71, 73). Both COX-1 and COX-2 are localized in the endoplasmic reticulum, whereas COX-2 also is localized in the nuclear membrane (74). COX-1 is constitutively expressed in many tissues and is thought to be involved in the regulation of physiological responses and homeostasis, whereas COX-2 is mostly inducible and involved in inflammation (73). The human lung is one of the organs with highest level of COX (75). The rat lung also expresses high activity of COX. Both COX-1 and COX-2 are constitutively expressed in the normal rat lung (76), suggesting that both enzymes have a crucial role in the regulation of pulmonary and vascular responses in the lung (77, 78).

Non-steroidal anti-inflammatory drugs (NSAID), such as aspirin, indomethacin, and celecoxib, inhibit the COX pathway with varying selectivity for the COX-1 and COX-2 enzymes. Since COX-2 has been described to be upregulated in inflammatory processes, and COX-1 in the gastric mucosa catalyzes formation of protective prostaglandins, selective COX-2 inhibitors (coxibs) were developed. The coxibs indeed initially displayed effective anti-inflammatory properties and less gastro-intestinal side-effects (79, 80). However, since COX-2 also is involved in normal vascular homeostasis, the predicted outcome with less deleterious side-effects has not been achieved in long-term treatments with the selective COX-2 inhibitors celecoxib (Celebra®), CLASS study) and rofecoxib (Vioxx®, VIGOR study). The favourable effects on gastric bleedings were outweighed by an increased rate of serious cardiovascular events as myocardial infarctions (80). However, selective COX-2 inhibitors are valuable and well tolerated in aspirin-intolerant asthmatics (81).

#### *Effects of prostanoids in the lung*

Prostanoids are involved in various physiological and pathophysiological processes in the lung (64) and the local levels of prostanoids are high in the lower respiratory tract (82). The half-life of most PGs in the circulation is less than 1 minute. TXA<sub>2</sub> hydrolyses rapidly to the biologically inactive TXB<sub>2</sub> (69). TXA<sub>2</sub> is involved in

allergen-induced asthmatic responses by the activation of the thromboxane prostanoid (TP) receptor (6). Cytokine-induced bronchoconstriction is dependent upon COX-2 and TP receptor activation (83). PGD<sub>2</sub> is a pro-inflammatory mediator of allergic asthma (84) and recognized as a marker of mast cell activation (85). PGD<sub>2</sub> and PGF<sub>2α</sub> are causing ASM contractions via the TP receptor (86-88) and induce airway inflammation via the CRTH<sub>2</sub> receptor (84). In the lung, PGE<sub>2</sub> is produced by a variety of different cells, such as airway smooth muscle (89) and epithelial cells (90). PGE<sub>2</sub> is implicated to have a beneficial role in the lung (91, 92), since this prostanoid may attenuate bronchoconstriction (93) and allergic airway responses (94), and regulate airway tone (95). However, owing to the existence of various EP-receptors, the potential actions of PGE<sub>2</sub> in the lung are multiple: bronchodilation *via* the EP<sub>2</sub> and EP<sub>4</sub> receptor mediated cAMP elevation and bronchoconstriction *via* the EP<sub>1</sub> and EP<sub>3</sub>-mediated responses that involve increased intracellular Ca<sup>2+</sup> and decreased cAMP levels (93, 95). In addition, EP<sub>1</sub> receptors may interact with β<sub>2</sub>-receptors (96), resulting in desensitisation of the receptors and decreased responsiveness to β<sub>2</sub>-agonist. The role of PGI<sub>2</sub> or prostacyclin is less defined in airways but PGI<sub>2</sub> is a potent vasodilator (97).

### *Leukotrienes*

In 1938 Feldberg and Kellaway discovered, during studies of histamine release in the perfusate of guinea pig lung treated with cobra venom, the release of another factor from the lung that produced a contraction of isolated intestines that was slow in onset and sustained. This distinguished the factor from the rapid contraction caused by histamine. They therefore named the smooth-muscle-contracting factor slow-reacting substance (SRS) (98). Brocklehurst confirmed in 1960 the release of SRS-like activity following anaphylactic challenge of sensitised tissue in a perfused guinea pig lung model and he named the biological activity slow reacting substance of anaphylaxis, SRS-A (99). Following studies indicated that SRS-A was important as a mediator in asthma and other types of immediate hypersensitivity reactions. In 1979-80 Samuelsson and co-workers discovered the leukotrienes (66, 100, 101) and established that SRS-A was made up of three cysteinyl leukotrienes (CysLT), LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> (29). In confirmation of the generation of SRS-A from asthmatic lung by Brocklehurst, it was possible to show that allergen challenge of asthmatic lung

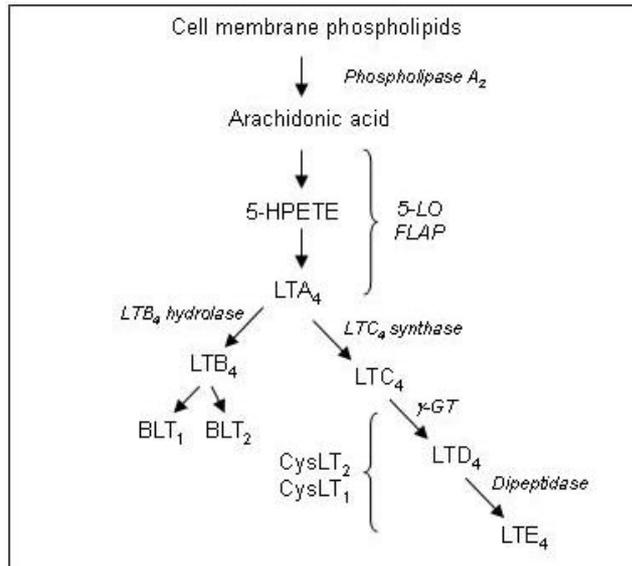
tissue released these three leukotrienes (102), and that the Schultz-Dale contraction of human asthmatic bronchi predominantly was due to the release of the CysLTs. The response in the anaphylactic contraction of human airways *in vitro* is nowadays established to be mediated predominantly by histamine, CysLTs and prostanoids (5, 103). CysLTs are 1000 times more potent than histamine to induce bronchoconstrictions (66, 104).

#### *Leukotriene generation by lipoxygenase enzyme*

Lipoxygenases are soluble enzymes located in the cell cytosol and found in most mammalian tissues and cells, including the lung, platelets, mast cells and leukocytes. After cell activation and in response to  $\text{Ca}^{2+}$  fluxes, 5-lipoxygenase translocates to the nuclear envelope and catalyzes, in interaction with 5-LO activating protein FLAP, the transformation of arachidonic acid to leukotriene A<sub>4</sub> (LTA<sub>4</sub>) via the unstable intermediate 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) (105, 106). LTA<sub>4</sub> is an unstable epoxide intermediate and further converted to leukotriene B<sub>4</sub> (LTB<sub>4</sub>) by LTA<sub>4</sub>-hydrolase or to cysteinyl leukotriene C<sub>4</sub> (LTC<sub>4</sub>) by LTC<sub>4</sub>-synthase. LTB<sub>4</sub> and LTC<sub>4</sub> are exported from the cell by different carrier systems (63). Extracellularly, the glutamic acid residue of LTC<sub>4</sub> is released from glutathione moiety by  $\gamma$ -glutamyl-transpeptidase ( $\gamma$ -GT) to generate LTD<sub>4</sub> from which the glycyl residue is cleaved by a dipeptidase to form LTE<sub>4</sub> (*fig 3*) (106, 107). Inhibitors of FLAP function prevent translocation of 5-LO from the cytosol to the membrane and inhibit 5-LO activation (106).

#### *Effect of leukotrienes*

Leukotrienes are involved in host defense reactions and play an important role in asthma, but may also contribute to other inflammatory diseases, such as inflammatory bowel disease, arthritis and atherosclerosis (106, 108, 109). Leukotrienes are released from granulocytes, mast cells, macrophages and platelets and acts via GPCR (110). LTB<sub>4</sub> is a potent chemoattractant for neutrophils, eosinophils and monocytes and has proinflammatory properties (111). LTB<sub>4</sub> acts via the BLT<sub>1</sub> (112) and the BLT<sub>2</sub> receptor (113). LTB<sub>4</sub> has been shown to cause constriction in the peripheral guinea pig lung via the BLT<sub>2</sub> receptor and this effect was indirect mediated by the release of histamine and thromboxane (114, 115).



**Fig 3.** Leukotriene biosynthesis from arachidonic acid. Specific GPCR receptors are included.

CysLTs mediate bronchoconstriction, increased vascular permeability and mucus production via the CysLT<sub>1</sub> and CysLT<sub>2</sub> receptor. In the guinea pig lung, LTD<sub>4</sub> and LTE<sub>4</sub> mediate contractions mainly via the CysLT<sub>1</sub> receptor, whereas LTC<sub>4</sub> mediates its effects via the CysLT<sub>2</sub> receptor. In human bronchi, each of LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> act on the CysLT<sub>1</sub> receptor (107). CysLTs are potent contractile agonists of ASM but have also a central role in airway inflammation and ASM hypertrophy and hyperplasia in severe asthma. CysLTs may contribute to airway remodelling with effects such as increase of airway goblet cells, mucus, blood vessels, smooth muscle and airway fibrosis (65). CysLT<sub>1</sub> receptor antagonists are used as therapy for asthma and have been shown to reduce airway inflammation in asthma (28, 116).

Interactions between 5-LO and 15-LO also generate lipoxins in the airways. Certain lipoxins have been implied to prevent airway hyperresponsiveness, dampen allergic pulmonary inflammation and inhibit eosinophil tissue infiltration (117). Interestingly, in severe asthma there appears to be a diminished biosynthesis of lipoxins in some inflammatory cells (118).

#### *G-protein coupled receptors*

Serotonin, histamine, leukotrienes and prostanoids mediate bronchoconstriction or dilations of ASM via activation of GPCR. The type of activation depends on receptor

expression, ligand affinity, signal transduction pathway and cell type. The GPCR is a seven transmembrane spanning protein and are generally located in the plasma membrane. Common regulations of the GPCR are desensitisation of the activated receptor and cross talks i.e. activation of other receptors via cellular pathways (119, 120).

## **Nitric Oxide**

### *The discovery of Nitric Oxide*

When Nobel was taken ill with heart disease, his doctor prescribed nitroglycerin. Nobel initially refused to take it. In a letter, Nobel wrote: “It is ironical that I am now ordered by my physician to eat nitroglycerin”. It has been known since last century that the explosive nitroglycerin has beneficial effects against chest pain. However, it would take 100 years until it was clarified that the nitroglycerin acts by releasing nitric oxide. ([http://nobelprize.org/nobel\\_prizes/medicine/laureates/1998/press.html](http://nobelprize.org/nobel_prizes/medicine/laureates/1998/press.html)).

In 1980 Furchgott and Zawadski described an endogenous factor that relaxed arterial smooth muscles in response to acetylcholine (121). The factor was later named endothelium-derived relaxing factor (EDRF). A few years later, in 1987, EDRF was demonstrated to be nitric oxide (122). A whole new area of research about nitric oxide began and NO is nowadays known to be an important mediator and regulator of many vital functions in the body (123).

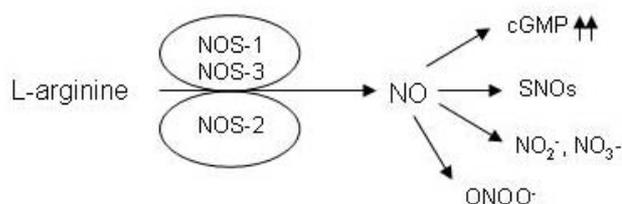
### *NO synthesis by different nitric oxide synthase*

NO is a small and reactive lipophil molecule that easily passes through the cell membrane and exerts its effect independently of cell surface receptors. NO have diverse effects on physiological and pathological responses (124). NO is a potent vasodilator, it increases vascular permeability and regulates function, death and survival of various cells involved in immunity and inflammation (125). NO is enzymatically generated in many different cells when nitric oxide synthase (NOS) catalyses the oxidation of L-arginine to NO and L-citrulline (*fig 4*). The NOS enzymes contain cytochrome P-450-like heme proteins that require molecular oxygen, NADPH, flavins and tetrahydrobiopterin as co-factors (124). At present, three known isoforms of NOS have been identified in the lung; NOS-1 (neuronal NOS, nNOS), NOS-2 (inducible NOS, iNOS) and NOS-3 (endothelial NOS, eNOS). These enzymes

are encoded by distinct genes and expressed in a wide range of pulmonary cells. NOS-1 is expressed in airway nerves localized in the ASM. NOS-2 is present in pulmonary macrophages, lung fibroblasts, alveolar type II cells, airway and vascular smooth muscle, endothelial cells and airway epithelial cells (126). Asthmatics have an increased expression of NOS-2 in airway epithelial cells (127). NOS-3 is expressed in endothelial cells of pulmonary circulation (126), in bronchial epithelium and in type II alveolar epithelial cells (128) and is thought to contribute to the regulation of ciliary beat frequency (129). NOS-1 and NOS-3 are constitutively expressed and calcium and calmodulin-dependent. In response to receptor stimulation by agonists that increases intracellular  $\text{Ca}^{2+}$ , picomolar concentrations of NO is released within seconds. The released NO mediates different effects, including relaxation in smooth muscle, through primary the activation of soluble guanylyl cyclase, which increase cyclic guanosine monophosphate (cGMP) in the target cell (130, 131). NOS-2 is calcium-independent and requires a number of co-factors. The enzyme is regulated at the level of transcription and can be induced by different cytokines like interferon- $\gamma$ , NF- $\kappa$ B and tumour necrosis factor- $\alpha$  (126). However, NOS-2 is also constitutively expressed in airway epithelium (132). The amount of NO produced by the inducible NOS is much larger (nanomolar) than after activation of the constitutive NOS and the induction of NOS-2 takes several hours (133).

NO has a short half-life of 1-5 seconds in biological tissues. This is due to the reactive properties of NO being a radical and the presence of oxyhemoproteins in the tissue. In intact cells, tissues and animals, NO has been shown to be oxidized to both nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ). Nitrate is the major metabolite (134). However, nitrite has been proposed to play an important role in signalling, blood flow regulation and nitric oxide homeostasis (135, 136). NO regulate its formation by competing with oxygen for a common binding site on the heme iron of the NOS (123). NO may interact with superoxide anion produced by the inflammatory cells. The superoxide anion production is significantly enhanced after the early and late phase allergic reactions. Superoxide rapidly inactivates NO, which leads to the formation of peroxynitrite ( $\text{ONOO}^-$ ) and nitrate tyrosine (137). Peroxynitrite is a highly reactive anion, it reacts and oxidizes many cellular components such as lipids and proteins, thereby disturbing their function and thus cellular homeostasis and aggravating the inflammation (123,

138). NO may also interact with thiols and form stable S-nitrosothiols (SNO) in the tissue. The role of SNOs is proposed to play a central role in airway physiology and regulation (139-141).



**Fig 4.** Generation of NO by NOS enzymes and intracellular pathways.

#### *Physiological role of NO in the airways*

NO is detectable in the exhaled air of guinea pigs, rabbits and humans (142). Asthmatics have increased levels of NO in their exhaled air (7), and exhaled NO is reduced shortly after an asthmatic bronchoconstriction (11). Exhaled NO is used as a biomarker of airway inflammation (143). However, administration of exogenous NO has also received considerable attention, mainly due to its therapeutic ability to exert haemodynamic effects (144). Nitric oxide (NO)-releasing NSAIDs, NO-NSAID, is a new class of anti-inflammatory and analgesic drugs generated by adding a nitroxybutyl moiety to the parent NSAID. The combination of balanced inhibition of the two main COX isoforms with release of NO confers to NO-NSAIDs reduced gastrointestinal and cardiorenal toxicity (145). More recently, the strategy of linking NO-releasing functional groups to a parent molecule has been extended to compounds that are chemically and pharmacologically unrelated to NSAIDs. The common goal is either to enhance the therapeutic efficacy or to improve side effects.

#### *Nitric oxide and potential interactions with histamine and eicosanoids*

Endogenously produced NO has been reported to have an inhibitory effect on bronchial obstruction in guinea pigs (146). Mast cells produce NO and express NOS-3 (12). NO has been implicated as a regulator of mast cell activation and mast cell mediated inflammation (147). NO has been shown to inhibit the release of histamine from rat mucosal mast cells (14). A co-localisation of NOS with 5-LO along the nuclear membrane has recently been suggested, providing a potential interaction

between NO and leukotriene synthesis in human mast cells (15). Alveolar macrophages produce NO via constitutively expressed NOS-3 (148) and the released NO may act on 5-LO and suppress leukotriene synthesis (149, 150). Inducible NOS has been implicated to mediate NO production leading to PGH<sub>2</sub> synthase nitration, and suppressed eicosanoid productions in vascular cells (151). NOS-inhibition decrease the formation of prostaglandins (152-154) and the mechanism behind this interaction of PGs and NO has recently been proposed since NOS-2 S-nitrosylates and activates COX-2 and thereby increases the synthesis of prostaglandins (155).

Thus many different actions of NO with relevance to antigen-induced responses have been described. The overall question remains whether NO is beneficial or deleterious in the pulmonary system.

## **AIMS OF THE THESIS**

The overall aim of this experimental study was to analyse the actions and interactions of NO and different eicosanoids on the early allergic airway response in the peripheral part of the lung.

- ❖ Nitric oxide is produced in high amounts in the airways of asthmatics and measured in exhaled breath as a sign of inflammation. The function of NO in the distal airways and lung is however not completely understood. The aim of paper I was to characterise the role of endogenous NO on antigen-induced contractions and mediator release in the peripheral lung by characterising the effects of inhibiting NO synthesis in the GPLP ovalbumin model.
- ❖ NO donors are widely used as vasodilators and have been suggested to be bronchodilators. However, it was hypothesized that in the peripheral lung the function of NO may be to regulate the release of inflammatory mediators during anaphylaxis rather than to relax smooth muscle. The aim of the study reported in paper II was to determine the effect of two structurally different NO donors, NCX 2057 and sodium nitroprusside (SNP), on antigen-induced contractions and mediator release in the peripheral lung, also using the GPLP ovalbumin model.
- ❖ A new class of antihistamines coupled to NO donors has been developed with the hypothesis that such compounds may have anti-allergic and anti-inflammatory properties beyond those of the parent antihistamine. The aim of the study reported in paper III was threefold: to establish if the compounds retained the antihistamine action of the parent compound cetirizine; to assess the efficacy of the new compounds as NO donors; and to test if they had broader anti-allergic activity than cetirizine in the lung. In addition to the GPLP ovalbumin model, tracheal, intestinal and vascular smooth muscle preparations were included in this study.

- ❖ The lung parenchyma is a complex tissue involving airways, vessels and airway smooth muscles. The aim of the study reported in paper IV was to establish the guinea pig precision cut lung slices (GP PCLS) and thereby better characterise the responses in the antigen-induced contractions in the peripheral guinea pig lung. The study included comparison of responses to mediators and antigen in GP PCLS and human PCLS.
  
- ❖ Although serotonin is the major mediator of the antigen-induced contractions of the rat lung, prostanoid mediators of the COX pathway have been implicated to play a role in the early allergic airway response. The aim of the study reported in paper V was to determine the role of the COX pathway and the influence of prostanoids during the early allergic airway response in the rat PCLS.

## **METHODS**

### **General**

To study the impact of NO and eicosanoids on the early allergic airway response in the peripheral lung, *in vitro* studies were performed with airway preparations (lung parenchyma and tracheal rings) and vessel preparations (pulmonary arteries and aorta) in classical organ bath set ups (paper I, II, III). To better understand and characterise the contractile responses obtained in the lung parenchyma tissue, studies in cultured precision cut lung slices (PCLS) were performed (paper IV, V). The release of mediators after antigen challenge to the bath fluid/medium were analysed with enzyme immuno assays (EIA) (paper I, II and V). The generation of nitric oxide was measured indirect as nitrite by a chemiluminescence technique (paper II and III).

### **Ethical approval**

The animal experiments were approved by the local ethic committees in Stockholm, Sweden (N14/02 and N127/04) (papers I, II and III) and in Milano, Italy (N124/2003-A) (paper III). The animal experiments and access to human lung material were approved by the local ethic committee in Germany (papers IV and V).

### **OVA sensitisation**

There are two different ways of sensitisation procedures for studies of the early allergic airway response; *active* and *passive* sensitisation.

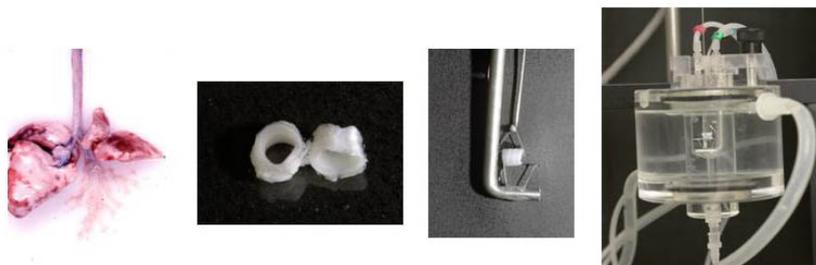
For studies of antigen-induced contractions in the GPLP and GPT, male *Dunkin Hartley* guinea pigs (300-350 g b.w.) were actively sensitised to Chicken Egg Albumin, ovalbumin, (OVA) at least four weeks prior to experiment. A stock solution of OVA was prepared by dissolving 500 mg OVA in 10 mL 0.9 % NaCl and 10 mL 2 % aluminium hydroxide gel and shaken for one hour. The guinea pigs were given a subcutaneous injection of 0.4 mL OVA (10 mg) in the neck and an i.p. injection of 0.4 mL OVA (10 mg). Studies of guinea pig sensitisation show that small amounts of antigen together with aluminium hydroxide produced both IgE and IgG1 antibodies (156). Antigen-induced contractions in the GPLP and GPT were studied by adding cumulative doses of ovalbumin (1-100,000 µg/L) to the organ bath preparations.

Passive sensitisation was used in the PCLS experiments. Passive sensitisation of human isolated bronchi has been shown to induce mast cell degranulation and release of the mast cell marker tryptase (157). For antigen studies in the rat PCLS, cell culture medium for overnight incubation was supplemented with 1% serum from actively sensitised Brown Norway rats (58). Guinea pig PCLS were treated and incubated overnight with 1% serum from guinea pigs, that had been actively sensitised with OVA as previously described. Human PCLS were incubated overnight with 1% serum from sensitised subjects with grass pollen allergy (158). The following day, slices were transferred into a 24-well plate with fresh medium and put under the microscope. A control image was taken, before the allergen was added. The medium was not changed until measuring occurred.

### **Organ bath experiments**

#### *Lung parenchymal strips, tracheal rings and vessel preparations*

The functional responses were studied in organ baths with airway and vessel preparations. Tracheal rings, lung parenchyma, aorta, pulmonary artery and ileum were prepared from sensitised and non-sensitised guinea pigs. Guinea pigs (500-900 g b.w.) were sacrificed by an overdose of inhaled CO<sub>2</sub> and the heart-lung-package was quickly removed and placed in ice-cold Tyrode's solution (prepared each day, containing NaCl 149.2 mM, KCl 2.7 mM, NaHCO<sub>3</sub> 11.9 mM, glucose 5.5 mM, CaCl<sub>2</sub> 1.8 mM, MgCl<sub>2</sub> 0.5 mM, NaH<sub>2</sub>PO<sub>4</sub> 0.4 mM). The lung parenchyma was cut parallel to the peripheral margins, yielding four to eight strips, each having a size of 2x2x20 mm. The trachea was gently cleared from connective tissue and cut into eight rings, referred as guinea pig tracheal rings (GPTR) (*fig 5*).

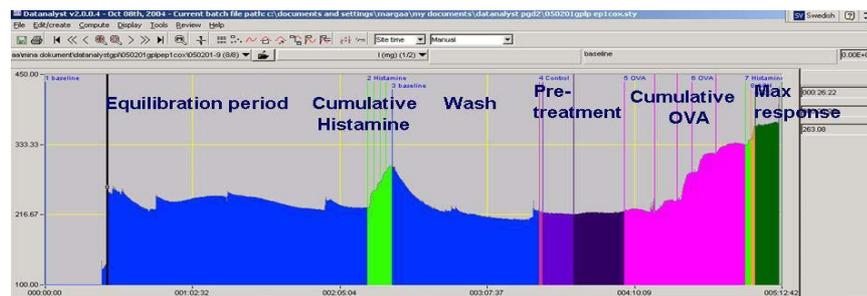


*Fig 5. The guinea pig lung en bloc and preparation of tracheal rings in organ baths*

The lung parenchymal strips were set up at a resting tension of 2.5 mN (0.25 g) and the tracheal rings at a resting tension of 30 mN (3 g) in 5 mL organ baths filled with Tyrode's solution, bubbled with carbogen gas (6,5 % CO<sub>2</sub> in O<sub>2</sub>) to keep a pH of 7.4, and the temperature was kept at 37°C. Changes in smooth muscle tension, i.e. contractions and relaxations, were recorded via isometric force-displacement transducers connected to a Grass polygraph, and responses were displayed via a chart-recorder or by using the IOX data acquisition system (EMKA, France). Data were analysed either manually from charts or by the software program Dataanalyst (EMKA, France) (*fig 6*).

After an equilibration period of 90 min and washes each 15 min, histamine (1-30 µM) was added cumulatively as a control of the GPLP and GPT reactivity. Another wash and equilibration period between histamine and treatment period was performed. Histamine (1-100µM), LTD<sub>4</sub> (0.1-100nM) or OVA (1-10,000 µg/L) were added as cumulative challenge of increasing concentrations without changing bath fluid. For study of relaxations, the GPLP was pre-contracted with a single dose of LTD<sub>4</sub> (10 nM). Maximum contractions of the preparation were determined with histamine (1 mM), acetylcholine (1 mM) and KCl (50 mM) at the end of each experiment, and other responses were expressed as percent of maximum contractions.

Guinea pig pulmonary artery (GPPA) and aorta (GPA) were prepared as rings with the endothelium gently removed, and then mounted in the organ baths under a resting tension of 15 mN (1.5 g). After an equilibration period of 60 min and washes each 10 min, noradrenaline (10 µM) was added and drugs were given at the plateau to study relaxations.



**Fig 6.** Representative tracing of a GPLP organ bath experiment.

### **Precision-cut lung slices (PCLS)**

Pharmacological and physiological studies were carried out in precision-cut lung slices (PCLS), an *in vitro* model for study of peripheral airway responses. Studies were done in passively sensitised and non-sensitised rat, guinea pig and human lung. The PCLS is a relatively new method to study airway and vessel pharmacology and physiology (58, 158). PCLS consist of very thin lung slices that have both airways and vessels in the same preparation. The slices are maintained alive in medium for three days having functional airway ciliar beating and mucus transport. Physiological studies of airways and vessels are done by comparing the area of the airway or the vessel before and after treatment with different drugs. The preparations are imaged and digitised with a digital video camera.

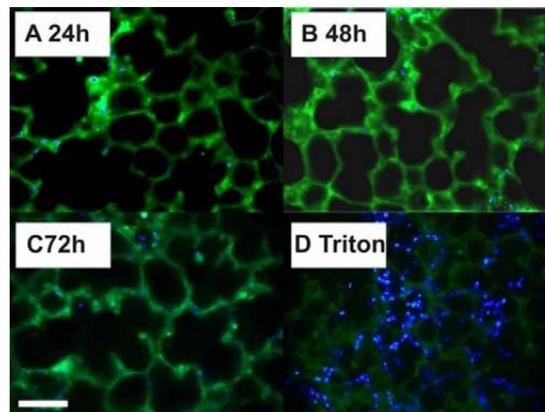
#### *Preparation of PCLS*

Female Dunken Hartley guinea pigs (350g±30g) and female Wistar rats (200g±20g) were obtained from Charles River (Germany). Guinea pig PCLS were prepared with the following modifications. After injection of pentobarbital (95 mg/kg) the trachea was cannulated and the animals exsanguinated by cutting the vena cava inferior. Through the cannula the lung was filled with a low-melting point agarose solution (0.75%, final concentration) containing isoproterenol (1 µM). In order to solidify the agarose and harden them for cutting, the lungs were placed on ice for 10 min. The lobes were separated and tissue cores prepared with a rotating sharpened metal tube (diameter 8 mm). These cores were cut into 220 µm thick slices with a Krumdieck tissue slicer (Alabama Research and Development, Al, USA). Human PCLS were prepared as recently described (158). The slices were incubated at 37° C in a humid atmosphere in minimal essential medium (pH 7.2) composed of CaCl<sub>2</sub> (1.8 mM), MgSO<sub>4</sub> (0.8 mM), KCl (5.4 mM), NaCl (116.4 mM), glucose (16.7 mM); NaHCO<sub>3</sub> (26.1 mM), Hepes (25.17 mM); sodium pyruvate, amino acids; vitamins; glutamine and isoproterenol (1 µM) for maximal 3h. Without the addition of isoproterenol there was a sustained post mortem bronchoconstriction of the guinea pig airways that was sustained over time. The isoproterenol was washed away and did not influence the experiments occurring the following day (paper IV). The medium was changed every 30 min during the first two hours followed by a change every hour for the next two hours, in order to remove the agarose and cell debris from the tissue. Subsequently, medium was further supplemented with penicillin and streptomycin and changed

every 24 hours. The rat and guinea pig slices were studied the following day, but remained functional within 72 hours.

#### *Viability of the slices*

Viability of the rat and guinea pig slices was assessed by measuring the relative amount of LDH released from the slices into the incubation medium as described (paper IV). Viability of the slices was expressed as the ratio of LDH in the supernatant to the total LDH (sum of LDH in slices and the supernatant). To visualize the viability of the guinea pig PCLS a 2-photon microscopy was used in combination with the LIVE/DEAD<sup>®</sup> viability/cytotoxicity assay kit (Molecular Probes, Eugene, Oregon, USA) as described (paper IV). The emission of Calcein AM for the cytoplasm (live staining, green) and the EthD for the staining of nuclei (dead staining, blue) were recorded separately. Slices were analyzed 24, 48, and 72h after preparation. To visualize the total amount of dead cells, some PCLS were treated with 1% Triton X-100 for 20min prior incubation with dyes (*fig 7*).

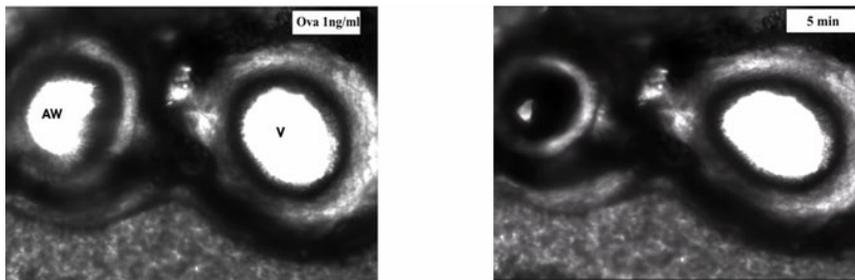


*Fig 7. LIVE/DEAD staining of GP PCLS to visualize viability over time. (Overlay images of Live staining, green and Dead staining, blue)*

#### *Measurement and Imaging*

The airways and vessels were imaged and digitized with a digital video camera. Airway or vessel area before addition of the mediators was defined as 100%. Bronchoconstriction or vasoconstriction was determined as the percentage of airway/vessel area of the initial area. For the measurements, slices with comparable airway size were selected. These slices were put on 24-well-plates and fixed with a nylon thread attached to a platinum wire. The slices were continuously covered with 1

ml incubation medium and kept at about 37°C. The 24-well plate was positioned on the stage of an inverted microscope. Images were recorded by analogue (JAI 2040, JAI Pulnix, Alzenau, Germany) or digital camera (IRB640, Visitron Systems, Munich, Germany). A control image was taken before addition of the mediator, frames were recorded every 30s for 5, 10 or 20 min depending on the study (fig 8).



**Fig 8.** Image of antigen-induced contractions after challenge with OVA 10ng/ml in the GP PCLS.  
*Notice the complete closure of the airway, whereas the vein remains unaffected.*

#### **Measurements of released mediators after antigen-challenge**

Bath fluid was collected from the tissue baths and immediately frozen at  $-20^{\circ}\text{C}$ . The samples were taken at the end of the equilibration period to obtain basal mediator release from the tissue and at the obtained contractile plateau to 100 $\mu\text{g/L}$  or 1000 $\mu\text{g/L}$  of OVA during challenge with cumulative doses of OVA (1-10,000 $\mu\text{g/L}$ ). Medium fluid was collected from well-plates containing six rat lung slices at three different time points; after incubation with medium, medium  $\pm$  pretreatment drugs and after challenge with OVA 10 $\mu\text{g/ml}$ .

#### *Enzyme immunoassay (EIA) analyses*

Determination of eicosanoids is based on competition of the eicosanoid and an eicosanoid-acetylcholinesterase (ACh) tracer. These substances compete for binding places on an antibody coated 96-well plate. A fixed amount of tracer is added, while the eicosanoid concentration differs in the test sample. 50 $\mu\text{l}$  of both tracer and test sample is added to the plate well. The more the eicosanoid binds the coated antibody, the less tracer will bind. To convert this to a spectrophotometrically visible signal, Ellman's reagent is added to the well. Ellman's reagent consists of acetylthiocholine which is converted to thiocholine. On its turn, thiocholine will be non-enzymatically

converted to 5-thio-2-nitrobenzoic acid, which strongly absorbs at 412 nm. If much tracer has been bound, there will be a strong yellow signal, i.e. there is an inverse relationship between the signal and the amount of the eicosanoid. EIA analyses were used for the different mediators CysLTs, LTB<sub>4</sub>, TXA<sub>2</sub>, PGD<sub>2</sub> and PGE<sub>2</sub> and performed according to the manufacture's instructions. TXA<sub>2</sub> was measured as the stable metabolite TXB<sub>2</sub>. CysLTs were measured as LTE<sub>4</sub>, the end metabolite of LTC<sub>4</sub> and LTD<sub>4</sub>. PGD<sub>2</sub> was measured as PGD<sub>2</sub>-mox. The assay detection limits for the different mediators were 7.8 pg/mL for TXB<sub>2</sub>, PGD<sub>2</sub>-mox and LTE<sub>4</sub> and 3.9 pg/mL for LTB<sub>4</sub>. Results below detection limits were set as zero in the statistical evaluation. The EIA specificity for the different mediators to interfere with each other was less than 0.01%, with the exception of the TXB<sub>2</sub> EIA that cross reacted with PGD<sub>2</sub> (0.53%) and with PGE<sub>2</sub> (0.09%). The LTE<sub>4</sub> EIA was performed with the cysLT antiserum and cross reacted with both LTC<sub>4</sub> (50%) and LTD<sub>4</sub> (100%). The PGE<sub>2</sub> EIA cross reacted with 8-iso- PGE<sub>2</sub> (37.4%).

#### *Histamine measurements*

Histamine was measured as described previously (159, 160). Briefly, 100µL of NaOH 1M was added to 500 µL sample and to histamine standards (1.9 - 1000 ng/mL). 25µl of OPT 0.1% (a fluorescence substance) was supplied to each sample and histamine standard after exactly 4 min the reaction was stopped by the addition of 50µL of HCl 3M. Duplicates of 300 µL were placed in 96-wells plates and the amount of histamine was analysed by a fluorometer at the wavelength 450 nM within 30 min and compared to the histamine standard response curve. The detection limit for histamine was 3.9 ng/mL.

#### *Serotonin measurements*

Serotonin was analysed with a standard serotonin ELISA-kit (RE59121) obtained from IBL-Hamburg, Germany and measured according to the instructions from the manufacture. Briefly, the sample preparation (derivatization of serotonin to N-acetylserotonin) is part of the sample dilution and is achieved by incubation of the respective sample with an acylation reagent. The assay procedure follows the basic principle of competitive ELISA whereby there is competition between a biotinylated and a non-biotinylated antigen for a fixed number of antibody binding sites. The

amount of biotinylated antigen bound to the antibody is inversely proportional to the analyte concentration of the sample. When the system is in equilibrium, the free biotinylated antigen is removed by a washing step and the antibody bound biotinylated antigen is determined by use of anti-biotin alkaline phosphatase as marker and p-nitrophenyl phosphate as substrate. Quantification of samples is achieved by comparing the enzymatic activity of samples with a response curve prepared by using known standards. The plate is analysed at the reading absorbance at 405 nm (reference wavelength 600-650 nm) and expressed as ng/mL.

### **Measurements of indirect NO formation**

Fluid was collected after 15, 30, 60, 90 and 120 minutes from the GPTR and GPLP organ baths after addition of 1 $\mu$ M or 100 $\mu$ M of different NO donors to assess the formation of nitrite over time as an indirect measurement of NO. The formation of nitrite without tissue preparations in the organ baths was also assessed. Nitrite and nitrate was analysed with a chemiluminescence method as previously described (161), where amount of NO in the samples are reduced to either nitrite or nitrate.

The kinetics of release of NO was studied for compounds NCX 2057, NCX 1512 and SNAP using HbFe(II)NO formation in rat whole blood, measured by EPR spectroscopy as previously described (162). The concentration of HbFe(II)NO in the incubation mixture reflects the quantity of nitric oxide accumulated at different times.

### **Calculations and statistics**

Organ bath data were expressed as percent of maximum contractions. The contractions obtained in PCLS were presented as area under the curve. Statistical analyses were made for paired and unpaired observations by Student's t-test or analyses of variances (ANOVA) followed by Tukey's t-test or Bonferroni's t-test. A p-value of less than 0.05 was considered significant. EC<sub>50</sub> values were calculated by linear regression and the pD<sub>2</sub>-value was calculated as the negative log of the EC<sub>50</sub>-value. The EC<sub>50</sub> value represents the drug concentration required to produce 50% relaxation or contraction of the tissue.

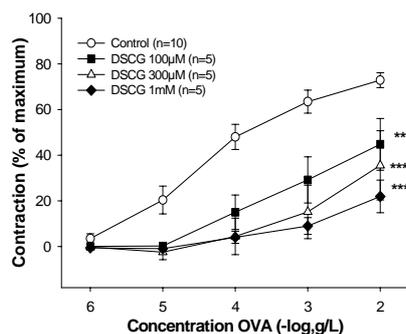
## RESULTS AND DISCUSSION

### Mediators involved in antigen-induced contractions

The work in this thesis has focused on the mediators that are involved in the early allergic airway response, i.e. the bronchoconstriction. However, also other important mediators are released upon mast cell activation. Most of these mediators, such as chemokines and cytokines, are primarily involved in the ongoing airway inflammation, or involved in the airway remodelling process (163).

### *Ovalbumin-induced contractions of the peripheral lung*

Cumulative concentrations of OVA (1-10,000 $\mu$ g/L) induced dose-dependent contractions of the GPLP and the GP PCLS. Furthermore, the generation of leukotrienes, prostanoids and histamine in the bath fluid was significantly increased in the GPLP after challenge with OVA (Paper I and II) with the following relative amounts: histamine  $\gg$  TXB<sub>2</sub>  $\gg$  PGD<sub>2</sub> > CysLTs > PGE<sub>2</sub> > LTB<sub>4</sub>. The contractions to OVA and the release of mediators from GPLP were dose-dependently inhibited by the mast cell stabiliser disodium cromoglycate (DSCG; 100-300-1000 $\mu$ M) (*fig 9, unpublished data*), supporting that the release of leukotrienes, prostanoids and histamine was due to mast cell activation. These concentrations of DSCG did not cause general depression of tissue contractility as the maximal response was unaffected.



**Fig 9.** Concentration-dependent contractions to cumulative doses of OVA (1-10,000 $\mu$ g/L) after pretreatment with the mast cell stabiliser DSCG (0.1, 0.3 and 1 mM) compared to control. Data are expressed as mean  $\pm$  SEM.

Likewise, the induced contractions to OVA in the GP PCLS were completely blocked by the combination of the antihistamine tripolidine, the CysLT<sub>1</sub> receptor antagonist montelukast and the TP receptor antagonist SQ29548 (paper IV). The data in GP

PCLS are similar to the results obtained in the GPLP (fig 2a, unpublished data) where only combined treatment of antagonist or synthesis inhibitors to histamine, leukotrienes and prostanoids reduced the contractile response to ovalbumin. These data confirm previous antagonist data in GPLP (17). The results obtained in the GP PCLS and GPLP are also consistent with data in antigen challenge of the perfused and ventilated guinea pig lung (18), indicating that histamine, CysLTs and several prostanoids are the dominant mediators of the antigen-induced airway constriction in this particular species.

In the rat lung, the antigen-induced contractions are mainly mediated by serotonin (paper V). Ketanserin, the 5-HT<sub>2A</sub> antagonist, completely blocked the early allergic airway response induced by both a single high dose (58) and a single low dose of OVA (paper V). The contractions to cumulative doses of OVA were also completely blocked by ketanserin. The critical role of serotonin was further substantiated by the increased release of serotonin from the slices after allergen challenge, a finding that had not been shown in PCLS before (paper V). These data confirm the view that serotonin is the major mediator in allergen-induced bronchial anaphylaxis in the rat (58, 59). In addition to serotonin, the antigen-induced contractions in the rat PCLS appear to be modulated by prostanoids but not by leukotrienes (paper V).

The PCLS technique allows direct evaluations of airway responses of guinea pigs to those from rats or humans in the same model. The similarity between humans and GP became evident during comparisons of mediators involved in the early allergic airway response in the guinea pig, rat and human PCLS (Table 1).

Mediators	Rat	Human	Guinea pig
Serotonin	X	-	-
Histamine	-	x	X
Prostanoids	x	X	X
CysLT	-	X	X

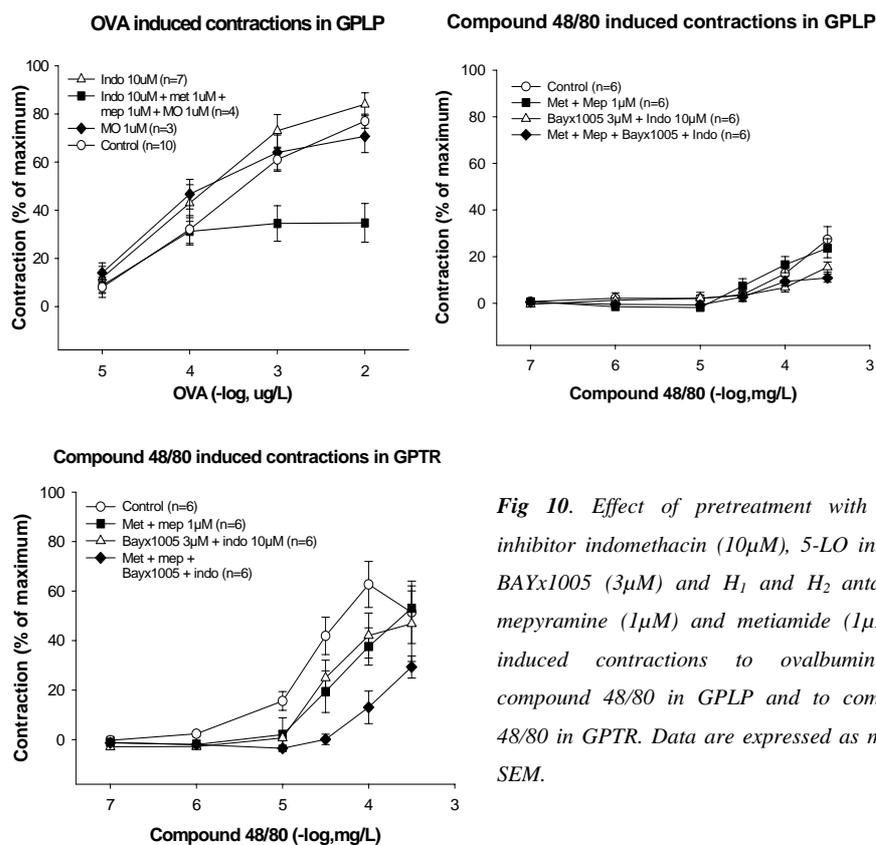
**Table 1: Mediators involved in the early allergic airway response of PCLS from different species.** Data from rat (58) (paper V), human (158) and guinea pig (paper IV). Effects of antagonists and synthesis inhibitors on mediators involved in antigen-induced responses. X, effective; x, partially effective, -, non effective.

The data show that the guinea pig is the species that is most similar to human in respect to lung pharmacology (paper IV). In guinea pig and human, both prostanoids and leukotrienes contribute to the early allergic airway response. The major difference relates to histamine which is more important in guinea pigs. However, also within the guinea pig lung there are differences in the response to histamine. During challenge with OVA (paper III) the distal lung emerges to be almost insensitive to antihistamine in comparison with more proximal airways. In the trachea, the histamine component of the antigen-induced contraction dominates (164), whereas in the GPLP, antihistamines alone have no inhibitory effect on the antigen-induced contraction (17). In contrast, in the rat lung, none of these mediators appear to play a role in the antigen-induced contractions. Instead they are almost exclusively mediated by serotonin (paper V); (58). Therefore, the established GP PCLS emerges to be a most relevant and valuable method for studies of the early allergic airway response in comparison with human lung pharmacology.

*Mast cell activation: Comparison of compound 48/80 and antigen challenge*

Compound 48/80 contracts the airways in a similar manner as antigen in guinea pig trachea and has been suggested as a useful tool (165) to study mediator release and airway smooth muscle contraction. Compound 48/80 has been implicated to be a potent inducer of mast cell degranulation, and histamine and leukotrienes are released from mast cells after stimulation in both guinea pig and rat airways (165, 166). However, the GPLP only weakly responded to compound 48/80 in comparison to challenge with OVA (*fig 10, unpublished data*). One plausible explanation would be that the distribution of mast cell in the lung is different between proximal and distal airways. Studies in the rat trachea showed that compound 48/80 caused degranulation of connective tissue mast cells, but not of mucosal mast cells (166), and guinea pig lung mast cells were refractory to the action of compound 48/80 but not to antigen challenge after active sensitisation (167). Pretreatments with compound 48/80 or with sodium cromoglycate (SCG) aerosolization significantly reduced OVA-induced contractions of rat trachea distal segments, whereas the contractions of proximal segments were reduced only by compound 48/80 (168). However, another study indicated that both rat connective tissue and mucosal mast cells are involved in the response to compound 48/80 (169). These results further emphasize the heterogeneity

of mast cells in the lung and between different species. It has been reported that the distribution of pulmonary mast cells increases along the bronchial tree towards the lung parenchyma in guinea pigs, whereas the highest concentration of mast cells are located in the central airways in the rat lung (170). Such variation in location of mast cell phenotype may account for variations in the intensity of contraction seen after the addition of OVA between proximal and distal airways and species.



**Fig 10.** Effect of pretreatment with COX-inhibitor indomethacin (10µM), 5-LO inhibitor BAYx1005 (3µM) and H<sub>1</sub> and H<sub>2</sub> antagonist mepyramine (1µM) and metiamide (1µM) on induced contractions to ovalbumin and compound 48/80 in GPLP and to compound 48/80 in GPTR. Data are expressed as mean ± SEM.

### The role of eicosanoids in antigen-induced contractions

*The impact of prostanoids in the early allergic airway response in the peripheral lung*  
 Prostanoids have been implicated to be involved in the early allergic airway response in different species. COX inhibition significantly enhanced the contractions to cumulative doses of OVA (1-10,000µg/L) in the guinea pig lung, suggesting a beneficial role of COX derived mediators in the peripheral lung. The enhancement was only seen in the presence of the unselective COX inhibitor indomethacin (10µM)

and the selective COX-1 inhibitor SC560 (10 $\mu$ M), whereas the selective COX-2 inhibitor celecoxib (10 $\mu$ M) had no effect on the antigen-induced contractions, implying a beneficial modulatory role of products formed by COX-1 in the peripheral lung (paper I).

In contrast, in the rat lung the antigen-induced contractions to OVA 10 $\mu$ g/ml were significantly attenuated by COX inhibition. This was furthermore caused by the unselective COX inhibitor indomethacin (10  $\mu$ M), the selective COX-1 inhibitors SC560 (10 $\mu$ M) and FR-122047 (5 $\mu$ M) and the selective COX-2 inhibitor celecoxib (10 $\mu$ M), implicating a role of products formed by both COX-1 and COX-2 in the peripheral rat lung (paper V).

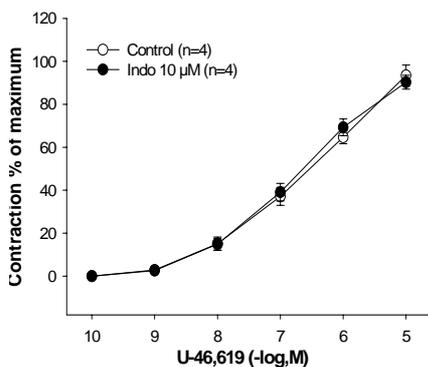
#### *Prostanoid formation due to antigen challenge*

Basal levels of prostanoids in the organ bath were not measurable (PGD<sub>2</sub>) or very low (TXB<sub>2</sub> and PGE<sub>2</sub>), whereas measurable levels were obtained for TXB<sub>2</sub>, PGE<sub>2</sub> and PGD<sub>2</sub> after challenge with OVA in GPLP. The release of TXB<sub>2</sub> was increased 15-fold and PGE<sub>2</sub> was increased five-fold above basal levels (paper I and II). Again, the situation was quite different in the rat PCLS, where medium contained relatively high basal levels of PGE<sub>2</sub>, TXB<sub>2</sub> and PGD<sub>2</sub>. Moreover, these high values were not affected by challenge with OVA (10 $\mu$ g/ml) (paper V). The release of serotonin was however significantly increased after addition of OVA and serotonin release was unaffected by the different COX-inhibitors. Both COX-1 and COX-2 inhibitors reduced the formation of TXB<sub>2</sub> and PGD<sub>2</sub>, whereas only COX-2 inhibition reduced the biosynthesis of PGE<sub>2</sub> (paper V). As the potent COX-1 inhibitor FR-122047 (171) did not affect the formation of PGE<sub>2</sub>, the data strongly suggests that the generation of PGE<sub>2</sub> was due to COX-2 activity in this particular lung preparation. The source of PGE<sub>2</sub> may be alveolar epithelium cells (172) and airway smooth muscle cells (89, 173), since PGE<sub>2</sub> is generated in high amounts by the COX-2 pathway in these cells. The COX-inhibitors did however not completely block basal biosynthesis of prostanoids in the rat PCLS (paper V). One reason for this may be the production of isoprostanes that are produced independent of the cyclooxygenase pathway (174).

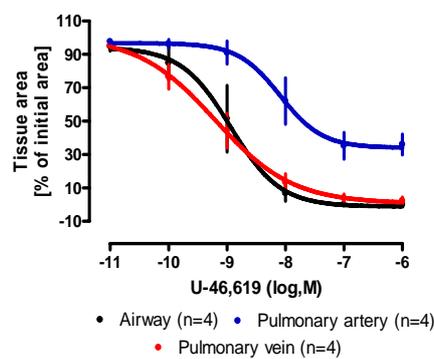
### *Effect of measured prostanoids in the peripheral lung*

The measured prostanoid mediators have been implicated in asthma. PGD<sub>2</sub> is a potent contractile agonist of both airways and vessels in the GP PCLS (paper IV). The synthesis of PGD<sub>2</sub> from the mast cells during the early allergic airway response (<30 min) is proposed to be generated by COX-1, whereas PGD<sub>2</sub> synthesised during the delayed phase has been ascribed to depend on COX-2 activity (175), implicating that PGD<sub>2</sub> released after challenge with OVA in the GPLP (paper I and II) was due to COX-1 activity.

TXA<sub>2</sub> may be involved in the early allergic airway response since the TP receptor antagonist SQ29548 caused attenuation of the antigen-induced contractions in both human (158) and GP PCLS (paper IV) and another TP receptor antagonist BAYu3405 attenuated the first five minutes of the OVA response in the rat PCLS (paper V). However, as the ASM contraction to PGD<sub>2</sub> also is mediated by TP receptors (86, 87), it remains possible that both PGD<sub>2</sub> and TXA<sub>2</sub> were involved in the early allergic airway response. The thromboxane receptor agonist U-46,619 potentially induced contractions of the airways in human PCLS (paper IV), in the GPLP (*fig 11a, unpublished data*) and of both vessels (pulmonary artery and vein) and airways in the GP PCLS (*fig 11b, unpublished data*), indicating that both vessels and airways are responding to the TP receptor agonist U-46,619 in the peripheral lung tissue.

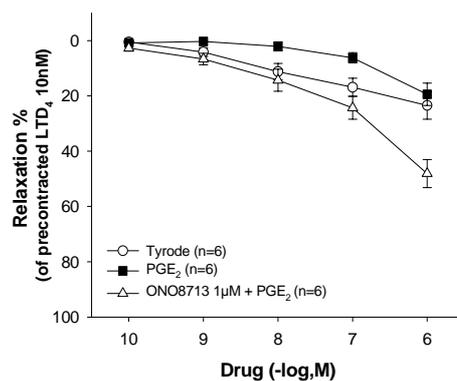


**Fig 11a.** COX-inhibitor indomethacin did not change the cumulative concentration response to the thromboxane agonist U-46,619 in GPLP. Data are expressed as mean  $\pm$  SEM.



**Fig 11b.** The thromboxane agonist U-46,619 induced concentration dependent contractions of airways, pulmonary vein and artery in GP PCLS. Data are expressed as mean  $\pm$  SEM.

The effects of PGE<sub>2</sub> on the investigated airway preparations were complex and related to species and the state of tissue (precontracted or at basal tone) and demonstrate a dual role of PGE<sub>2</sub> in the airways. Thus, in the rat PCLS (paper V) the antigen-induced contractions were significantly reduced by the selective EP<sub>1</sub> receptor antagonist ONO-8713 (176). In the GPLP, pretreatment with exogenous PGE<sub>2</sub> (100nM) significantly attenuated the contractions to OVA and reversed the enhancement induced by COX-inhibition (paper I), suggesting that endogenous PGE<sub>2</sub> inhibits the antigen response in this particular model. PGE<sub>2</sub> (0.1-1-10 $\mu$ M) did not induce contractions of either rat PCLS or GPLP (*unpublished data*). Precontracted lung parenchyma relaxed to cumulative doses of PGE<sub>2</sub> (0.1-1000nM) after pretreatment with the EP<sub>1</sub> receptor antagonist ONO-8713 (*Fig 12, unpublished data*), suggesting that PGE<sub>2</sub> is maintaining airway tonus via the EP<sub>1</sub> receptor and mediating dilations via the EP<sub>2</sub>/EP<sub>4</sub> receptors. The data obtained in the GPLP implicate a mainly beneficial role of PGE<sub>2</sub> during antigen-induced contractions in the peripheral lung. The modulatory effect of PGE<sub>2</sub> in the GPLP may accordingly in part relate to relaxation of airway smooth muscle, but presumably inhibition of mast cell mediator release is another important mechanism (92, 177, 178). In the rat PCLS, endogenous PGE<sub>2</sub> appears instead to contribute to bronchoconstriction via the EP<sub>1</sub> receptor during the early allergic airway response, and this as modulator rather than mediator of the antigen-induced contractions (see below).



**Fig 12.** Pretreatment with the EP<sub>1</sub>-antagonist ONO-8713 (10 $\mu$ M) caused relaxations to PGE<sub>2</sub> in the GPLP. Data are expressed as mean  $\pm$  SEM.

#### *Role of prostanoids and serotonin in antigen-induced contractions in the rat*

Despite the pharmacological evidence of prostanoid involvement in the early allergic airway response in the rat lung, there were no increased levels of TXB<sub>2</sub>, PGD<sub>2</sub> or PGE<sub>2</sub> after antigen-challenge (paper V). One explanation for this discrepancy may be

the high baseline levels of prostaglandin (2ng/slice), making it difficult to assess any additional prostanoid release with the current methods. The formation of prostanoids by rat mast cells, where both COX-1 and COX-2 were found (76) is altered by treatment with different COX inhibitors. This would indicate the possibility of a modulatory effect of the COX pathway on the antigen response at the level of the mast cell activation/degranulation. However, the data in this thesis do not provide direct evidence of such an effect.

Since the 5-HT receptor antagonist ketanserin completely blocked the antigen-induced contractions in rat PCLS (paper V), it was hypothesised that the effects of COX inhibition might involve interactions between serotonin and prostanoids. The effect of interventions with the COX pathway on serotonin-induced bronchoconstriction was therefore also studied in the rat PCLS. Treatment with the COX-2 inhibitor celecoxib and the EP<sub>1</sub> receptor antagonist ONO-8713 attenuated the serotonin-induced bronchoconstriction (paper V). In contrast to the antigen-response, COX-1 inhibition had no effect. These findings suggest that COX-2-derived PGE<sub>2</sub> acts on EP<sub>1</sub> receptors to contract airway smooth muscle. These findings also explain the effectiveness of COX-2 inhibitors against the antigen-induced bronchoconstriction. A plausible explanation of the contribution of the EP<sub>1</sub> receptor to serotonin-induced bronchoconstriction may be that serotonin and PGE<sub>2</sub> interact at the signalling level with other GPCR, for instance other prostanoid receptors (119, 120). This interaction appeared specific as methacholine-induced bronchoconstriction not was affected by either COX-inhibition or EP<sub>1</sub> antagonist. It is possible that new PGE<sub>2</sub> was formed in response to allergen or 5-HT receptor activation and that a specific interaction between PGE<sub>2</sub> and 5-HT<sub>2A</sub> receptor activation occurred (paper V), but the measurements of PGE<sub>2</sub> were inconclusive in this respect.

Thus, mechanisms by which prostanoids contribute to the early allergic airway response differ among species. In guinea pigs and humans, prostanoids are primary mediators of the antigen-induced bronchoconstriction (paper I and paper IV), whereas in the rat lung prostanoids do modulate airway responsiveness towards serotonin rather than being primary mediators of the antigen-induced contraction (paper V).

#### *Impact of leukotrienes in the early allergic airway response in the peripheral lung*

CysLTs are potent bronchoconstrictors in GPLP (paper I), GP PCLS and human PCLS (paper IV). There was however no effect of either 5-LO inhibition or the

CysLT<sub>1</sub> receptor antagonist montelukast on antigen-induced contractions in the GPLP (paper I) and GP PCLS (paper IV). It may seem surprising that 5-LO inhibitors alone did not significantly affect the contractile response to OVA. However, this lack of effect of 5-LO inhibition has been explained previously in detailed studies of antigen-induced contractions in the guinea pig airways (17, 18). Neither inhibition of LTs, histamine or prostanoids separately affected the antigen-induced contraction, whereas combined inhibition of these synergistically acting mediators almost completely abolished the antigen-induced contraction.

In contrast, in the rat PCLS, there was no contraction to LTD<sub>4</sub> (unpublished data) and 5-LO inhibition had no effect on the antigen response. It has also previously been shown that rat airways are insensitive to the contractile effects of CysLTs (179). LTD<sub>4</sub> has together with serotonin nevertheless been implicated to be involved in the early allergic airway response in the rat (56), but the effect appear to be microvascular leakage (180). The release of CysLTs and LTB<sub>4</sub> was generated upon stimulation with ovalbumin (paper I and II). Whereas CysLTs have a more distinct role as bronchoconstrictor (107), LTB<sub>4</sub> has a more proinflammatory role generating chemotaxis and indirectly inducing contractions of the lung parenchyma via release of histamine and thromboxane (114).

### **The role of nitric oxide in antigen-induced contractions**

#### *Effect of endogenous NO on antigen-induced contractions*

Pretreatment with the NOS-inhibitor L-NOARG caused an enhancement of the contraction evoked by challenge with cumulative doses of OVA (paper I). This enhancement by L-NOARG was abolished after co-treatment with the NOS substrate L-arginine. Moreover, the enhancement of the antigen response by L-NOARG was reproduced by three other NOS inhibitors, L-NAME, L-NMMA and 1400W (paper I). L-NAME, L-NMMA and L-NOARG are unselective inhibitors of NOS-1, NOS-3 and NOS-2, whereas 1400W has been documented to selectively inhibit NOS-2 (181). There was no significant difference in effect of the four different NOS-inhibitors tested. Taken together, the main enzyme generating NO in this particular model may be NOS-2, as supported by the effect of 1400W. The concentration of L-arginine did not by itself significantly affect the OVA-induced contractions; nor did a higher concentration of L-arginine (300 µM) (paper I). In contrast to the guinea pig lung, inhibition of endogenous NO with the unselective NOS-inhibitor L-NAME (100µM)

did not affect the antigen-induced responses to pollen extracts in human PCLS or to OVA in rat PCLS (fig 13, unpublished data).

The role of NO in the early allergic airway response appears to be protective by reducing the contraction response in the peripheral lung. Studies in a transgenic mouse model with an overexpressed NOS-2 have also indicated that NO had no proinflammatory effects on the lung and decreased the airway responsiveness (182). However, data from murine models with different deletions of NOS are conflicting (183, 184), and the latter mice studies also used measurements of pulmonary function that presumably at best measure central airway function.

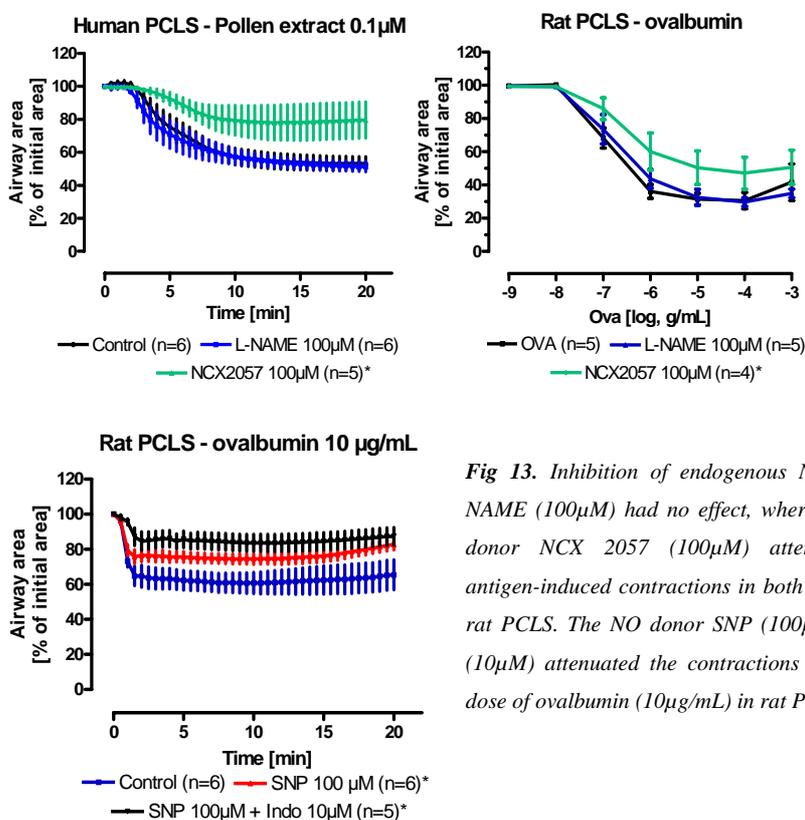


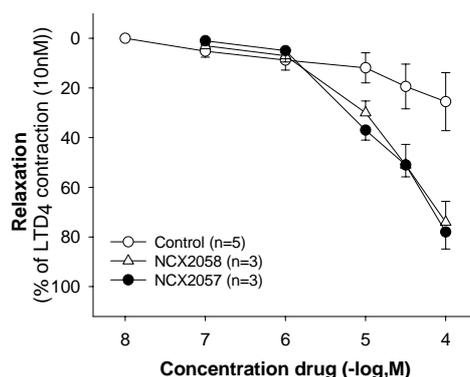
Fig 13. Inhibition of endogenous NO with L-NAME (100 μM) had no effect, whereas the NO donor NCX 2057 (100 μM) attenuated the antigen-induced contractions in both human and rat PCLS. The NO donor SNP (100 μM) ± Indo (10 μM) attenuated the contractions to a single dose of ovalbumin (10 μg/mL) in rat PCLS.

#### Effect of exogenous NO on antigen-induced contractions

Structurally different NO donors, NCX 2057 (structure in fig 1, paper III). SNP and NONOate were used to study the impact of exogenous NO on antigen-induced reactions. NCX 2057 is a newly developed NO donor that also may be attached to

different drugs, such as the antihistamine cetirizine (paper III). NCX 2057 has also been shown to have anti-inflammatory properties in chronic neuroinflammation (185). The NO donor SNP is a powerful vasodilator used for cardiovascular treatments to lower blood pressure and to improve cardiac function. The NO donor NCX 2057 dose-dependently inhibited the concentration-response to cumulative doses of OVA (1-10,000 $\mu$ g/L) in GPLP. The contractions to OVA were significantly attenuated by NCX 2057 at the doses (10 $\mu$ M) and (100 $\mu$ M) and by pretreatment with SNP (100 $\mu$ M). In contrast, NONOate (100 $\mu$ M) had no effect on the concentration-response to OVA in the GPLP (paper II). Unpublished data in rat PCLS (*fig 13*) support that SNP reduced the contractions to OVA, and NCX 2057 significantly attenuated the antigen-induced contractions in both human and rat PCLS (*fig 13*), suggesting a general mechanism of SNP and NCX 2057 in the peripheral lung.

Nonetheless and somewhat surprisingly, the two different NO donors NCX 2057 and SNP had different inhibitory profile on the released mediators. SNP blocked the immediate rise in histamine after challenge with OVA, whereas NCX 2057 blocked the synthesis of leukotrienes and PGD<sub>2</sub> (paper II). NCX 2057 is derived from the parent compound ferulic acid, which been described to have anti-inflammatory and anti-oxidant potentials (186). Nevertheless, ferulic acid (100 $\mu$ M) did not affect the induced contractions to OVA (paper II). However, compound NCX 2058, the bromine analogue to NCX 2057, also attenuated cumulative contractions to OVA (paper II) and relaxed precontracted GPLP in a similar way as NCX 2057 (*fig 14*), suggesting an NO-independent modulation by NCX 2057 in the peripheral lung.



**Fig 14.** Effect of NCX 2057 (0.1-100 $\mu$ M) and NCX 2058 (0.1-100 $\mu$ M) on LTD<sub>4</sub> (10nM) precontracted GPLP. Data are expressed as mean  $\pm$  SEM.

Nonetheless, in contrast to NCX 2057, NCX 2058 did not reduce the generation of leukotrienes (paper II) and did not induce any vasodilation (paper III). It seems that

the specific effects NCX 2057, such as vasodilation and inhibition of leukotriene biosynthesis are mainly caused by release of NO, but that additional compound related effects may contribute as shown with NCX 2058.

### **Direct effects of NO on vascular and airway smooth muscle**

#### *Effect of NO on vascular tissue and proximal airways*

The NO donors NONOate and NCX 2057 dose-dependently relaxed guinea pig aorta, pulmonary artery and tracheal rings, but consistently at higher concentrations in the trachea (paper I and III), indicating that the studied NO donors predominantly were effective as vasodilators. The new NO releasing derivatives of cetirizine, NCX 1512 and NCX 1514, also relaxed precontracted rabbit aorta (paper III). NO donor group substitution with a bromine group (NCX 2058), or pharmacological interventions with the guanylate cyclase inhibitor ODQ ablated the vasodilation induced by NCX 2057, NCX 1512 and 1514 (paper III). The compound NCX 2057 relaxed in similar way both intact and denuded aorta preparations, indicating that NO was cleaved from the compound and became bioavailable in the vascular tissue. It is noteworthy that in comparison with vessel preparations, the NO releasing derivatives of cetirizine, NCX 1512 and 1514, and the NO-donor NCX 2057 had comparatively weak relaxant effects in the tracheal preparation. For example, NCX 2057 had an EC<sub>50</sub> of 0.4 µM in the rabbit aortic rings whereas in guinea pig tracheal rings the EC<sub>50</sub> was almost 100-fold higher (30 µM) (paper III). It is therefore evident that airway smooth muscle appears much less sensitive to the relaxant effects of NO than blood vessels. The same conclusions has been made from experiments where NO has been inhaled in humans (187).

#### *Effect of NO in the peripheral lung tissue*

A plausible explanation of the inhibitory effect of the NO donors on antigen-induced contraction in the GPLP would be relaxation of airway smooth muscle via the cGMP pathway, as has been shown in more proximal airways (188) and in vascular system (131, 189). However, the NO donors (0.1-100µM) NONOate, NCX 2057 or SNP did not induce dose-dependent relaxation of the leukotriene D<sub>4</sub> (10nM) precontracted guinea pig lung parenchyma in contrast to the cGMP analogue, 8-bromo-cGMP (paper I, II). Only a high dose of NCX 2057 (100µM) significantly relaxed the lung parenchyma, which is presumable independent of NO mediated relaxation, since

pretreatment with the guanylyl cyclase inhibitor ODQ (30 $\mu$ M) did not affect the response to NCX 2057 (paper II) and the bromine analogue NCX 2058 (100 $\mu$ M) also induced some relaxation of the precontracted GPLP (*fig 14*). SNP mediated relaxation has been described to be unaffected by guanylyl cyclase inhibitors in the trachea (190). Together, the findings suggest that the tested NO donors possess bioactivity that is independent of the NO/cGMP pathway in the peripheral lung. The unselective NOS-inhibitor L-NOARG had no significant effect on contractions induced by either of the direct smooth muscle stimulants histamine and LTD<sub>4</sub> (paper I). The lack of effect on LTD<sub>4</sub> contractions was also seen in a previous study (191), and is in distinct contrast to the effect of NO inhibition on the responses induced by agonists in vascular preparations (192).

Neither endogenous nor exogenous NO induced any significant relaxant effect in the GPLP model, suggesting that the peripheral lung may be less responsive to the direct smooth muscle relaxant effects of NO (paper I and II). The finding that NO is devoid of smooth muscle relaxing properties in the peripheral lung was strengthened by the fact that NONOate had no relaxant activity even at 100  $\mu$ M bath concentration in LTD<sub>4</sub>-precontracted GPLP. In contrast, 30 nM of NONOate caused 50 % relaxation of guinea pig pulmonary artery precontracted by noradrenaline (paper I). Taken together, it is not likely that the inhibitory effect of NO on the antigen-induced contractions was related to effects at the level of the airway smooth muscle. Another NO donor, nitroglycerin, did not relax bovine lung parenchyma (193), suggesting that the lack of NO sensitivity on airway smooth muscle is a general characteristic of the peripheral lung.

#### *NO and nitrite generation from tested NO-donors*

Preliminary data on direct measurements of NO generated from the different NO donors in the organ bath with and without GPLP showed that neither NCX 2057 nor SNP did release detectable amounts of free NO; in contrast to NONOate that quickly increased the formation of NO in the bath fluid independent of tissue presence (paper II). The kinetics of release of NO was studied using HbFe(II)NO formation in rat whole blood. The NO releasing derivative of cetirizine NCX 1512 and the NO donor NCX 2057 displayed a slow and progressive release of NO during four hours, whereas another NO donor SNAP caused a release that was maximal within 30 minutes (paper III).

Measurements of nitrite generation in the organ bath fluid showed that nitrite was generated by the NO donor NONOate, SNP, NCX 2057 and the NO-releasing derivatives of cetirizine NCX 1512 and NCX 1514 (paper II and III). SNP caused a lower but sustained production of nitrite over time, whereas NCX 2057 caused greater generation of nitrite with a peak after 30 min (paper II). The compounds NCX 1512 and NCX 1514 generated low levels of nitrite over time (paper III). The formation of nitrite from SNP was decreased by more than 50% when the GPLP tissue was absent in the organ baths, while the generation of nitrite from NCX 2057 was only partially reduced in the absence of GPLP. These results were opposite to NONOate that produced the same amount of nitrite independent of the presence of GPLP tissue (paper II). These data imply that SNP require activation by the lung tissue to generate nitrite or NO, as also seen with NCX 2057 in rat blood plasma (162). Nitrite accumulation is generally interpreted as an indicator of NO release. However, it is important to realise that some drugs may be metabolised to nitrate and nitrite without apparent formation of NO and therefore one cannot rely on nitrate/nitrite measurements as altogether precise estimates of NO formation.

### **Interactions of NO and histamine in antigen-induced contractions**

#### *Effect of endogenous NO on histamine*

The NOS-inhibitor L-NOARG had no significant effect on the release of histamine after OVA challenge (paper I), and in agreement with the data obtained with NCX 2057 (see below) (paper II). Other studies have shown that inhibition of endogenous NO caused increased release of histamine from activated mast cells (14) and that NOS inhibition enhanced the antigen-induced mast cell degranulation (147). However, a recent study indicated that NOS inhibition had no effect on the immediate on-set of mast cell degranulation (194).

#### *Effect of exogenous NO on histamine*

The NO donor SNP completely reduced the generation of histamine during challenge with ovalbumin, whereas the other tested NO donor NCX 2057 had no such effect (paper II). Previous findings in the rat mast cell confirm that both SNP and nitrite may inhibit the release of histamine (195, 196), suggesting that SNP has a specific mode of action on histamine degranulation in mast cells. SNP and NCX 2057 differ notably in the kinetics of nitrite formation but also in the release of other nitrogen oxides. Thus,

in contrast to NCX 2057, SNP also liberates NO<sup>+</sup> equivalents, which can react with sulphhydryl groups (-SH), to form S-nitrosothiols. S-nitrosylation serves to regulate the activity of a vast number of proteins in different cell types, including mast cells (141). However, the mechanisms behind the distinct inhibitory effect of SNP on the early allergic airway response need to be further elucidated.

*Effect of the NO releasing derivatives of the antihistamine cetirizine*

A new class of antihistamines coupled to NO donors was developed with the hypothesis that such compounds may have anti-allergic and anti-inflammatory properties beyond those of the parent antihistamine. The aims of the study reported in paper III were to establish if the compounds retained the antihistamine action of the parent compound cetirizine, to assess their efficacy as NO donors, and to test if they had broader anti-allergic activity than cetirizine in the lung. Accordingly, both cetirizine and the NO-releasing derivative NCX 1512 equally inhibited the contractions to cumulative concentrations of histamine in the GPLP and GPTR, indicating that the novel compound NCX 1512 preserved the pharmacological activity as a H<sub>1</sub> antagonist in the respiratory system, as well as in a standard assay for histamine antagonism, the isolated guinea pig ileum. There was however not an improved anti-allergic effect of NCX 1512 compared to cetirizine, as cetirizine and NCX 1512 had similar pronounced inhibitory effects on antigen-induced contractions in the GPTR. Furthermore, in the GPLP, it was confirmed that cetirizine by itself had no significant effect on the antigen-induced contraction. The same major degree of inhibition or lack of inhibition remained when higher concentrations of NCX 1512 were tested (10-100 µM) in GPTR and GPLP, with the intention to promote higher levels of NO release. The reason for the negative results in these acute models is probably that the new compounds did not release sufficiently high and sustained levels of NO at the therapeutic dose of cetirizine. Compound NCX 1512 was also a weaker vasodilator than NCX 2057 that may contribute to the different effect obtained by these two substances on antigen-induced responses in the GPLP (paper II and III).

The new cetirizine derivative with a NO-releasing spacer group (NCX 1512) kept the anti-histamine properties in the GPLP while acquiring the added function to release NO. Although this dual mode of action conferred no added benefit in the currently tested models of the early allergic responses in guinea pig airways, it might

be of value in other settings of inflammation where delivery of NO may synergise therapeutically with H<sub>1</sub> receptor antagonism. In particular, the compound NCX 1514 that have a slower kinetic profile of NO release may be of value in other systems with an ongoing inflammation, such as upper airways and eyes in allergic rhinitis and conjunctivitis, respectively.

There are finally lessons to be learned from the studies of some proposed NO donors in this thesis. First, the mode of action of NO in the target tissue must be defined. Clearly, bronchodilation only occurs at very high concentrations of NO whereas the endogenous levels appear to modulate release of mediators from mast cells and perhaps also other inflammatory cells. Second, other actions than supply of NO may contribute to the effects of prospective NO donors. The diverse results obtained with the tested NO donors in the guinea pig lung and vessels (paper I, II and III), implicates that different NO donors act with distinct and selective profiles. These studies highlight the importance of characterising the selected NO donors (197) before they are used as pharmacological tools in ongoing studies.

### **Interactions between NO and eicosanoids in antigen-induced contractions**

#### *Influence of NO and prostanoids on ovalbumin-induced contractions*

The release of PGE<sub>2</sub> was significantly decreased in preparations treated with L-NOARG, which raised the possibility that endogenous NO stimulated PGE<sub>2</sub> release, as been shown in other models (151, 152, 154). Nevertheless, on the functional level, when L-NOARG was given to indomethacin treated preparations, the enhancement of the concentration response relation to OVA caused by L-NOARG was the same. In contrast, the attenuating effect of the NO donor SNP on antigen-induced contractions in rat PCLS, was improved by co-treatment with indomethacin (*fig 13*). However, the current observations suggests that the two regulatory mechanisms (NO and PGE<sub>2</sub>) in this particular models may represent both parallel (198) and connected pathways (153, 187) in the peripheral lung.

The NO donor SNP did not affect the release of prostanoids. However, the NO donor NCX 2057 significantly reduced the formation of PGD<sub>2</sub> (paper II), suggesting an inhibitory role of NCX 2057 on mast cell degranulation. However, both NCX 2057 and the bromine analogue NCX 2058, inhibited the release of TXB<sub>2</sub> (paper II). One explanation of the inhibitory effect of the NCX compounds on prostanoids may relate to the observation that other derivatives of the parent compound ferulic acid

suppressed the activity of COX-2 (199). The data suggest that NCX 2057 may have a dual anti-inflammatory role in the peripheral lung. Nevertheless, further studies are required to resolve the relation between COX and NO pathways in this particular model.

*Effect of NO and leukotrienes on OVA-induced contractions*

Inhibition of endogenous NOS with L-NOARG (100  $\mu$ M) significantly increased the release of LTE<sub>4</sub> after challenge with ovalbumin in the GPLP (paper I). Pretreatment with FLAP inhibitor BAYx1005 (3 $\mu$ M), as expected, abolished the release of LTE<sub>4</sub>. Moreover, the enhancement of the OVA-response by L-NOARG was not seen in preparations also treated with the BAYx1005 (paper I), suggesting that the effect of NO inhibition is exerted at the level of 5-LO and leukotriene synthesis. The NO donor NCX 2057 significantly reduced the generation of LTB<sub>4</sub> and CysLTs (paper II), suggesting that NO may act as a potential leukotriene synthesis inhibitor and regulator in the peripheral lung. This suggestion is further supported by the findings that NOS-3 is co-localised with 5-LO in human mast cell nucleus (15). Likewise, NO appears to be able to suppress 5-LO activity in airway macrophages (149, 150). Alveolar macrophages have been implicated to contribute to antigen-induced hyperreactivity through release of thromboxane, PGE<sub>2</sub> and CysLTs upon immune stimulation (200-202). The findings in this thesis strengthen the idea that NO has a protective role during allergen challenge of the peripheral lung. Inhibition of endogenous NO enhanced the antigen-induced contractions in the GPLP (paper I), whereas addition of different NO donors attenuated the antigen-induced contractions (paper II). The inhibitory effect of NO on antigen-induced contractions apparently involves modulation of the release of mediators, rather than direct relaxation of airway smooth muscle (paper I, II and III). Those effects of NO on mast cell function may be a regulator of allergen-induced responses. Consequently, obtained data implies that NO is modulating antigen-induced contractions by reducing the release of leukotrienes in the peripheral lung. The exact molecular mechanism behind NO and eicosanoid interactions remains to be established.

### **Species differences and methodology**

#### *Comparison of trachea and lung parenchyma*

Both guinea pig and rat show regional differences in the reactivity of the airways (203, 204). Rat and guinea pig parenchymal strips are for example more sensitive to histamine than tracheal preparations (31, 204). Isolated lung parenchymal strips were established by Lulich et al (205). The contractile response in lung parenchyma is more complex than in the trachea, since the lung parenchyma consists of vessels, bronchioles, connective tissue and smooth muscles (31, 206). In a previous study, the responses of histamine in tracheal spirals and parenchymal strips were compared and the responses of three different thicknesses of parenchymal strips were also compared. All strips demonstrated the same relative responsiveness to histamine. In the thickest strips (1.5mm square) conducting airways and blood vessels were numerous; in the thinnest strip (0.2 mm square) no such structures could be found (207). The differences found between tracheal and parenchymal strips according to the histamine response suggest that there is an intrinsic difference in the pharmacologic response between central airways smooth muscle and parenchymal contractile elements, assuming that alveolar duct smooth muscle and/or alveolar contractile elements were responsible for the parenchymal contraction (206, 208, 209). As there is considerable evidence that also the peripheral lung is important in asthma, the GPLP model has specific value to gain understanding of this segment.

#### *Use of the precision cut lung slices (PCLS)*

The GPLP model is acting as a bioassay reflecting the bioactivity of released mediators in the peripheral lung. However, the GPLP model has a limitation in that it is not possible to distinguish if the induced contractions are occurring in the airways or the vessels. This can be established in the PCLS using videomicroscopic recording of contractions in the peripheral lung. Compared to classical models of studying airway functions *in vitro* such as tracheal rings or parenchymal strips, PCLS offer in addition many advantages such as economic use of expensive agents, longevity and, in particular, the possibility to simultaneously study airway and vascular responses; PCLS even permit to differentiate between pulmonary arteries and pulmonary veins in the same slice.

The rat PCLS was already established as a model of the peripheral lung but had important differences in involved mediators compared to the GPLP preparation and human PCLS during the early allergic airway response. For pharmacological and mechanistic studies relevant to human disease, differences in airway pharmacology between rodent models and human lungs remain an important issue. Guinea pigs are widely used in pulmonary pharmacology, because the airway responsiveness to mediators and drugs is thought to resemble human airways more closely than those of mice and rats (18) during the early allergic airway response. Therefore, to be able to further characterise the agonist and antigen-induced contractions of GPLP, and to elucidate proposed differences and similarities between human, rat and guinea pig peripheral airways, the GP PCLS preparation was established (paper IV).

#### *Influence of vessels and airways in the lung parenchyma*

In the GP PCLS, the airways were completely closed after challenge with OVA. The pulmonary vein was also affected, but not to the same degree, whereas the pulmonary artery remained unaffected. The response to the TP receptor agonist U-46,619 induced dose-dependent contractions of the GPLP and the airway visualized in GP PCLS (*fig 11*). However, the vessels appear to significantly contribute to the induced contractions of thromboxane and PGD<sub>2</sub> in the GPLP, since both the pulmonary artery and vein contracted to U-46,619 and PGD<sub>2</sub> in the GP PCLS (*fig 11 and front page, unpublished data*). Endothelin-1 induced substantial contractions of both airways and vessels in the GP PCLS (paper IV). The influence of the vasculature in the peripheral lung appear to be an important contractile contributor and needs to be further studied during early allergic airway responses and inflammatory disorders of the lung.

#### *Species differences and similarities*

Because the preparation of PCLS is essentially the same in all species, this model provided the possibility to compare the airway pharmacology of different species. PCLS allow investigation of single airways and vessels under cell culture conditions, and has already been used to elucidate important mechanisms of airway contraction in rat, mouse and human peripheral lungs (58, 158, 210, 211). The contractile response of mediators relevant to asthma was in this thesis for the first time compared between guinea pig and human PCLS. The EC<sub>50</sub> and pD<sub>2</sub> values for human and guinea pig bronchoconstriction were almost identical for LTD<sub>4</sub> and methacholine (paper IV).

Airways of both species responded to the TP receptor agonist U-46,619 and histamine, with human airways being more sensitive to prostanoids, and guinea pig airways being more sensitive to histamine. The most significant difference was obtained for serotonin which was quite effective as bronchoconstrictor in guinea pig and completely ineffective in humans (paper IV). In general, guinea pig airways narrowed to a greater extent than human airways. The reason for this is not clear, but may, at least in part, relate to age differences tissue of (young healthy guinea pigs versus lung tissue from middle-aged adults undergoing pulmectomy). Of note, the human airways were all from small airways (diameter <2 mm), and thus free of cartilage. Overall, the comparison between guinea pig and human airways showed that guinea pig provide a reasonable conformity to humans. This is indeed true in comparison to mouse and rat airways that do not or only weakly respond to leukotrienes and histamine (58, 204, 210, 212), both of which mediators are documented to play a role in human asthma (4). In guinea pig and humans (paper IV); both prostanoids and leukotrienes contribute to the allergen-induced bronchoconstriction. However, none of these mediators plays a role in rats, where the allergen-induced bronchoconstriction is almost exclusively mediated by serotonin (paper V). The findings indicate that guinea pigs are more suitable for studies relevant to human airway pharmacology during the early allergic airway response.

## CONCLUSIONS

- ❖ Endogenous NO exerts an inhibitory effect on antigen-induced contractions in the peripheral lung. The action of NO apparently involves inhibition of the release of mediators, especially CysLTs, rather than direct relaxation of airway smooth muscle. These findings further support that endogenous NO has a protective role in the peripheral lung as a beneficial modulator of the early allergic airway response.
- ❖ The tested NO donors may attenuate antigen-induced contractions in the GPLP by different mechanisms. The common mode of action is however inhibition of the release of specific inflammatory mediators rather than bronchodilation. The findings support that different NO donors may have selective and protective anti-inflammatory effects in the peripheral lung tissue.
- ❖ The new cetirizine derivatives with a NO-releasing spacer group, NCX 1512 and NCX 1514, act as antihistamines and NO donors. However, there was no improved effect compared to cetirizine on antigen-induced constriction of the central and peripheral lung, presumably because these particular compounds did not cause sufficiently sustained release of NO.
- ❖ GP PCLS was established and represents a new model to simultaneously measure airway and vascular responses under cell culture conditions. The study documents that the pulmonary pharmacology of the guinea-pig PCLS closely resembles that of the corresponding human tissues, making it superior to mouse and rat preparations.
- ❖ The mechanisms by which eicosanoids contribute to the early allergic airway response differ among species. In guinea pigs and humans prostanoids and CysLTs are primary mediators of the antigen-induced bronchoconstriction, whereas in the rat lung prostanoids, in particular COX-2 derived PGE<sub>2</sub> acting on EP<sub>1</sub> receptors, do modulate airway responsiveness towards serotonin.

## POPULÄRVETENSKAPLIG SAMMANFATTNING (Swedish)

Astma är idag en av de mest förekommande lungsjukdomarna i västvärlden och antalet astmatiker ökar kontinuerligt. I Sverige har cirka 8 procent av befolkningen astma. Astma är en kronisk inflammatorisk sjukdom som påverkar de nedre luftvägarna. Astmaanfall kan utlösas av olika stimuli, till exempel av allergen såsom pollen och kvalster. Allergisk, eller så kallad atopisk astma, innebär att kroppen vid första kontakt med ett allergen börjar bilda antikroppar (IgE-antikroppar) mot detta allergen (kallas för sensibilisering). Vid ett senare tillfälle och påföljande kontakter utlöses en allergisk reaktion; en reaktion mellan antigenet (allergen) och de tidigare bildade IgE-antikropparna och inflammatoriska celler, mastceller, i lungan. Under ett pågående astmaanfall dras musklerna i luftvägarna ihop, samtidigt uppstår det en svullnad och ökad slembildning i luftvägarna, vilket resulterar i akut andnöd. Sammandragningen eller kontraktionen av luftvägarna beror på att mastceller har aktiverats och frisatt inflammatoriska och kontraherande ämnen, histamin och eikosanoider.

Eikosanoider är fettsyror. De bildas i de flesta celler och verkar som lokala hormoner. Eikosanoider består av leukotriener, tromboxan och prostaglandiner. Prostaglandiner upptäcktes i prostatan, tromboxan i blodplättar (thrombocytes) och leukotriener i vita blodceller (leukocytes), därav deras namn. De är involverade i många inflammatoriska processer i kroppen. Leukotriener orsakar bland annat kraftiga kontraktioner i luftvägarna.

Kväveoxid (NO) är kanske mer känd som en giftig gas som bildas vid förbränning. Men denna reaktiva molekyl bildas även i kroppens olika celler och är en viktig signalsubstans. NO frisätts i blodkärl och reglerar blodflöde och blodtryck. NO bildas också i celler inom immunförsvaret och har betydelse för skyddet mot mikroorganismer. NO kan mätas i vår utandningsluft. Höga nivåer i utandningsluften av NO avspeglar ofta en pågående inflammation i luftvägarna. Astmatiker har förhöjda halter av NO i sin utandningsluft. Frågan är vad NO gör i luftvägarna. Har NO en skyddande effekt under ett pågående astmaanfall?

Under de senaste åren har nya forskningsresultat visat att den perifera lungan, där syresättningen av blodet sker, är kraftigt påverkad vid astma. Dels är sammandragningarna i luftvägarna kraftigare och mer långvariga, dels verkar den

pågående inflammationen vara värre. Forskningen har tidigare varit fokuserad på de centrala luftvägarna (bronkerna) då det har varit svårt att undersöka de perifera luftvägarna med lämplig metodik.

Syftet med min avhandling har därför varit att studera allergen-utlösta kontraktioner i den perifera lungan och betydelsen av NO, eikosanoider och histamin i denna reaktion. Det finns studier som visar att NO kan interagera med eikosanoider. Därför studerades även om NO påverkade frisättningen av prostaglandiner och eikosanoider.

Studier har gjorts i olika experimentella modeller. För att studera allergen-utlösta sammandragningar i perifera lungan användes lungvävnad från marsvin och råtta som sensibiliserats för allergenet ovalbumin (äggprotein). Råtta och marsvin är de djurmodeller som generellt används inom luftvägsforskning. Men marsvin är den djurmodell som uppvisar mest likheter med den allergen-utlösta kontraktion som sker i humana luftvägar.

Den perifera lungvävnaden består inte enbart av luftvägar utan även blodkärl och det är därmed svårt att säga om den allergen-utlösta kontraktionen sker i både blodkärl och luftvägar. Därför etablerades i samarbete med Borstel Research Institute i Tyskland en ny marsvinsmodell. I denna modell studerades blodkärl och luftvägar i lungvävnaden med videomikroskopi och den pågående kontraktionen i den perifera lungan kunde därmed bättre förstås. Detta gav oss även möjlighet att jämföra skillnader och likheter mellan marsvin, råtta och människa i allergen-utlösta reaktioner i den perifera lungan.

Studierna i denna avhandling visar att NO ger mindre kraftiga allergen-utlösta kontraktioner i den perifera lungvävnaden. Denna effekt beror på att NO interagerar med leukotriener och minskar frisättningen av dessa kontraherande ämnen. Därmed har NO en skyddande roll i den perifera lungan under ett allergen-utlöst astmaanfall.

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**ORIGINAL PUBLICATIONS, PAPER I-V**

