Responsiveness of Human Airway Smooth Muscle:

Modulation by the Epithelium and Peptidergic Nerves

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Responsiveness of Human Airway Smooth Muscle:

Modulation by the Epithelium and Peptidergic Nerves

Reactiviteit van Humaan Bronchiaal Glad Spierweefsel: Beïnvloeding door Epitheel en Peptiderge Zenuwen

Proefschrift

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aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof. Dr P.W.C. Akkermans M.A.
en volgens het besluit van het College van Promoties

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"Do not assume that I am the right person in whose hands you might place your body and soul for treatment. Heaven be my witness that I myself know well that I am one of those who are not perfect in the art of medicine and who shrink from it because it is enormously difficult to attain its vastness..."

Moses Maimonides (1135-1204), court physician of sultan Saladin, when he presented his 'Treatise on asthma' to the sultan.

Ter nagedachtenis aan mijn vader

Table of Contents

Part 1:

Introduction

Chapter 1

Introduction and Aims of the Studies

1.1 Asthma

Asthma is one of the most common chronic disorders in the Western world, particularly in children, and its prevalence, morbidity and mortality seem to increase [12 33 38 44]. The Greek term ασθμα means short-drawn breath or panting and was used to embrace several disorders characterized by breathlessness or a pain in the chest. Although the meaning is now limited to airway disease, asthma has proven difficult to define, probably because of lack of understanding of the disease mechanism(s). In a recent international consensus report on diagnosis and treatment of asthma, however, the different aspects of the disease were drawn together into an operational definition:

'Asthma is a chronic inflammatory disorder of the airways in which many cells play a role, including mast cells and eosinophils. In susceptible individuals this inflammation causes symptoms which are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment, and causes an associated increase in airway responsiveness to a variety of stimuli' [38].

The increased airway responsiveness of the tracheobronchial tree mentioned in the above definition is defined as an increased tendency of the airways to narrow to a variety of clinical, pharmacological or physical stimuli [38]. The major clinical symptoms of asthma are recurrent episodes of cough, dyspnoea, tachypnoea, wheezing, and chest tightness, often provoked by exogenous factors such as allergens, irritants, exercise, and viral infections [36-38]. Asthma symptoms are episodic, and many patients have a poor perception of the severity of their disease [43]. In addition, signs and symptoms do not correlate with the intensity of the attack [13]. Therefore, objective measures of airflow obstruction and its variability are crucial in establishing the diagnosis [36 38]. Demonstration of allergen-specific IgE, and a positive bronchoprovocation test may support the diagnosis [36 38].

Histologic evaluation of asthmatic airways shows a chronic inflammation in the airway wall [16 25]. There is a complex interaction between mediators and cytokines and inflammatory cells leading to a local reaction that may be neurally amplified [3 5 6 16 25 41 42]. The mediators involved cause epithelial shedding, smooth muscle contraction, enhanced mucus production, and enhanced vascular permeability with mucosal edema; pathological changes that contribute to the airflow obstruction in asthmatic patients. In addition, some of these mediators and cytokines elicit an influx of inflammatory cells [3 16 25 42]. A subset of $CD4^+$ helper T-cells, termed Th2-cells, may play a central role in the patho-physiology of asthma by the production of cytokines that contribute to aberrant IgE production, growth and differentiation of eosinophils, isotype-switching of B-lymphocytes and the evolution of naïve T-lymphocytes into the Th2 phenotype [3 14 25 42].

Asthmatic airways are chronically inflamed, even when patients are asymptomatic [32], and therefore antiinflammatory drugs such as glucocorticoids should be used early in the course of the disease [11 37 38 40 44]. Indeed, treatment with inhaled steroids not only improves clinical signs of asthma, but also reduces the frequency of acute episodes of asthma, the requirements for $ß_2$-adrenergic receptor agonists and oral steroids, the level of bronchial responsiveness, the number of mast cells and eosinophils in bronchial biopsies, and the number of hospital admissions [8 17 28 29 48]. The long-term effects of inhaled corticosteroids on the morbidity and outcome of asthma, however, await further observation.

1.2 BACKGROUND AND AIMS OF THE STUDIES

In 1900 the German pathologist Fränkel described epithelial shedding in the airways of patients who died during an asthmatic attack [21]:

> *'..ein gemeinsames, die Fälle verknupfendes Band:*
> *das ist die reichliche Epitheldesquamation.'*

His observations were disregarded until the early sixties, when Dunnill reported similar findings in asthmatic patients at autopsy [18]. More recent studies involving both bronchoalveolar lavage and endobronchial biopsies showed epithelial damage or -shedding and infiltration of inflammatory cells in the airway epithelium in most patients, even when they appeared to be asymptomatic [8 9 27 31 32 39].

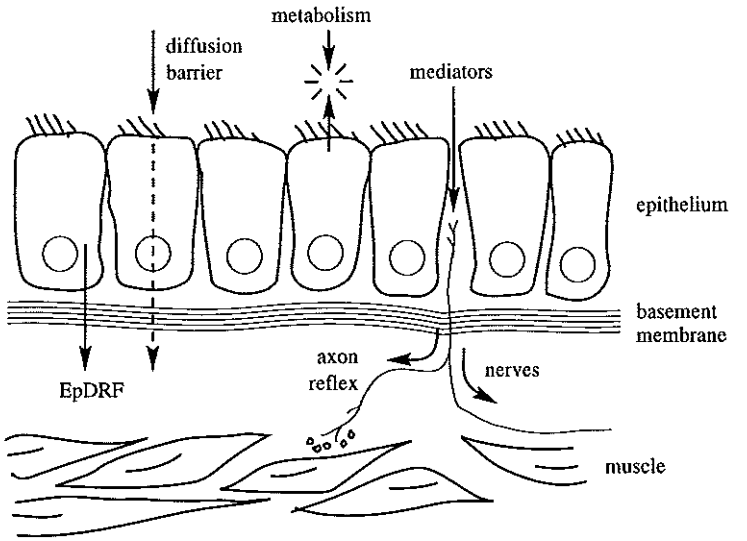

Figure 1. Putative mechanisms explaining the relationship between epithelial damage and airway hyperresponsiveness. EpDRF = epithelium derived relaxing factor.

One of these inflammatory cells, the eosinophil, may play an important role in these pathophysiological changes in asthma: The presence of eosinophils and their products correlates with the degree of epithelial damage and with the level of bronchial hyperresponsiveness in a given patient [5 10 27 39]. The eosinophil produces cationic proteins such as major basic protein (MBP) and eosinophil cationic protein (ECP) and reactive oxygen species which are highly toxic to respiratory epithelium [1 5 22]. Reactive oxygen species are also produced by neutrophils [258] and macrophages [37]. Exposure of airways to these products causes epithelial damage and airway hyperresponsiveness in vivo and in vitro [19 23 47]. It is not clear how epithelial damage causes airway hyperresponsiveness. Several mechanisms have been proposed such as a reduced production of epithelium-derived relaxing factors [2 20] or decreased metabolization of contractile agonists [15 30] by the damaged epithelial cells (figure 1). Alternatively, loss of epithelial integrity may increase airway permeability, and provide easy access of bronchoactive mediators to the airway smooth muscle [24 46 49]. Finally, epithelial damage may expose intraepithelial, nonmyelinated, sensory C-fibers which contain neuropeptides (tachykinins) such as substance P (SP) and neurokinin A (NKA) [7 34 35]. Excitation of C-fibers by inflammatory mediators such as bradykinin might produce a retrograde conduction with local release of neuropeptides, a hypothetical mechanism called the 'local axon reflex' [4] (figure 1). After re-

lease, neuropeptides may cause smooth muscle contraction, microvascular leakage and tracheobronchial vasodilatation, a process called 'neurogenic inflammation' [7 35 45]. Axon reflex mechanisms have been demonstrated in the rodent trachea [26], but it is not known whether these reflexes occur in human airways, and whether or not they are relevant to asthma.
Because of the supposed modulatory role of the airway epithelium and peptidergic nerves in airway responsiveness in human asthma, our studies addressed the following questions:

1. To what extent is human airway responsiveness to contracting and relaxing agonists modulated by the airway epithelium?
   What is the mechanism of this epithelial modulation?
   What is the effect of epithelial damage on airway responsiveness?
2. Is it possible to demonstrate the presence of sensory (peptidergic) nerves in human isolated airways by functional studies?
   Is it possible to measure tachykinin release from these peptidergic nerves?
   Can the so-called 'axon reflex mechanism' be demonstrated in isolated human airways?

Part 1 of this thesis encomprises a review of the literature. Part 2 contains a review on the methodology of studies on human airways in vitro. In addition, we describe a new model that we developed to answer our questions. Part 3 is a compilation of original articles which address the questions raised above. Part 4 includes the general discussion, directions for future research, and a summary of the thesis.

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Chapter 2

Airway Mucosa: Structure, Physiology, and Pathophysiology in Asthma

2.1 Introduction

The airway mucosa consists of a lining layer of ciliated and nonciliated cells, the basement membrane and the lamina propria [6]. The submucosa of the airways contains cartilage, glands and smooth muscle (figure 1). In large bronchi, ducts of submucosal glands penetrate the mucosa and open at the mucosal surface. This chapter describes the histological and ultrastructural features as well as functional aspects of the epithelial cells, the basement membrane and the lamina propria of the human airway. In addition, pathophysiologic changes in asthma are discussed.

2.2 Histology and Ultrastructure of the Airway Epithelium

In man, the stratified squamous epithelium lining the larynx becomes pseudostratified columnar ciliated epithelium in the trachea and cuboidal ciliated epithelium in the bronchioles [101]. The term pseudostratified is used because basal cells are interposed between columnar cells and the basement membrane [71].
The tubulo-acinar submucosal glands in the airways are derived from mucosal cells that migrate during foetal development beneath the lamina propria into the submucosa [253]. At least eight different epithelial cell types have been identified in the conducting airways of mammals [32 96 118]. In addition, inflammatory cells, which migrate through the epithelial basement membrane, and terminal processes of sensory nerve fibres can be found within the airway epithelium [117 123 147 201 240]. In the following paragraphs, the ultrastructure and putative functions of the different airway epithelial cells will be described in some detail.

Ciliated cells
The ciliated cells are end-stage epithelial cells that presumably originate from

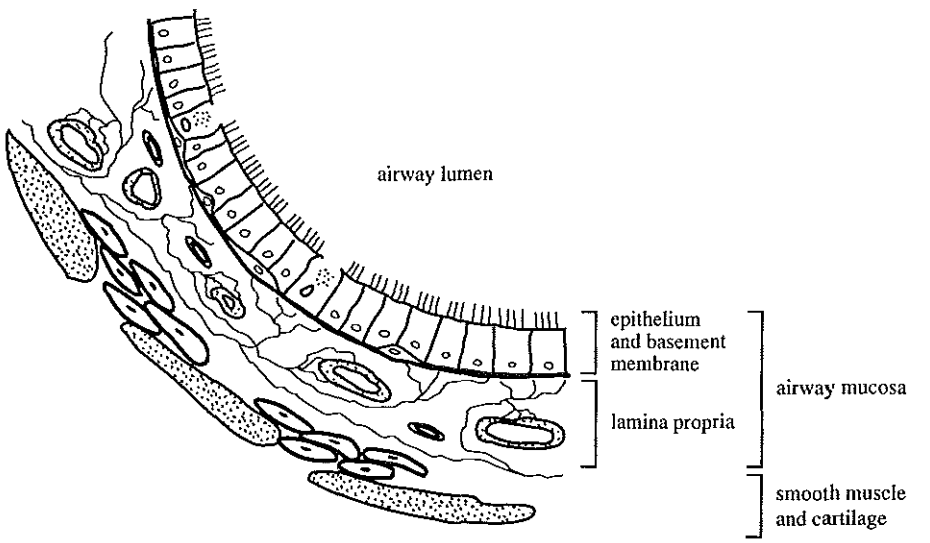

Figure 1. Schematic diagram of a medium-sized bronchus.

basal or secretory cells [4 112]. The primary function of the ciliated cells is to generate mucus flow with motile cilia, 200-300 of which cover the luminal surface of each cell. Each cilium beats about 1000 times per minute in a cranial direction [228 237]. A cilium is composed of a basal body embedded in the apical cytoplasm of the cell and a ciliary shaft (axoneme) that contains a central pair of singlet tubules surrounded by nine doublet tubules. The motility of the cilium is provided by extensions of the tubules composed of a protein called dynein that contains ATPase [153 228 237]. The overlying mucous layer is moved by the tips of the cilia, facilitated by minute little hooklets [120]. Long microvilli interspersed between the cilia expand the luminal surface of the cell and may be important in ion translocation and fluid exchange.

Secretory cells

Non-ciliated bronchiolar cells were originally described by Clara in 1937 and their morphology has been further documented in later studies [5 118 159 222 274]. Five secretory cell types have been identified in the tracheobronchial surface and the submucosal glands of the conducting airways.

Mucous or goblet cells make up 15-25% of the surface epithelium and can be easily recognized by their abundant electron-lucent secretory granules. Production and secretion of airway mucus is the most important function of the mu-

cous (goblet) cell and is controlled by autonomic nerves [96 250]. In normal human airway epithelium, ten ciliated cells to one goblet cell have been described [240]. The number of mucous cells may increase in asthma and chronic bronchitis, and increases following exposure to ozone and cigarette smoke [119 124 165 215].

Clara cells contain discrete electron-dense granules and produce antiproteases and pulmonary surfactant apoproteins [57 92 192]. In addition, Clara cells may have ion-absorbing and secreting properties [256].

The *serous cell* may also produce antiproteases and is believed to undergo a serous to mucous shift [222]. The mucous cell, the Clara cell and the serous cell may all be stem cells capable of division and differentiation into both ciliated and secretory cells [4 128]. The mucous cell is the predominant secretory cell in the larger airways [118], whereas the Clara cell is predominant in the bronchioles [4 274]. Recently, serous cells, well-represented in the submucosal glands, were described in adult human airway surface epithelium [222].

Neuroendocrine (NE) cells usually lie in a basal position in the airway epithelium, often with a cytoplasmic projection to the airway surface [53 243]. They occur individually or as clusters (neuroepithelial bodies), preferentially at bifurcations of the bronchi, and their presence increases towards the periphery [164]. The small dense granules of NE cells contain amines and peptide hormones which may modulate airway smooth muscle tone [152 254]. Because NE cells are sometimes associated with sensory nerves and degranulate during hypoxia, these cells may have a chemoreceptor function regulating shunting of blood to better ventilated zones of the lung [122 152].

Kultschitsky cells are common in the fetal lung but their number diminishes with lung development. These argyrophilic cells may be a variant of the polypeptide hormone-secreting cells of intestinal mucosa (APUD cells). They may be involved with secretion of growth and differentiation factors [80].

Basal cells

Basal cells are small, flattened cells, containing bundles of tonofilaments. They are closely attached to the basement membrane by hemidesmosomes [181]. The basal cell has been considered as the stem cell of the airway epithelium [4 113]. In addition, several studies indicate that basal cells anchor columnar cells to the basement membrane by hemidesmosomes and basal lamina proteins such as laminin, collagen, and proteoglycans [71 84]. The number of basal cells, their tonofilament content, and the number of desmosomes per basal cell increase with epithelial height [72].

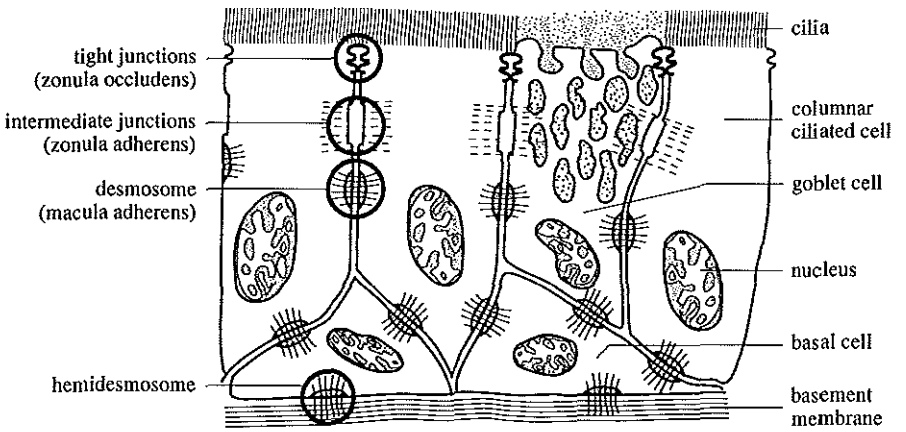

Figure 2. Schematic representation of the adhesion of the bronchial epithelium.

2.3 PHYSIOLOGY OF THE AIRWAY EPITHELIUM

The airway epithelium forms the interface between the respiratory system and the external environment. The epithelium acts as a barrier which restricts access of inhaled noxious stimuli and is also involved in a variety of other airway functions such as ion and fluid transport, enzymatic degradation of neurotransmitters and chemicals, generation of bronchoactive mediators and cytokines, and recruitment and activation of inflammatory cells. These epithelial functions, except ion and fluid transport, will be discussed in the following sections.

Epithelium as a diffusion barrier

Experimental work on human and animal isolated airways and studies in vivo have shown that the airway epithelium forms a barrier against leakage of water and solutes into the airways and prevents penetration of inhaled material to the airway interstitium [10 83 89 95 126 244 280]. There are two pathways by which substances can cross the epithelial barrier: the transcellular route, through lipid cell membranes with ion channels for transport, and the paracellular pathway through which large and hydrophilic molecules pass between epithelial cells [90 275]. The barrier function of the epithelium is achieved through several mechanisms which are shown in figure 2 [181 229]. The desmosome (macula adherens) and the intermediate junction (zonula adherens) maintain cell-to-cell adhesion. Desmosomes are prominent electron-dense plaques consisting of unique proteins called

desmoplakins and desmogleins [246]. Tonofilaments, made up of filamentous proteins, insert at the cytoplasmic edge of these plaques and extend into the adjacent cytoplasm [48]. Intermediate junctions form a ring-like adhesive mechanism around to which adhesion molecules have been localized [229]. Hemidesmosomes consist of integrins and anchor the basal cells to the basement membrane [181 184]. The tight junction (zonula occludens) is a narrow belt-like structure surrounding each cell at the apical pole. The composition of the tight junction is unknown [229]. It assists in the maintainance of surface polarity and appears to be responsible for paracellular transport [162]. Tight junctions are dynamic, regulated structures, that depend on intracellular calcium levels and cAMP levels and protein kinase C activation in epithelial cells [64 199].

Enzymatic degradation of neurotransmitters and chemicals

Autonomic nerves in the airways release acetylcholine, catecholamines, and several neuropeptides such as tachykinins and vasoactive intestinal peptide (VIP) (see chapter 3). Removal of the airway epithelium or addition of specific peptidase inhibitors enhanced the responsiveness of both animal and human airways to these neurotransmitters suggesting inactivation of neurotransmitters by peptidases present in the epithelium [21 59 74 75 105 140 190 232]. The membrane-bound neutral endopeptidase (NEP, CD10, EC 3.4.24.11), present on human airway epithelium [127 151], inactivates neuropeptides and may modulate the responses to these peptides [24]. Angiotensin converting enzyme (ACE), also present on human airway epithelium [127], may not be important in degrading neuropeptides since the enzyme inhibitor captopril did not affect tachykinin-induced responses in man [105]. Human airway epithelium may also inactivate inflammatory medi-ators: In the guinea-pig trachea, histamine and the pro-inflammatory peptide bradykinin are metabolized by the enzymes N-methyltransferase and NEP, respectively [31 82 156 196]. Although there are no functional studies on the role of N-methyltrans-ferase in human airways, N-methyltransferase mRNA has been localized to human airway epithelium [197]. Oxygen free radicals, important molecules that injure cells and tissues [40 93 270], can be inactivated by intra- and extracellular antioxidant activities of the airway mucosa: Human epithelial lining fluid contains molecules with antioxidant capacity [36] and cultured airway epithelial cells have potent oxidant-scavenging ability due to the enzymes catalase, superoxide dismutase and gluthatione peroxidase [41 98 134 154]. Oxidant stress leads to upregulation of these antioxidant systems [98].

Generation of bronchoactive mediators and cytokines

Human airway epithelial cells are the source of a variety of biologically active compounds which may modulate the function of neighbouring cells and tissues. The capacity of epithelial cells to metabolize membrane bound arachidonic acid to both cyclooxygenase and lipoxygenase products has been studied extensively and is schematically shown in figure 3 [39 104 108 224]. The lipoxygenase product 15-hydroxyeicosatetraenoic acid (15-HETE) and the cyclooxygenase product prosta-glandin E_2 (PGE_2) are the major metabolites and their release is stimulated by arachidonic acid, acetylcholine, H_2O_2 and bradykinin, but not by histamine [39 97 211 224]. The cyclooxygenase products thromboxane A (TxA_2), prostaglandin D_2 (PGD_2), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) constrict human airways both in vivo and in vitro probably through activation of the thromboxane prostanoid (TP) receptor [12 42 97]. Prostaglandin E_2 (PGE_2) may relax or contract airway smooth muscle depending on which receptor subtype is involved [43 44]. Although prostaglandin I_2 (PGI_2, prostacyclin) usually relaxes smooth muscle, PGI_2-induced release of tachykinins may result in bronchoconstriction [163]. Prostaglandins are also involved in the regulation of tracheobronchial blood flow [58 268] and mucus and ion secretion [166 167 272]. PGI_2 and $PGF_{2\alpha}$ stimulate sensory nerve endings and cause reflex bronchoconstriction [163 191]. PGE_2 inhibits neuronal acetylcholine release and may play a role in the regulation of airway cell growth and differentiation [79 104 264]. Activation of the lipoxygenase pathway leads to the formation of 5S-, 12S-, or 15S-hydroxyperoxyeicasotetraenoic acids (HPETE's); instable intermediates which are further metabolized to 5-, 12-, and 15-hydroxyeicosatetraenoic acid (HETE's) [104]. The physiological role of the 12-, and 15-lipoxygenase products is unclear [104]. 5-Lipoxygenase catalyzes the formation of the unstable leukotriene LTA_4 which is further converted to LTC_4, LTD_4, and LTE_4 [143 225]. Leukotrienes, especially LTC_4 and LTD_4, are potent constrictors of airway and vascular smooth muscle and increase microvascular permeability and mucus secretion [62]. LTB_4 and LTE_4 are potent chemoattractants for inflammatory cells [150 170] but human airway epithelium appears unable to produce these leukotrienes in significant amounts [104].

Human airway epithelial cells not only release prostanoid and eicosanoid chemoattractants but also other chemotactic factors for lymphocytes, monocytes, eosinophils and fibroblasts [14 142 219 236 241 261]. Although the precise identity of these chemotactic factors is unclear the airway epithelium has been shown to produce the interleukins (IL)-1, IL-6, IL-8, granulocyte/macrophage colony-stimulating factor (GM-CSF), transforming growth factor (TGF)-ß, endothelin,

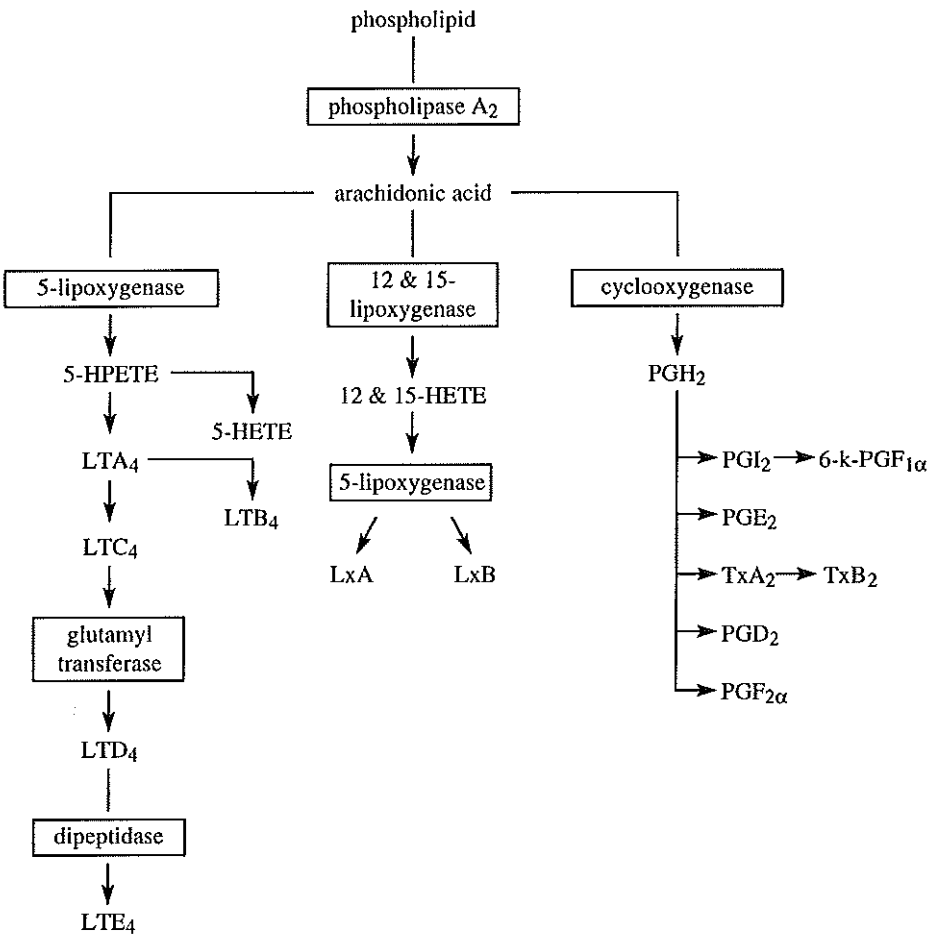

Figure 3. Metabolism of arachidonic acid.

substance P, and fibronectin [20 50 174 213 223 236 245]. The epithelial cell products TGF-ß, fibronectin and endothelin may play an important role in the regulation of tissue repair: they promote proliferation of epithelial cells and fibroblasts in the injured area [216 247]. Smooth muscle hyperplasia, a characteristic finding in airways of asthmatic subjects, may be a side-effect of this repair mechanism [247]. The regulation of tissue inflammation and repair not only involves recruitment of cells by cytokines but also requires interactions between cells. Specific receptors on the surface of epithelial cells called adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and HLA-DR molecules participate in these cell-to-cell interactions [22 259].

Airway epithelial cells are also a source of the highly reactive gas nitric oxide (NO) [8 85 189]. NO is formed as a product of the conversion of L-arginine and oxygen to L-citrulline [180]. Cultured airway epithelium can produce nitrite and immunocytochemical staining has demonstrated the expression of constitutive and inducible NO-synthase (cNOS and iNOS) in human airway epithelial cells [94 139 233]. cNOS can be stimulated by several mediators such as histamine and bradykinin whereas expression and activity of iNOS is increased by certain cytokines and oxidants and can be inhibited by glucocorticoids [1 85 94 138 220]. NO may relax airway smooth muscle in vitro and in vivo through activation of soluble guanylate cyclase either directly or after its reaction with thiol to the more stable S-nitrothiol [67 86 187]. In addition, endogenous NO may be an important modulator of mucociliary clearance by increasing ciliary beat frequency of epithelial cells [116]. Epithelial NO may not be the so-called 'epithelium-derived relaxing factor (EpDRF)' [8 77 187]. In fact, epithelium may metabolize NO or act as a diffusion barrier to NO [8].

2.4 STRUCTURE AND FUNCTION OF THE BASEMENT MEMBRANE

The airway epithelium is separated from the lamina propria by the basement membrane (BM) which is about 10 μm in thickness. The BM provides elastic support to the epithelium, attaches epithelial cells, and constitutes a barrier to macromolucules and noninflammatory cells [51]. In addition, the BM may influence cellular behaviour. The BM consists of a electron-dense layer of 50 to 100 nm called the basal lamina positioned immediately above a loose array of collagen fibrils forming the reticular lamina [171].

The basal lamina contains type IV collagen, laminin, and heparan sulphate proteoglycan (HPS) whereas the reticular lamina consists of type III collagen, type V collagen, and fibronectin [171 202 221 249]. It is generally believed that the components of the BM are synthesized by cells resting on the BM [171].

The reticular lamina may be produced by a subpopulation of fibroblasts in the lamina propria[221]. Type IV collagen is found exclusively in BM. Each collagen molecule consists of a triple helical structure and is linked to three other molecules via terminal structures [168 249]. These interactions between type IV molecules provide a strong flexible three-dimensional framework containing numerous sites where other components of the BM can bind.

Laminin is the most abundant glycoprotein in BM [169 249], and may play a role in

attachment, differentiation, and motility of a variety of cells [136 137 249]. The glycoprotein enactin, unique to BM, may play a role in linking laminin to collagen IV [248]. HPS may prevent the passage of negatively charged molecules [129]. The function of the protein fibronectin is unknown; it may represent plasma fibronectin trapped during filtration [171].

2.5 STRUCTURE AND FUNCTION OF THE LAMINA PROPRIA

The lamina propria consists of a dense network of arterioles, capillaries and postcapillary venules embedded in collagenous, elastic and reticular fibers [23 107 145 146]. Figure 4 shows a photograph of a vascular cast of this subepithelial microvascular plexus. The collagenous, elastic, and reticular fibers provide mechanical stability to the lamina propria [23]. In bronchioles with a diameter of 1 mm the volume of mucosal blood vessels is about 20% of the total volume of the lamina propria suggesting that a considerable part of the total airway blood flow is directed to the mucosa [265]. Indeed, in sheep as much as 85% of the total tracheal bloodflow is directed to the mucosa [200]. The arterioles have a diameter of 10-50 μm and have a single layer of smooth muscle that envelopes the endothelium [100 145]. The capillaries have a diameter of 10 μm and are orientated parallel to the long axis of the airway [107 145 176]. Their endothelium consists of the continuous type although in some species fenestrations have been described [100 176]. The postcapillary venules, 10-50 μm in diameter, are the most numerous of the mucosal blood vessels [107 146 176]. Although they have a continuous endothelium enveloped by a basement membrane, the tight junction belt between adjacent endothelial cells is discontinuous which makes the venules vulnerable [11]. Inflammatory mediators like bradykinin, histamine, leukotrienes, and platelet-activating factor (PAF) increase vascular permeability by opening of these endothelial gaps [73 204 205 208]. In addition, vascular permeability can be increased by tachykinins released by peptidergic nerves [161]. This process called 'neurogenic inflammation' is described in chapter 3.

Histochemical studies showed dense innervation of arterioles by cholinergic, adrenergic and peptidergic nerves whereas no nerve profiles were found near the postcapillary venules [60 160 177 201]. This suggests that the arterioles are involved in the neural regulation of mucosal blood flow [146 176]. Activation of adrenergic nerves causes vasoconstriction by release of noradrenaline and neuropeptide Y whereas the parasympathetic nerve products acetylcholine and

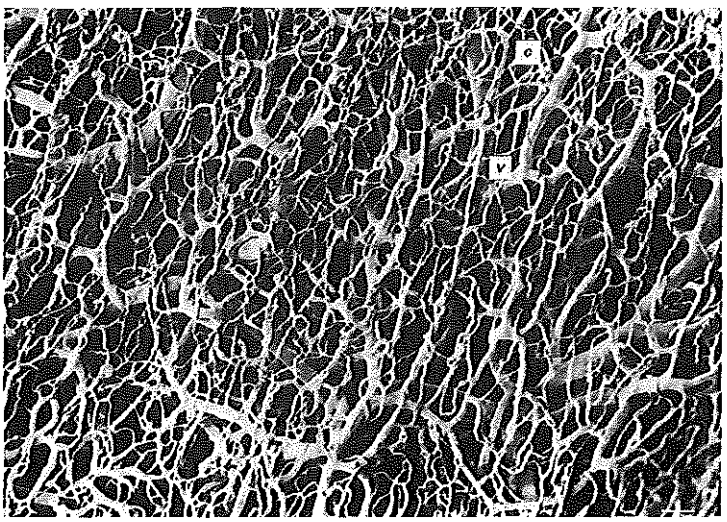

Figure 4. Scanning electron micrograph taken from the luminal side of a vascular cast from the wall of the trachea of a dog. A rich vascular network is shown in the tracheal mucosa. Small capillaries (C) are draining into deeper veins (V). Bar = 100 µm. From reference 145, with permission.

vasoactive intestinal peptide (VIP) produce vasodilation [53 173].
The main function of the mucosal microcirculation is to provide nutrients to the epithelium [273]. In addition, the mucosal microvascular plexus may have an important role in the conditioning of inspired air, and the clearance and distribution of inflammatory mediators [178 213 273]. Finally, in pathologic conditions such as asthma, the mucosal microcirculation is the source of plasma and inflammatory cells, that pass into the lamina propria and the epithelium [205].

2.6 PATHOPHYSIOLOGY OF THE AIRWAY EPITHELIUM IN ASTHMA

Histological examination of bronchial tissue of patients who died during an asthmatic attack and of bronchial biopsies obtained from patients with asthma showed epithelial damage and- shedding, and infiltration of eosinophils and lymphocytes [13 66 121 124 141 148 149 193]. Because the degree of epithelial damage correlated with the presence of eosinophils and their products and with the level of bronchial reactivity, eosinophils have been proposed as the major effector cells in asthma [9 13 28 121 124 193]. Eosinophils produce cationic proteins such as

major basic protein (MBP) and eosinophil cationic protein (ECP) and reactive oxygen species which are highly toxic to respiratory epithelium [2 9 81 87 186]. Exposure of human and animal airways to cationic proteins and oxidants indeed produced epithelial damage and an increase in airway responsiveness [78 91 130 198 255]. Recently, it was shown that neutrophils may also contribute to detachment of epithelial cells by release of proteases and oxidants [258]. It is not clear how epithelial damage causes an increase in airway responsiveness. Several mechanisms have been proposed:

Loss or damage of a diffusion barrier

In 1979 Boucher et al. described a relationship between airway hyper-reactivity and airway permeability to histamine following specific allergen challenge in sensitized monkeys [26]. In asthmatic patients, increased airway permeability to inhaled radiolabeled tracer molecules was found [111] and electronmicroscopic studies of the epithelium revealed opening of tight junctions and widening of intercellular spaces in these patients [70 193]. The opening of tight junctions correlated with eosinophil infiltration and the degree of bronchial responsiveness, suggesting a causal relationship [193]. Indeed, in experimental models exposure to eosinophils or their products increased both airway permeability and responsiveness [99 198 281]. Similarly, increased airway permeability and responsiveness have been found following exposure to oxidants [18 19 125 175 270]. The effects of cationic proteins and oxidants on human airway permeability have not been studied.

Loss of a metabolic site

In paragraph 2.3 the inactivation of neurotransmitters by epithelial neutral endopeptidase (NEP) was discussed. NEP-inhibitors like thiorphan and phosphoramidon increase bronchial responsiveness to tachykinins and this led to the hypothesis that NEP-activity may be decreased in asthmatics. Several studies suggested that environmental pollutants and pathogens can decrease airway NEP: Inhalation of cigarette smoke or toluene diisocyanate, an agent used in the manufacture of plastics, increased airway responsiveness to the tachykinin substance P and decreased NEP activity [69 235]. Similarly, viral infections and chronic airway inflammation may decrease NEP activity [68 115 155]. Glucocorticoids, used in the treatment of asthma, increase NEP expression by airway epithelial cells by enhancing transcription and protein synthesis [25 151]. In a recent study of mild asthmatic subjects, however, no evidence for NEP-dysfunction was found [38].

Thus, although NEP activity seems to be decreased during viral infections, airway hyperresponsiveness in asthmatic patients cannot be explained by NEP dysfunction only.

Impaired release of relaxing substances
Following the discovery of a vascular smooth muscle relaxing factor released by endothelial cells (endothelium derived relaxing factor, EDRF), much research has been spent in order to identify a similar factor released by airway epithelium (epithelium derived relaxing factor, EpDRF). It was hypothesized that damaged epithelium would not be able to produce the relaxing factor, resulting in bronchoconstriction. Despite the use of various biotechniques including superfusion-cascade, coaxial bioassays, and perfused airway tubes the existence and the putative role of EpDRF remain controversial [76 103 187 238 244]. Recently, EDRF has been identified as nitric oxide (NO) [180]. Although human airway epithelial cells can produce NO [94 139] epithelial NO is probably not EpDRF [8 77 187].

Exposure of sensory nerves with release of tachykinins
In animal and human airways, superficial tachykinin-containing sensory nerves have been identified that terminate in the airway epithelium [45 147 172 226]. In 1986, Barnes proposed that epithelial damage might expose these nerve endings to inflammatory stimuli, facilitating axon reflexes with local release of tachykinins [7]. Tachykinins are potent inducers of airway constriction, airway oedema, and mucus secretion, all characteristics of asthma [172 242]. Evidence that local axon reflexes may contribute to bronchoconstriction has thusfar only been found in guinea pig isolated airways: Bradykinin-induced bronchoconstriction was reduced in airways which were depleted of tachykinin by pretreatment with capsaicin [114]. The inflammatory peptide bradykinin can stimulate sensory nerve fibers, resulting in release of tachykinins [172 227]. In asthmatic patients inhalation of a tachykinin antagonist protected against bradykinin-induced bronchoconstriction [110]. However, this does not prove local axon reflexes, because a central cholinergic reflex could explain these results as well. Until now, the relevance of axon reflexes in the pathophysiology of asthma remains to be shown.

2.7 PATHOPHYSIOLOGY OF THE BASEMENT MEMBRANE IN ASTHMA

Light-microscopic studies revealed a thickened basement membrane (BM) in asthmatic subjects and this abnormality has long been considered pathognomonic

for asthma [35 49 66 171 239]. The thickening was thought to reflect increased basal lamina production by injured epithelial cells [171 207]. However, later studies using transmission electronmicroscopy and monoclonal antibodies to differentiate collagen subtypes revealed that the basement membrane has a normal thickness in asthmatics [54 221]. In these studies, dense collagen fibrils were seen beneath the BM which were considered as the BM in the earlier light microscopical studies. Immunohistochemistry revealed the presence of collagen IV, fibronectin, and laminin in the 'true BM' of asthmatic patients which is not different from that of normal subjects [222]. The subepithelial collagen fibrils appeared to consist mainly of collagen III and V and fibronectin but not laminin [222]. The thickness of the subepithelial collagen layer correlated with the number of myofibroblasts beneath the epithelium, suggesting that these cells may be responsible for the surplus of collagen fibrils [33 102]. Similar pathophysiological changes in the basement membrane zone have been observed in collagenous colitis [109]. It seems, therefore, that thickening below the basement membrane zone is a nonspecific sequel of mucosal inflammation.

2.8 PATHOPHYSIOLOGY OF THE LAMINA PROPRIA IN ASTHMA

Inflammation of the mucosa produces hyperemia in the lamina propria [205 273]. Increased hydrostatic pressure and mediator-induced permeability of post-capillary venules cause plasma exudation [204 205]. This exudate decreases the colloid osmotic pressure gradient, which leads to further plasma leakage. The process of vasodilation and plasma leakage may increase airway wall thickness which may directly contribute to airway narrowing [55 262 273]. In addition, the exudate contains inflammatory mediators and peptides that promote migration of leukocytes from the venules into the surrounding tissue. This infiltration of leukocytes in the airway mucosa had already been recognized more than 50 years ago in patients who died from asthma. Eosinophils, neutrophils, lymphocytes and plasma cells were also present in the airways in these fatal cases [35 54 65 66]. After the introduction of bronchoscopic biopsy techniques it appeared that increased numbers of inflammatory cells were present in all asthmatic patients even in those with mild or newly diagnosed disease [13 28 121 148]. These inflammatory cells are not only present in the lamina propria but also in the airway epithelium to which they migrate during the inflammatory response.

Eosinophils

Eosinophils are the predominant inflammatory cells in the airways in fatal asthma. Their presence is less prominent in stable asthma [121 149]. Eosinophils in asthmatic airways are in an activated state. They not only release cationic proteins and oxygen metabolites, (described earlier in this chapter) but also lipoxygenase products, primarily LTC_4 [131 234], and the interleukins IL-3, IL-5, IL-6, GM-CSF and TGF-$ß_1$ [34 135 158 195]. The migration, differentiation, and activation of eosinophils is regulated by cytokines mainly produced by lymphocytes of the Th2-subtype [27].

Neutrophils

The role of the neutrophil in human asthma is controversial. Increased numbers of neutrophils have been found in bronchoalveolar lavage (BAL) fluid of asthmatics and chronic asthma may be associated with mucosal neutrophil influx [56 179 214]. However, neutrophils can also be found in the BAL fluid of normal subjects; in a bronchial biopsy study, neutrophils were more numerous in the control subjects than in mildly asthmatic patients [13 52 266]. Recent evidence suggests, however, that neutrophils may cause detachment of epithelial cells by the production of proteases and oxidants [258].

Mast Cells

In BAL fluid from asthmatic patients the percentage of mast cells and the amounts of mast cell derived mediators such as histamine and tryptase are slightly higher than in BAL fluid from non-asthmatics [206 266 271]. The epithelium of asthmatics also contains more mast cells than the epithelium of normal subjects. No differences in subepithelial numbers of mast cells were found [206]. Most of the mast cells in human airways are of the tryptase-containing subtype [230].

The finding of mast cell mediators in BAL fluid and their degranulated appearance on electronmicroscopy suggests that tissue mast cells are in an activated state [206 212]. Apart from histamine and tryptase, mast cells may release PGD_2, LTC_4 and small amounts of other arachidonic acid metabolites and the interleukins IL-3, IL-4, IL-5, IL-6, GM-CSF and TNF-α [29 267 279]. Because IL-4 stimulates differentiation of T-lymphocytes into the Th2-subtype [203 276] and IL-5 promotes recruitment and activation of eosinophils [132 158 257] it has been suggested that mast cells may be important in the initial stage of the disease [61 144].

Macrophages

Macrophages account for 80-90% of the airway cells in BAL fluid in both asth-

matic and normal subjects [133 266]. The macrophages in asthmatic airways, however, are hypodense and appear more immature, suggesting increased recruitment of circulatory monocytes [209]. Macrophages from asthmatic airways have enhanced superoxide production and an increased release of IL-1 [37 210]. In addition, they are able to release LTB_4, $PGF_{2\alpha}$, TxB_2, and TGF-ß in response to IgE, suggesting their involvement in the early asthmatic response (EAR) [61 188]. Finally, macrophages have been shown to produce the so-called macrophage inflammatory proteins (MIPs), members of a superfamily of cytokines called chemokines [278]. These proteins exhibit proinflammatory and immunoregulatory activities; increased MIP expression has been observed in oxidant-induced lung injury [63].

Lymphocytes

Immunohistochemical analysis of BAL fluid and bronchial biopsies failed to show differences in either T-cell numbers or the $CD4^+/CD8^+$ ratio between asthmatic and nonasthmatic patients [30 132 277]. However, the number of epithelial lymphocytes is increased in asthma [148]. In addition, surface expression of IL-2 (CD25), HLA-DR, and very late antigen (VLA-1) is enhanced, suggesting activation of T-cells [46 263 277]. $CD4^+$ lymphocytes have been further categorized in two distinct subsets, Th1 and Th2, because of their different cytokine profile [185]. The Th2-subpopulation produces IL-3, IL-4, IL-5, IL-10, IL-13, and GM-CSF and may play a central role in airway inflammation [3 47 218]. IL-4 stimulates the differentiation of naïve T cells into Th2 cells and IL-4 and IL-13 are able to induce a B-cell immunoglobulin-isotype switch leading to IgE production [203 276 283]. T-lymphocytes in the lavage fluid of asthmatics have increased mRNA expression for IL-4 and IL-5 [3]. The development of selective inhibitors of Th2 cells and their products and animal experiments where specific genes are knocked out by genetic enginering may give more insight in the precise role of these cells in the pathogenesis of airway inflammation.

Cellular events in allergic airway inflammation

Local endobronchial challenge techniques have been used to study the processes of chemoattraction, adhesion and transendothelial migration of inflammatory cells in allergic asthma. Within minutes after challenge, elevations of histamine, tryptase, prostaglandins, leukotrienes and ß-glucuronidase have been found in lavage fluid, suggesting mast cell, macrophage, and endothelial cell activation [157 231 252]. Apart from their chemoattractant effects some of these media-

tors will contribute to airway obstruction. At this stage a reduction in lavage $CD4^+$ T-lymphocytes is found, probably because they are more adherent to the epithelial cells [88]. Endobronchial biopsies six hours after challenge show an accumulation of leukocytes expressing ß2-integrin and lymphocyte function-associated antigen (LFA)-1 in the epithelium and lamina propria [182]. ß2 integrin and LFA-1 are the counter ligands for leukocyte cell adhesion molecule (LeuCAM) and intercellular adhesion molecule (ICAM)-1, respectively, which are expressed by endothelial cells [183 184]. LeuCAM's are a group of membrane-bound proteins of which the selectins P-, E-, and L-selectin and vascular adhesion molecule (VCAM)-1 are the best known members. Expression of selectins by endothelium is an early event in response to inflammatory mediators and has been demonstrated prior to a spontaneous asthmatic attack followed by expression of ICAM-1 2 to 4 h later [17 194]. Up-regulation of VCAM-1 is a slow process and is believed to be responsible for eosinophil recruitment [15 282].
The abovementioned cell-to-cell interactions (cell adhesions) probably enable transendothelial and transepithelial migration [269]. T-lymphocyte activation is apparent 24 h after local endobronchial allergen challenge [16 260]. Production of cytokines by these T-lymphocytes will contribute to further eosinophil accumulation [260]. In addition, cytokine production by other leukocytes, tissue fibroblasts and epithelium may also contribute to influx of inflammatory cells [106].

2.9 CONCLUSIONS

The airway epithelium consists of ciliated and secretory cells that form a diffusion barrier which restricts access of inhaled noxious stimuli and leakage of solutes into the airways. In addition, the epithelium has important metabolic functions. Epithelial cells degrade neurotransmitters, inflammatory mediators and oxygen radicals and produce bronchoactive substances such as cyclo-oxygenase and lipoxygenase products and nitric oxide. The epithelium also releases chemotactic factors for inflammatory cells and growth factors that stimulate epithelial repair after damage. In asthma, the epithelium is damaged probably by cationic proteins and oxidants produced by inflammatory cells. The degree of epithelial damage is correlated with the level of bronchial reactivity in asthmatic patients. It is not clear how epithelial damage causes airway hyperresponsiveness. Loss of barrier or metabolic function, exposure of sensory nerves with release of tachykinins and a decreased release of relaxing substances have

been proposed. Evidence for a contributory role of each of these putative pathophysiological mechanisms has been found.

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Chapter 3

Autonomic Innervation of Human Airways: Structure, Physiology, and Pathophysiology in Asthma

3.1 Introduction

The human tracheobronchial tree is innervated via efferent and afferent autonomic nerves. These nerves regulate many aspects of airway function including airway smooth muscle tone, airway secretions, bronchial circulation, microvascular permeability and the migration and the release of inflammatory cells [16 21 22 134 174]. Apart from the classical adrenergic- and cholinergic nervous systems, a third non-adrenergic, non-cholinergic (NANC) component has been described in human airways [16 24 25 202]. This NANC nervous system has inhibitory (iNANC) and excitatory (eNANC) components and neuropeptides and nitric oxide (NO) are the neurotransmitters [24 25 27 70 71 239]. Alexander and Paddock were the first to suggest a defect in the autonomic control of the airways in asthma [6]. Since then, several abnormalities of the autonomic control in airway disease have been proposed including enhanced cholinergic-, α-adrenergic- and eNANC mechanisms and, on the other hand, reduced ß-adrenergic- or iNANC mechanisms. The hypothesis that abnormal neural control of the asthmatic airway may be responsible for the airway narrowing in these patients is supported by in vitro studies of asthmatic airways. In these studies no correlation was found between responsiveness in vivo and in vitro. In addition, no abnormalities of the smooth muscle itself has been found [8 47 57 233]. In this chapter the autonomic innervation of the human lung is described. In addition, possible abnormalities of the autonomic innervation in asthma are discussed.

3.2 Cholinergic Innervation

The cholinergic or parasympathetic nervous system is the dominant neural pathway in the control of airway smooth muscle tone and secretion in human airways [17 19 39 203 222]. Stimulation of cholinergic efferent nerves causes broncho-

constriction, mucus secretion, and bronchial vasodilatation. These effects are mediated via the release of the excitatory neurotransmitter acetylcholine from agranular vesicles in cholinergic nerve terminals which rapidly diffuse to cholinergic receptors on the target cell. Acetylcholine is broken down by the enzyme cholinesterase which can be inhibited by cholinesterase inhibitors, such as eserine or pyridostigmine.

Distribution

Cholinergic efferent nerves arise in the vagal nuclei of the brainstem and pass down the vagus nerve to synapse in ganglia localized external to smooth muscle and cartilage in the airway wall [16 187]. These ganglia, which also receive adrenergic and NANC innervation, may adjust neural input to airway smooth muscle and submucosal glands [16 22 51 223]. Short postganglionic unmyelinated axons pass to airway smooth muscle, vascular endothelium, submucous glands, and probably, mast cells [22 53 187]. The epithelium and the bronchial vessels are sparsely supplied [16 187] although functional studies suggest cholinergic control of bronchial blood flow [92 167]. Cholinergic innervation is denser in medium-sized bronchi (generation 4 to 7) than in the trachea and in the bronchioles and is absent in alveolar walls [22 53]. Close contacts between nerve endings and individual muscle cells are rare and neuronal excitation may be distributed by myogenic coupling [53 87].

Cholinergic receptors

Acetylcholine released from preganglionic vagal fibers activates nicotinic (N)-receptors on ganglionic nerve cells. Apart from mediating cholinergic transmission, these nicotinic receptors may facilitate the release of neuropeptides from sensory nerves [128 214]. When released from postganglionic nerves, acetylcholine stimulates muscarinic (M)-receptors. Receptor binding studies and autoradiography demonstrated muscarinic receptors on human airway smooth muscle, vascular endothelium, submucosal glands, airway ganglia, peribronchial nerves and alveolar walls [161 237]. Although muscarinic receptors are only weakly expressed on human airway epithelium, a strong in situ hybridization signal with a M_3-receptor cDNA probe was found, indicating a rapid turnover of receptors on human airway epithelium [162]. In animal airways five M-receptor subtypes have been cloned [35 36 131]. However, only three subtypes of muscarinic receptors have been demonstrated pharmacologically and four with specific cDNA probes [19 66 69 109 162 172]. In human airways only three muscarinic receptor subtypes have

Table 1. Muscarinic receptor subtypes in human airways *

	Localization	Function
M_1	Parasympathetic ganglia	Facilitation of neurotransmission
	Submucosal glands	Increased secretion
	Alveolar walls	?
M_2	Postganglionic cholinergic nerves	Inhibit acetylcholine release
	Airway smooth muscle	Antagonism of bronchodilatation?
	Sympathetic nerves	Inhibit noradrenaline release
M_3	Airway smooth muscle	Contraction
	Submucosal glands	Increased mucus secretion
	Epithelial cells	Increased ciliary beating frequency?
	Goblet cells	Increased secretion
	Endothelial cells	Vasodilation via NO-release
M_4	?	?
M_5	?	?

*based on references 19, 109, 243, and 245.

been demonstrated [243]. The localization and function of these receptors in the lung is shown in table 1. The M_2-subtype is the predominant muscarinic receptor subtype in most smooth muscle types [69]. In human airway smooth muscle M_2-receptor mRNA and functional M_2-receptors have been detected [162 245 246]. Blockade of smooth muscle M_2-receptors in animal airways augmented isoprenaline-induced relaxation suggesting a modulatory role of this receptor in airway smooth muscle relaxation [76]. However, in human airways a functional role of smooth muscle M_2-receptors in bronchoconstriction remains to be established [207 209]. Muscarinic receptors of the M_3-subtype have been de-monstrated on airway smooth muscle of human central and peripheral airways and stimulation of these receptors results in bronchoconstriction [161 162 208]. Endothelial cells of the bronchial and pulmonary circulation also express M_3-receptors which may mediate the vasodilator response to acetylcholine [167 169]. Human submucosal glands have both M_1- and M_3-receptors whereas in alveolar walls M_1-receptors are found [161 162]. The muscarinic receptors in human airway ganglia are of the M_1-subtype and may facilitate neuronal conduction [22 140].

Prejunctional M_2-autoreceptors have been demonstrated in animal and human airways and may play an important role in the regulation of cholinergic neuro-

Table 2. Modulation of cholinergic neurotransmission by prejunctional receptors

Receptor/mediator	Rodent	Human	References
α_2-Adrenergic	–	–	7, 14, 100
β_2-Adrenergic	–	–	5, 116, 200
Muscarinic M_2	–	–	64, 171
Histamine H_3	–	–	110, 112
5-HT_2	+	?	231
PGE_2	–	+/–	33
Thromboxane	?	+	215
Opioid	–	–	26, 30
Tachykinin	+	+	4, 34, 242
VIP	–	?	229
Neuropeptide Y	–	–	86, 228
Nitric oxide	–	–	217, 241
Diuretics	?	–	74

– = inhibition, + = stimulation

transmission by inhibiting the release of acetylcholine from cholinergic nerves and noradrenaline from sympathetic nerve terminals [22 84 117 171 194]. In the rabbit lung, M_4-receptor mRNA has been localized to alveolar walls and vascular and airway smooth muscle [126]. No M_4-receptors have been found in human airways [162].

Prejunctional modulation of cholinergic neurotransmission

Acetylcholine release from cholinergic nerve terminals in animal and human airways is modulated by many prejunctional mechanisms that may either inhibit or facilitate cholinergic neurotransmission. These mechanisms, most of which are receptor-mediated, are listed in table 2.

Abnormalities of the cholinergic innervation in asthma

The benefial effect of anticholinergic drugs in acute asthma and COPD indicates that cholinergic mechanisms contribute to bronchoconstriction. Although an increase in vagal cholinergic tone may contribute to nocturnal asthma [191], there is no direct evidence for an increase in basal cholinergic tone in asthma and COPD [21]. However, there may be an increase in cholinergic reflex bronchoconstriction due to stimulation of nonmyelinated sensory nerve fibers (C-fibers)

by inflammatory mediators such as histamine, prostaglandins, and bradykinin [21 52 127 175]. Bronchoconstriction may also result from dysfunction of prejunctional (inhibitory) M_2-receptors resulting in increased postganglionic acetylcholine release [9 140]. Indeed, in virus-infected guinea pigs dysfunction of prejunctional M_2-receptors has been found resulting in potentiated bronchoconstriction [82]. It appears that viruses have both a direct and an indirect, leukocyte-dependent effect on M_2-receptors [83]. Loss of M_2-receptor function has also been found after exposure of guinea pigs to ozone or cyclooxygenase inhibitors [85 216]. Acetylcholine release may be facilitated by pre- and postjunctional actions of inflammatory mediators such as tachykinins and arachidonic acid metabolites [102 113]. Finally, inflammatory mediators may directly increase the sensitivity of human airway smooth muscle to cholinergic stimulation resulting in an enhanced bronchoconstriction [121].

3.3 ADRENERGIC INNERVATION

The adrenergic or sympathetic nervous system may control the airways via sympathetic nerves which release noradrenaline and by circulating catecholamines (mainly adrenaline) released from the adrenal medulla [16 98]. In contrast to the dense parasympathetic innervation, the sympathetic innervation to the human airways is sparse. Functional studies did not show sympathetic innervation of human airway smooth muscle [53 56 205 234]. However, sympathetic nerves may influence airway tone indirectly by the regulation of airway blood flow [245] and by interacting with the cholinergic innervation at the ganglionic or postganglionic level [5 14 55 100 200]. Sympathetic nerves supplying blood vessels may have an important role in the control of tracheobronchial blood flow and airway secretions [92 163 183]. Finally, adrenergic receptors may have a role in airway smooth muscle proliferation: Stimulation of α-adrenergic receptors induced airway smooth muscle cell mitogenesis, whereas ß-adrenergic receptor agonists inhibited smooth muscle growth [180].

Distribution

Preganglionic sympathetic nerves originating from the upper six thoracic segments of the spinal cord synapse in the middle and inferior cervical ganglia and upper four thoracic prevertebral ganglia. Postganglionic fibers travel to the lung where they mix with cholinergic nerves to form a dense plexus around airways

and vessels [16 203]. Morphological studies of human lungs have shown that sympathetic nerves supply bronchial vessels, submucosal glands, and parasympathetic ganglia. Airway smooth muscle receives only few sympathetic nerves [53 55 65 136 186 221]. Neuropeptide Y (NPY) is a cotransmitter in adrenergic nerves and may have a synergistic effect on the actions of sympathetic nerve stimulation [192].

Adrenergic receptors

Noradrenaline and adrenaline exert their effects through stimulation of α- and ß-adrenergic receptors. Adrenergic receptors belong to a large family called the G-protein-coupled receptors. The genes for all of the subtypes have been isolated [63 144]. Radioligand binding studies and autoradiography demonstrated a low density of α-adrenergic receptors in the human lung whereas ß-adrenergic receptors are abundant [15 20 43 225 226].

Stimulation of α_1-adrenergic receptors causes smooth muscle contraction in pig and canine vascular smooth muscle and secretion from submucosal glands [182 190]. After blockade of ß-adrenergic receptors in human airways, adrenergic stimulation caused only weak contractions suggesting an insignificant role of α-adrenergic receptors in human airway smooth muscle responsiveness [226]. Prejunctional α_2-receptors (autoreceptors) may inhibit the release of both noradrenaline and of neuropeptide Y from adrenergic nerves and the release of tachykinins from C-fibers [99 133 153]. Cholinergic neurotransmission may also be inhibited via prejunctional α_2-receptors [100].

On the basis of pharmacological criteria, ß-adrenergic receptors have been subdivided into $ß_1$- and $ß_2$-subtypes [141]. The ß-adrenergic receptor consists of a single polypeptide chain containing seven regions of hydrofobic amino acids suggesting the forming of seven membrane-spanning helices with the hydrophobic segments as extra- and intramembranous loops [64]. The gene for the human $ß_2$-adrenergic receptor has been cloned and sequenced [129] and ß-receptors and mRNA encoding ß-adrenergic receptors have been localized by autoradiography in human airways from trachea to alveolus [43 103 226]. Expression of mRNA encoding $ß_2$-adrenergic receptors is increased in subjects with asthma [10]. ß-Adrenergic receptors have also been localized on other cell types including epithelium, endothelium, nerve fibers, and inflammatory cells [43 74 75 96 147]. Although $ß_1$-adrenergic receptors have been demonstrated in the human lung [212 226], ß-adrenergic receptors on human airway smooth muscle are entirely of the $ß_2$-subtype and their number increases towards the peripheral airways [43 103 226 246]. Stimulation of these $ß_2$-adrenergic receptors results in relaxation of airway

smooth muscle. The physiological role of these $ß_2$-adrenergic receptors is unclear. Although circulating adrenaline can dilate normal and asthmatic airways [16], plasma adrenaline levels in asthmatics are not elevated, not even during an attack or bronchoprovocation [143 213]. The epithelial and mast cell ß-adrenergic receptors are also of the $ß_2$-subtype whereas in human submucosal glands and alveolar walls, receptors of the $ß_1$-subtype have also been found [43]. Prejunctional $ß_2$-adrenergic receptors present on postganglionic cholinergic nerves inhibit cholinergic output [5 14 200]. They are also present on sympathetic nerve fibers that supply human pulmonary arteries [96]. Alveolar type 2 cells express both $ß_1$- and $ß_2$-adrenergic receptor subtypes [54]. ß-adrenergic receptor agonists increase surfactant production, primarily mediated by the $ß_2$-adrenergic receptors [48]. Recently, evidence for the presence of ß-adrenergic receptors of the $ß_3$-subtype in airways has been found. In canine airway smooth muscle these receptors may mediate bronchodilation [232]. In guinea pig airways $ß_3$-adrenergic receptors may exert a prejunctional inhibitory effect on eNANC responses [163] and epithelial $ß_3$-adrenergic receptors may mediate active transport of albumin across the epithelial cell layer [241]. In man, the gene for the $ß_3$-adrenergic receptor has been isolated and although functional studies have located these receptors in white fat cells they were not present in the human lung [130 145 152].

Abnormalities of the adrenergic innervation in asthma

Szentivanyi postulated a reduced respiratory ß-adrenergic receptor function in atopic disease and bronchial asthma [229]. This would lead to impaired relaxation of airway smooth muscle and perhaps also increase cholinergic tone and mediator release from mast cells. Investigations of ß-adrenergic receptor function in asthmatics, however, have shown conflicting results. Several investigators reported decreased ß-adrenergic receptor function in isolated airways from asthmatic patients [12 47 93], whereas others found normal relaxations of airway smooth muscle from asthmatics [58].

After cloning and sequencing of the gene encoding the human $ß_2$-adrenergic receptor, it appeared that experimentally induced mutations, involving small regions of the $ß_2$-adrenergic receptor, markedly altered the functional properties of the receptor [148]. This led to the hypothesis that mutations might be present in the $ß_2$-adrenergic receptor of asthmatic patients. Indeed, the Gly16 (glycine at position 16) polymorphism, which causes enhanced downregulation of $ß_2$-adrenergic receptors, is overrepresented in patients with nocturnal asthma [236]. However, the frequency of most polymorphisms of the $ß_2$-adrenergic receptor

gene is not different between asthmatic patients and control subjects [193 199]. Thus, there seems to be no genetic defect in the $ß_2$-adrenergic receptor. This was, however, unlikely because ß-agonists have excellent bronchodilatory effects in asthmatic patients.

3.4 EXCITATORY NON-ADRENERGIC, NON-CHOLINERGIC (eNANC) INNERVATION

Excitatory NANC (eNANC) bronchoconstrictor responses are believed to be mediated via the release of sensory neuropeptides from a subpopulation of non-myelinated sensory C-fibers in the airways. Six sensory neuropeptides have been identified in mammalian sensory nerves: calcitonin gene-related peptide (CGRP) and the tachykinins substance P (SP), neurokinin A (NKA) neurokinin B (NKB), neuropeptide K (NPK), and neuropeptide Y (NPY) [25 105]. The peptides may be colocalized in the same secretory granules [170] and can be released from sensory nerve endings by a range of chemical and physical stimuli [22 224].
Sensory neuropeptides have been shown to induce airway smooth muscle contraction in vitro [2 158 177] and in vivo [50 122], mucus secretion [195 210], vascular hyperpermeability [40 189 219], and tracheobronchial vasodilatation [244]. In addition, SP modulates chemotaxis of inflammatory cells and the production of immunoglobulines and cytokines by these cells [38 42 181].
The effects of neuropeptides are limited because they are rapidly degraded by neutral endopeptidase (NEP, CD10, EC 3.4.24.11), a membrane-bound enzyme present in human airways [120 142], angiotensin-converting enzyme (ACE, kininase II, EC 3.4.15.1), mast cell tryptase and chymase [48], and other peptidases [31 37].

Distribution
Tachykinin-containing, nonmyelinated C-fibers have been localized in human airways by immunohistochemistry. Terminal varicosities are located within the epithelium, airway smooth muscle, submucosal bronchial glands, bronchial blood vessels, and intramural tracheobronchial ganglion cells [91 155 156 157 159 165 166]. In the rat lung an association between peptidergic nerves and mast cells has been found and this may also be the case in the human lung [179]. Sensory C-fibers from the airways travel up the vagal nerves to neuronal bodies in the jugular and nodose ganglia. In addition, SP-containing nerves pass with sympathetic nerve fibers via the stellate ganglion to terminate in thoracic spinal dorsal root gan-

glion cells [16 224]. Both SP, NKA and NPK but not NKB have been demonstrated by radioimmunoassay in human bronchi [156 165].

Tachykinin receptors

Tachykinins exert their action through activation of specific receptors in the target tissue. In human airways, radiolabeled binding studies with ^{125}I-labeled SP have shown a high density of tachykinin receptors in vascular endothelium of microvessels of the lamina propria, whereas airway smooth muscle, pulmonary vascular smooth muscle, submucosal glands and the epithelium were scarcely labeled [45 94 238]. In contrast, recently a high density of SP-receptors was reported in airway smooth muscle, submucosal glands and airway epithelium of the human lung, which is in accordance with the pharmacological action of SP [78]. Three classes of tachykinin receptors have been demonstrated pharmacologically in mammalian airways: the neurokinin 1 (NK_1-), NK_2- and NK_3-receptors [160 196 197 198]. In addition, a species-dependent heterogeneity has been described for the NK_1- and NK_2-receptor based on differences in affinity for specific nonpeptide antagonists [123 160 196]. The tachykinin receptors differ in their affinity for natural tachykinins: NK_1-receptors are activated preferentially by SP, NK_2-receptors by NKA, and NK_3-receptors by NKB [101 198].

In animal airways the NK_2-receptors and in some species the NK_1-receptors have been shown to be involved in bronchoconstriction, whereas the NK_1-receptor is involved in mucus secretion, microvascular leakage, vasodilation, and mucociliary activity [81 124 151 160]. In human airways the NK_2-receptor mediates bronchoconstriction [3 72 177]. The functions of NK_1- and NK_3-receptors in human airways have not been elucidated.

The three tachykinin receptors from animals and humans have been cloned and sequenced [90 97 176]. The receptors belong to the superfamily of G-protein-coupled receptors characterized by seven transmembrane regions and the homology among species is high [176]. mRNA for the NK_1- and the NK_2-receptor has been detected in central and peripheral human lung and is predominantly localized in airway epithelium and vascular endothelium [1 13].

Specific binding studies with radiolabeled CGRP have shown that CGRP-receptors are widely distributed in human lung. Bronchial and pulmonary blood vessels and alveolar walls were densely labeled whereas smooth muscle, epithelium and submucosal glands were sparsely labeled [224]. There are two receptors for CGRP which are probably coupled to G-regulatory proteins [25].

Table 3. Factors modulating tachykinin release*

Factors promoting release	Factors inhibiting release
Capsaicin	Capsaicin (systemic dose)
Bradykinin (B_2-receptor)	Opioids (μ receptor)
Histamine (H_1-receptor)	Histamine (H_3 receptor)
Leukotriene D_4	Neuropeptide Y
Hydrochloric acid	Vasoactive intestinal peptide
PGI_2 (IP receptor)	PGE_2 (EP receptor)
PAF	Adenosine (P_1 receptor)
Cholinergic agonists (N-receptor)	α_2-, β_2-adrenergic agonists
Ammonia vapor	Ruthenium red
Electrical field stimulation	Sodium cromoglycate
Mechanical probing	Lidocaine
Hypertonic aerosol	ω-Conotoxin
Cationic proteins	Tetrodotoxin

* based on references 22 and 224.

Prejunctional modulation of tachykinin release

A variety of chemical and mechanical factors can modulate C-fiber conductivity and neuropeptide release (table 3). Capsaicin, the pungent agent in chili peppers, is commonly used as a pharmacologic tool to stimulate sensory nerves [106 158]. It acts on a nonselective cation channel resulting in release of tachykinins from C-fibers [32]. Addition of this agent to the perfusate of isolated, perfused guinea pig lungs increases SP-immunoreactivity in the effluent [214]. Chronic systemic administration of capsaicin partially depletes the lung from tachykinins [24 119], a characteristic that is often used in studies of laboratory animals in vivo.

Abnormalities of the eNANC innervation in asthma

Because the effects of tachykinins in the airways resemble the pathophysiology of asthma, Barnes postulated his 'axon reflex hypothesis' in 1986 [23]. This states that in the damaged epithelium of asthmatic patients, epithelial C-fibers will be exposed. Excitation of these fibers will produce a retrograde local axon reflex that results in release of tachykinins. This local release of tachykinins causes smooth muscle constriction, microvascular leakage and tracheobronchial vasodilatation, a process called 'neurogenic inflammation'. Until now evidence for this mechanism has only been found in experimental animals [115 153 247]: Bradykinin-induced contractions of the guinea pig trachea were reduced by

tetrodotoxin or pretreatment with capsaicin, suggesting that bradykinin-induced contraction was potentiated by the release of tachykinins [115]. In asthmatic patients, inhalation of the tachykinin antagonist FK-224 partly prevented bradykinin-induced bronchocontriction [111]. Although the authors speculated that local axon reflexes were involved, their findings can also be explained by a reduced cholinergic reflex bronchoconstriction after blockade of tachykinin receptors on cholinergic nerve terminals.

In 1991, Ollerenshaw reported a greater number of SP-containing nerve fibers in lung tissue of patients who died from asthma [185]. Later studies reported increased SP-immunoreactivity in bronchoalveolar fluid and sputum of asthmatic patients [178 235]. Although these studies favour a role of neuropeptides in the pathophysiology of asthma there are some controversies: In bronchial biopsies of asthmatic patients no difference in substance P contents was found between asthmatic and control subjects [108]. Moreover, in a recent study, resected lung tissue from asthmatics contained less SP-immunoreactivity than tissue from nonasthmatic patients [150].

Neurogenic inflammation may also result from an increased production or release of neuropeptides or from a reduced degradation of neuropeptides. Environmental pollutants such as sigarette smoke or toluene diisocyanate decrease airway NEP activity and increase airway responsiveness to SP [68 221]. Similarly, viral infections and chronic airway inflammation may decrease NEP activity and increase the effects of (endogenous) neuropeptides [67 118 149]. In a recent study of mild asthmatic subjects, however, no evidence for NEP dysfunction was found [49]. Despite all the evidence that neuropeptides are involved in asthmatic airway inflammation, the precise role of neuropeptides in the pathophysiology of asthma remains to be elucidated.

3.5 INHIBITORY NON-ADRENERGIC, NON-CHOLINERGIC (iNANC) INNERVATION

Because functional studies did not show important sympathetic innervation of human airway smooth muscle [53 56 205 233], the inhibitory NANC (iNANC) nervous system is believed to be the only neural bronchodilator pathway in human airways [24 138 204]. iNANC nerves have been demonstrated in vitro in human airways by electrical field stimulation [204 233] and in vivo by laryngeal stimulation [139]. Stimulation of iNANC nerves results not only in bronchodilation but also in

increased mucus secretion [188] and tracheobronchial vasodilation [59 211 244]. Although vasoactive intestinal peptide (VIP) has long been considered to be the neuro-transmitter of the iNANC nerves [71 168], evidence is accumulating that nitric oxide (NO) is the major iNANC neurotransmitter in human airways [11 28 29 71]. Although exogenous VIP can be degraded by neutral endopeptidase (NEP, CD10, EC 3.4.24.11) and angiotensin-converting enzyme (ACE, kininase II, EC 3.4.15.1), the NEP inhibitor phosphoramidon did not affect iNANC responses [201]. It is likely, therefore, that mast cell tryptase and chymase may be mainly responsible for the breakdown of VIP [46 80 231].
The neurotransmitter NO is formed during the conversion of L-arginine and oxygen to L-citrulline by the enzyme nitric oxide synthase (NOS). After production NO is released by simple diffusion. NO is a highly reactive, unstable molecule with a half-life in tissue of less than 5 sec, and may behave in a paracrine fashion [88].

Distribution
Immunohistochemical procedures have been used to localize VIP-containing nerves in human airways [60 135]. VIP appeared to be present in nerve fibers supplying airway smooth muscle, blood vessels and submucosal glands. The VIP-containing nerves were more abundant in larger airways [135]. In addition, a dense network of VIP-immunoreactive nerve fibers is present around nerve cell bodies in local microganglia [135]. VIP-immunoreactivity was also found in the vagus nerve [154] and appeared to coexist with acetylcholine-containing granules [135], suggesting a functional relationship between VIP and classic cholinergic neural control. Indeed, studies in rodent tracheas showed that endogenous VIP modulates cholinergic neurotransmission and airway smooth muscle contraction [27 218]. In human airways, however, these mechanisms could not be confirmed [239]. Other peptides with the same immunohistochemical distribution and similar characteristics as VIP have been identified in mammalian nervous tissue such as peptide histidine isoleucine (PHI) and peptide histidine methionine (PHM), encoded by the same gene as VIP, peptide histidine valine (PHV), and helodermine [24].
The NO producing enzyme NO synthase (NOS) has been demonstrated in the human lung by immunohistochemical staining and is present in epithelial cells, endothelial cells, vascular smooth muscle cells, iNANC-neurons, and inflammatory cells [41 77 88 104]. cNOS in NANC-nerves is colocalized with VIP [61] and is present in airway smooth muscle, blood vessels, submucosal glands and symphatic and sensory ganglia [77]. It has been suggested that NO may be co-

released with acetylcholine from parasympathetic nerves. However, iNANC responses are absent in heart-lung transplant recipients whereas cholinergic responses are preserved [18].

VIP receptors

VIP receptors have been localized in human airways by binding studies using ^{125}I-VIP. In two studies a high density of labeling over airway smooth muscle in the human bronchus was found and blood vessels were very densely labeled [44 206]. These findings are in contrast, however, with another study in which lack of specific binding over airway smooth muscle and a high density of binding in pulmonary artery smooth muscle was found [146]. In all studies dense labeling was also found over submucosal glands, the epithelium and alveolar walls [44 95 146 206].

Nitric oxide does not act through binding with a specific receptor but simply diffuses through the cell membrane to react with intracellular cyclic guanosine 3',5'-monophosphate (cGMP) [19 88 173]. Alternatively, NO may react with thiol to form S-nitrothiols which are potent smooth muscle relaxants [89].

Abnormalities of the iNANC innervation in asthma

In 1990 Ollerenshaw and colleagues described absence of VIP-immunoreactive nerves in patients with asthma [184]. Their observations suggested that bronchial hyperresponsiveness in asthma might be due to a loss of bronchial relaxation mediated by the release of VIP by iNANC nerves. However, normal VIP-immunoreactive nerves were found in biopsies from asthmatics [107], suggesting that the results of Ollerenshaw and coworkers may have been influenced by degradation of VIP when sections of lung tissue were cut [21]. Alternatively, the VIP-degrading enzyme tryptase [46], elevated in asthmatic airways [242], may degrade VIP before it exerts its bronchodilatory and antiinflammatory actions.

The importance of these observations can be questioned because NO is now regarded as the principle neurotransmitter of iNANC nerves. Therefore, a defect in airway iNANC responses seems possible. This could result from breakdown of NO by superoxide anions produced by inflammatory cells [88] or, alternatively, from dysfunction or reduced expression of NOS. Evidence for a NOS dysfunction was found in tracheas from virus-infected guinea pigs: In vitro the tracheas were hyperresponsive to histamine and this was accompanied by a decrease in NO release [89]. Functional studies in vivo and in vitro, however, showed no differences in iNANC responses between asthmatic and normal subjects [12 137].

3.6 CONCLUSIONS

It is clear that the cholinergic (parasympathetic) nervous system is the dominant neuronal pathway in the control of airway smooth muscle tone in human airways. Stimulation of cholinergic nerves causes bronchoconstriction, mucus secretion and bronchial vasodilation. Although abnormalities of the cholinergic innervation have been suggested in asthma, thusfar the evidence for cholinergic dysfunction in asthmatic subjects is not convincing.
Adrenergic (sympathetic) nerves have been demonstrated by immunohistochemistry in human airways. Although the adrenergic nervous system may control tracheobronchial blood vessels, functional studies could not demonstrate sympathetic innervation of human airway smooth muscle. ß-adrenergic receptors, however, are abundant on human airway smooth muscle and activation of these receptors causes bronchodilatation. The physiological role of ß-adrenergic receptors is unclear and their function seems normal in asthmatic patients.
Recently, non-adrenergic, non-cholinergic (NANC) nerves with inhibitory (iNANC) and excitatory (eNANC) components have been described in human airways. iNANC nerves may be the only bronchodilatory nerves in human airways. Although a dysfunction of iNANC nerves has been proposed in asthma, thusfar no differences in iNANC responses were found between asthmatic and normal subjects. In animal airways, eNANC activation causes bronchoconstriction, mucus secretion, vasodilation, vascular hyperpermeability and chemotaxis of inflammatory cells, a process called 'neurogenic inflammation'. Although eNANC nerves and their neurotransmitters have been demonstrated in human airways, their functional relevance and their role in the pathophysiology of asthma remains to be elucidated.

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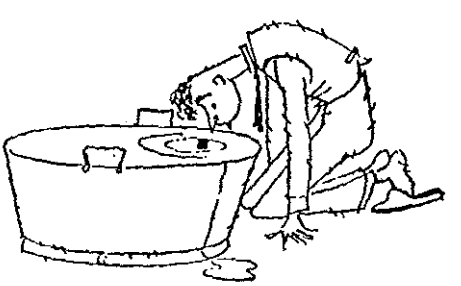
Part 2

Methods



Chapter 4

Studies of Human Airways in Vitro: A Review of the Methodology

4.1 Introduction

The pathophysiology of human airway narrowing that characterizes asthma and chronic obstructive pulmonary disease (COPD) is only partly understood. Inflammation of the lung, especially of the airways, is found both in asthma [32] and in COPD [20] and may play a pivotal role in the development of bronchial hyperresponsiveness in asthma [17 75] and COPD [5 152].

In order to gain more insight into the underlying processes of human lung diseases, animal models have been developed. In most of these animal models, the immediate (IAR) and the late asthmatic reaction (LAR) can be induced by allergen challenge [61 62 95 129 142]. However, important differences between species exist [129]. Therefore, it is important to study pathophysiological and pharmacological aspects of human airway disease in human isolated airways. Most in vitro studies of human airways have been performed on tissue derived from smokers with or without COPD who were operated on because of bronchial malignancy. These studies have shown that the sensitivity of isolated airways is not related to the sensitivity to inhaled histamine or methacholine in nonasthmatic subjects indicating that airway hyperresponsiveness may not result from an intrinsic abnormality of airway smooth muscle [15 23 135 140]. In the rarely available airways of asthmatic patients increased [6 24 125] as well as decreased [52 147] responses to contractile agonists have been reported. These apparently contradictory results, that may well be due to methodological factors, and the lack of correlation between in vitro and in vivo measurements illustrate that in vitro data should be interpreted with caution [28].

Nevertheless, the study of pharmacology of human airways in vitro may provide insight into the pathogenesis of human airway disease. For instance, the role of the airways epithelium [3 40 45] and the non-adrenergic, non-cholinergic

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(NANC) nervous system [25 36 118] in airway responsiveness have been studied in detail in isolated airways. Furthermore, studies in vitro offer the opportunity to test the effects or side effects of novel pharmacological compounds potentially acting on lung tissue. The relaxant effects of the potassium channel opener cromokalim [19], originally developed for the treatment of hypertension, and of the phosphodiesterase inhibitors rolipram and SK&F 94120 [8] on human isolated airways are examples of such in vitro studies.
A variety of models for animal and human isolated airways in vitro has been described since Williams [149] demonstration of the contractile mechanisms in isolated lungs. This review will briefly discuss these models, their historical backgrounds, their applications, and their restrictions. Furthermore, methodological aspects such as preparation and overnight preservation, epithelium removal, and electrical field stimulation are discussed, emphasizing human airways.

4.2 ISOLATED AIRWAY PREPARATIONS

Airway strip and ring preparations
In 1912 an isolated large airway preparation was described by Trendelenburg [138]. Isotonic recordings were made in bovine tracheal rings with or without cartilage, and the bronchodilator effects of caffeine, adrenaline, and atropine were demonstrated [138]. In order to measure the responses of tracheal muscle of small animals in vitro, Castillo and De Beer [14] used a chain of 10 to 12 tracheal rings. By means of the additive responses of the rings in the chain preparation, they were able to demonstrate bronchoactive effects of various drugs. The same principle was applied to human central bronchi [60 120]. However, the tracheal chain is a laborious preparation that requires a lot of airway tissue, and each connecting knot is a potential source of tissue damage and mechanical instability. Several years later, spirally cut airway strips from animals [18 112 114] and humans [12 50 114] were described. These were easier to prepare, but the preparation also caused tissue damage. The development of sensitive transducers made it possible to record isotonic shortening or isometric force development in strips or rings of dissected animal [1 63 114] and human [22 43 73 114] small airways. In figure 1 the different airway strip and ring preparations are shown schematically. Theoretically, airway ring preparations have advantages over airway strips: the contraction of a ring is directly related to airway narrowing and, furthermore, the con-

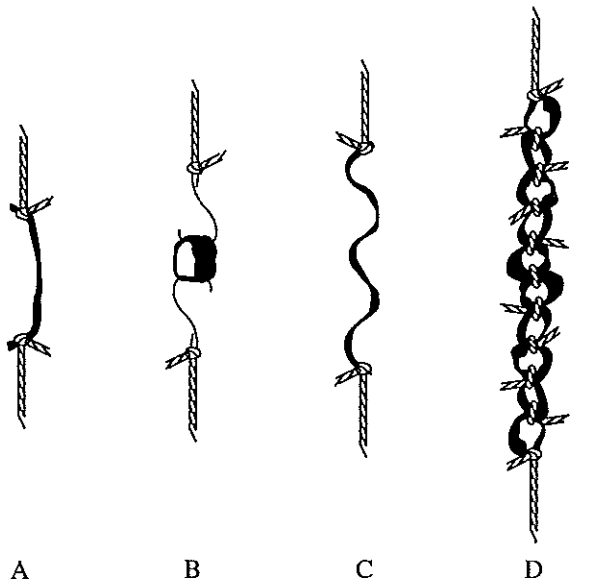

Figure 1. Schematic representation of the airway strip (A), airway ring (B), spiralized airway strip (C), and airway chain preparation (D).

figuration of the smooth muscle bundles is largely preserved. A practical advantage is that only a small piece of tissue is needed. A disadvantage of the airway strips and ring preparations is the inability to stimulate the mucosal or the serosal side selectively. Figure 2 displays an airway ring preparation in a conventional double-jacketed organ bath.

Lung parenchymal strips

The lung parenchymal strip was developed by Lulich and colleagues [88] as an in vitro preparation to evaluate the actions of drugs on peripheral airways. Thin strips (approximate dimensions 20 x 3 x 3 mm) were dissected from a lung lobe and studied in a conventional organ bath. The preparation has been widely used in studies of both laboratory animal [16 33 77 108] and human [42 49 123] lungs. Marked differences in responsiveness to various agents of the smooth muscle of central airway strips, and lung parenchymal strips were found, and it was assumed that the drug-induced effects in parenchymal strips reflected the responses of smooth muscle of small airways present in the bronchioles and alveolar ducts [16 33 42 49 88]. However, since the responses of parenchymal strip preparations to sympathomimetic drugs were not consistent between species and even within a single

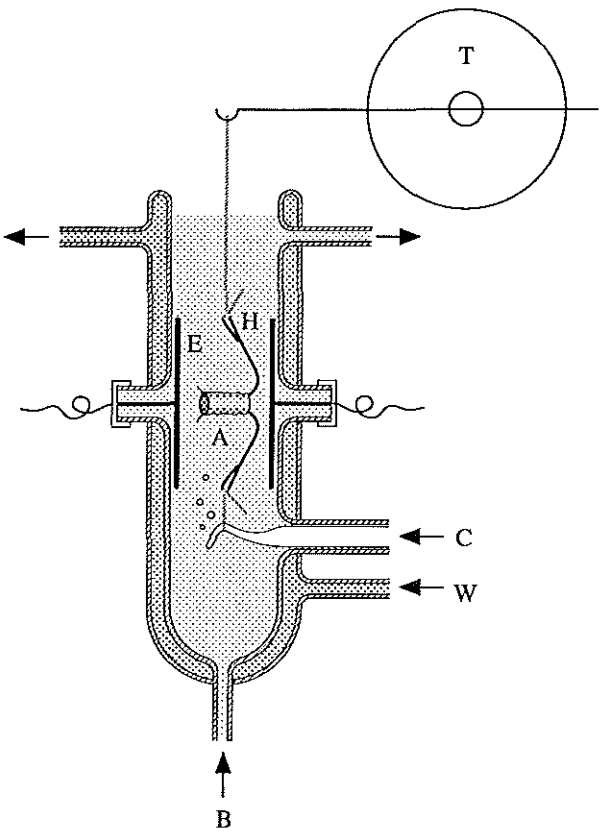

Figure 2. Schematic representation of an airway ring preparation mounted in a double-jacketed, glass organ bath. A = airway preparation; B = buffer supply; C = carbogen supply; E = platinum electrodes; H = stainless steel hooks; T = isotonic transducer; W = warm (37°C) water supply.

animal, the involvement of non-airway components such as vascular smooth muscle [50 96] and, probably, interstitial contractile cells [76] was suggested. Indeed, Bertram and coworkers [10] showed that the type and size of responses of human parenchymal strips to the sympathicomimetic drugs serotonin and noradrenaline depended on the relative amounts of blood vessels and larger airways present in the airway preparation. Thus, noradrenaline will induce a contractile response in parenchymal strips containing more than twice as much vascular smooth muscle as airway smooth muscle, and noradrenaline will induce a relaxation when this ratio is lower than twofold (figure 3). These relative amounts of the contractile components were determined with stereological analysis, a method that enables estimates of different parameters in a 3-dimensional body [143], and a

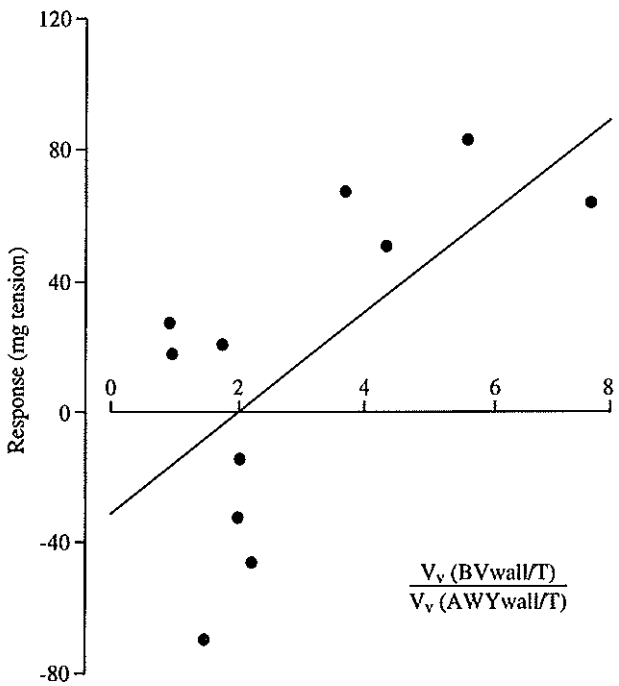

Figure 3. Relationship between the ratio of the volume densities of blood vessel wall to airway wall in tissue, V_v (BVwall/T)/V_v (AWYwall/T), and responses of human isolated lung parenchyma strips to noradrenaline ($r=0.65$, $p<0.05$). From reference 10, with permission.

large variability in composition was shown between strips obtained from the same lung [10]. Similarly, the disparity of responses in different species can be explained by differences in composition; a slice of rat lung will comprise larger airways and blood vessels than would a similarly sized slice of human lung [38 51]. It appears, therefore, that the anatomical complexity of the parenchymal strip restricts its value as a method to study small airway pharmacology.

Isolated perfused airways

The airway epithelium may modulate airway responsiveness through the release of relaxing factors [39], the breakdown of agonists [2 31], and by acting as a physical barrier [103]. In human airway strips and rings, however, only a 1.5- to 2.5-fold increase in sensitivity to contracting agonists is found after mechanical removal of the epithelium [3 74 116], probably because the stimulus reaches the smooth muscle not only via the mucosal side but also via the serosal side and the cut surface.

Therefore, models have been developed that allow independent stimulation of rodent intact tracheas and pig and human bronchial segments from the serosal and the mucosal side selectively. These airway 'tube preparations' were perfused under conditions of constant flow [40 97 102 154] or constant pressure [97 107 130], and responsiveness was measured as a change in perfusion pressure or flow, respectively. In these intact perfused airway preparations, the sensitivity to luminally applied contractile and relaxing agonists was much lower (over 30-fold) than that to serosally applied agonists. These differences were abolished after mechanical rubbing of the epithelium, indicating that the effect was caused by the presence of epithelium. We developed a similar model to investigate the modulatory role of the epithelium in human peripheral airways [64]. Human isolated peripheral airway tubes were perfused with Krebs-Henseleit (K-H) solution at a constant pressure of 6 cm H_2O (figure 4), and responsiveness was measured as a change in flow. Accurate and reproducible measurements of sensitivity to methacholine were obtained. With this method we demonstrated a much greater modulatory role of the epithelium in human perfused peripheral airways than in peripheral airway strips [65].

Apart from studying the modulatory role of the epithelium, airway tube preparations have been used to study other factors that determine airway narrowing such as preload and airway compliance. The effect of preload on airway narrowing has been studied in rabbit, pig, and human isolated airways. The transmural pressure in closed airway segments was varied between -10 and +30 cm H_2O, and the pressure change to field stimulation was recorded. It appeared that both the presence of cartilage and the transmural pressure determine the preload (and hence force) of the smooth muscle (figure 5) [100 132].

Gunst and Stropp [57] determined pressure-volume relationships in canine bronchi by measuring bronchial transmural pressure changes during inflation and deflation of the airway preparation with K-H solution. The compliance of contracted airways was lower than that of relaxed airways. Large airways contracted with acetylcholine (10^{-3}M) developed pressures >30 cm H_2O only near their maximal volumes, whereas small airways developed similar pressures at a much wider volume range (figure 6). Furthermore, small airways were able to constrict to closure but large airways constricted only to 30% of maximal volume. These differences are probably due largely to differences in orientation of the smooth muscle tissue and in the amount of cartilage between large and small airways [57].

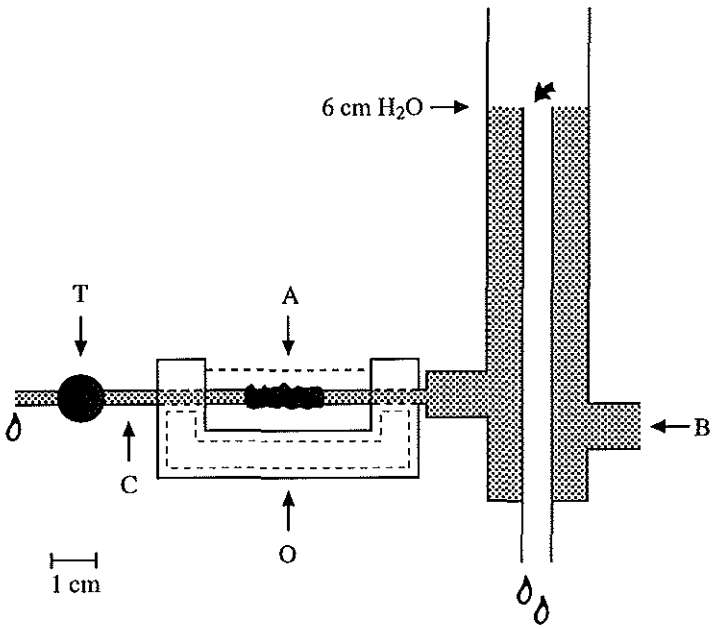

Figure 4. Schematic presentation of the human perfused airway preparation. The transmural pressure is maintained at 6 cm H_2O. B = buffer supply; C = stainless steel cannula; O = organ bath; A = airway segment; T = electromagnetic transducer. From reference 64, with permission.

Isolated perfused lungs

The lung is supplied by both the pulmonary circulation and the tracheobronchial circulation. The tracheobronchial circulation may be important in the pathogenesis of asthma because of its involvement in the influx of inflammatory cells into the airways, in the development of airway wall edema, and in the clearance of bronchoactive mediators and inhaled drugs [29 30 115 148]. In addition, hyperemia and hyperpermeability of bronchial vessels may increase airflow resistance and airway responsiveness to bronchoconstricting agents [87 115]. These aspects can not be studied in isolated airway preparations. In isolated whole-lung preparations, however, relationships between airways and the vascular systems are preserved. Models of dog, rat, rabbit, and guinea pig perfused and ventilated lungs have been described [37 59 84 90 104 122 146]. After anesthesia, animals are tracheotomized, and a cannula is inserted into their trachea and connected to a ventilator. Then the thorax is opened, and heparin sodium is injected into either the right ventricle or intravenously. The pulmonary artery and pulmonary vein or the left atrium are cannulated, and the blood is flushed from the pulmonary

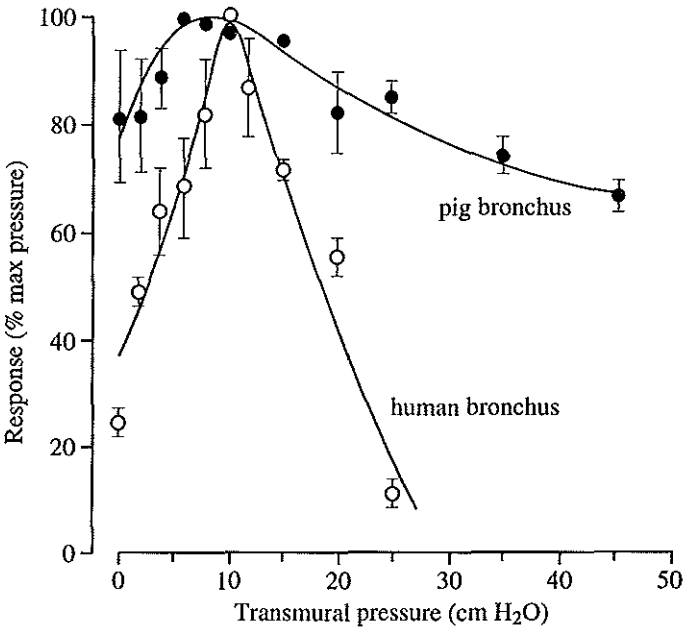

Figure 5. Relationship between transmural pressure and response to EFS of bronchial segments from pig (3 mm i.d.) and human (4 mm i.d.) lung. Closed segments were activated by electrical field stimulation (20 Hz, 0.5 ms, 60 V) and the increase in pressure was recorded via a transducer attached to a T-piece at one end of the preparation. The transmural pressure was changed by introducing different volumes of Krebs-Henseleit solution into the segment. Means ± SEM, n=3-4 animals. From reference 131, with permission.

circulation with K-H solution at 37°C. The lungs are either left in situ [141] or removed from the thorax and placed in a water vapor-saturated glass or perspex chamber warmed to 37°C. During the experiment the lungs are perfused via the pulmonary artery with oxygenated K-H buffer (37°C) containing 4%-5 % bovine albumin or with whole blood under either constant pressure or constant flow conditions. In most studies the preparation is ventilated with preheated and humidified gas by creating a rythmically varying negative pressure (-3 to -12 cm H_2O) in the thorax chamber. Alternatively, positive pressure ventilation has been used [59 71 84 92]. Weight gain during the experiment, indicating extravasation of the perfusate, is continuously monitored or measured before and after the experiment. In figure 7 the experimental set-up of an isolated perfused and ventilated guinea pig lung is displayed.

The absence of perfusion of the bronchial circulation and lymphatic drainage

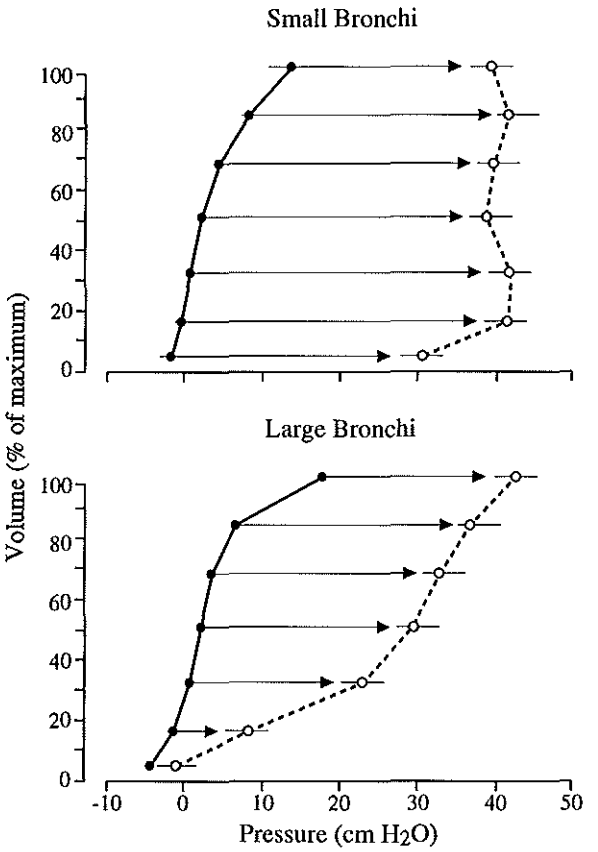

Figure 6. Maximal pressures developed by small (n=6) and large (n=7) bronchi. Closed circles represent passive pressure obtained 1 min after inflation. Open circles represent pressures obtained after contraction with acetylcholine (10^{-3} M). From reference 57, with permission.

are considered as major drawbacks in these preparations. However, after perfusion of the pulmonary artery with fluorescein isothiocyanate (FITC-D, MW 150,000) Kröll and coworkers found abundant presence of FITC-D in tracheobronchial tissue [82]. This indicates functioning anastomoses between the pulmonary and the bronchial circulation, and this increases the validity of this model. In order to ensure oxygenation of medium-sized and large airways, Kröll and colleagues ventilated the isolated lung with supranormal O_2 tension [81]. The nonfunctioning lymphatic system, fluid extravasation, and the use of an artificial perfusion medium may lead to a weight gain during perfusion [44 81]. Nevertheless, preparations can be used for several hours during which the lung function remains

stable [81]. The model can be used for the measurement of lung resistance (R_L) and dynamic compliance (C_{Dyn}) and for metabolic and pharmacological studies [81]. Furthermore, Wang and coworkers [141] showed that it is possible to measure capillary transit time in isolated rabbit lungs by fluorescence video microscopy. No studies in perfused ventilated human lungs have been described, probably because a fresh whole-lung preparation is rarely available.
It might be possible to develop a model for the perfusion and ventilation of human lung lobes or segments. However, the fact that the blood supply of a given ventilatory unit comes from several vascular units [144] may provide a major problem in preparations of human lung segments.

4.3 PREPARATION AND OVERNIGHT STORAGE

Animal airways tissue can usually be studied immediately after removal. Human airway tissue, however, is often obtained with some delay because pathological examinations have to be performed. In case of autopsy there may be even many hours of delay. In a study by Ferguson and Richardson [41], lung tissue was obtained at autopsy within five hours of death. Electronmicroscopy of epithelium and smooth muscle cells showed swelling of mitochondria and endoplasmatic reticulum, condensation of cell nuclei and blebs in the cell membrane. In addition, in the smooth musle cells disorganization and clumping of the contractile filaments was seen [41]. These changes were largely reversible after incubation of the tissue in organ baths containing carbogenated K-H buffer solution. Although this may indicate recovery of the preparation from the anoxic period, only brief functional studies were performed by the authors. Bronchial tissue obtained at thoracotomies seems preferable because the anoxic damage and autolysis can be largely avoided when the tissue is submerged in cooled (0 to 4°C) K-H buffer solution immediately after surgical resection. De Jongste and colleagues described a technique for preparation and storage of human lung tissue [22]. After washing to remove blood, bronchi are identified on the cut surface of the excised lung tissue and cannulated with polyethylene tubes. Air is gently inflated to ascertain that an airway and not a blood vessel is cannulated. The airway is carefully separated from the surrounding tissue guided by the cannula. Thereafter, blood vessels, lymphatic tissue, and parenchyma are removed from the airway with iris scissors under a binocular preparation microscope [22]. The airway tubes can be cut into helical or transversal strips or rings.

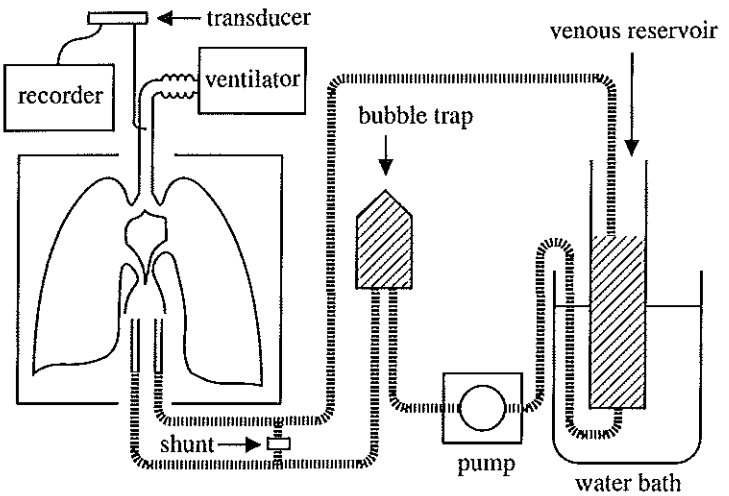

Figure 7. Experimental set-up for studies in isolated perfused lungs.

Tissue preparations can be stored overnight in cooled (4°C) Ringer's solution [60] or, preferably, cooled, and carbogenated K-H solution [12 22 117]. With K-H buffer, the response to methacholine remains unchanged until up to 55 hours after resection [22]. To prevent bacterial overgrowth, antibiotics, e.g. penicillin ($3x10^{-5}$ g/l) and tobramycin ($5x10^{-3}$ g/l) should be added.

4.4 PHYSIOLOGICAL SALT SOLUTIONS

In vitro studies are performed in glass or plexiglass organ baths filled with a physiological salt solution. Plexiglass organ baths can be a problem because interaction of several drugs with synthetics has been described [80].
A physiological salt solution is a solution of inorganic salts in which an isolated organ or tissue survives for some time and displays most of its normal functions. The critical ions in any salt solution are sodium, potassium, calcium, and bicarbonate. Sodium and chloride are the main osmotic ions, potassium, calcium, sodium, and magnesium are important for contractility. Bicarbonate is part of a bicarbonate - carbon dioxide buffer system. Ringer was the first to use a physiological salt solution in his studies on the frog heart [119]. The first salt solution for mammalian tissues was devised for the heart by Locke [86]. He increased the salt concentration of Ringer's solution to increase the osmotic

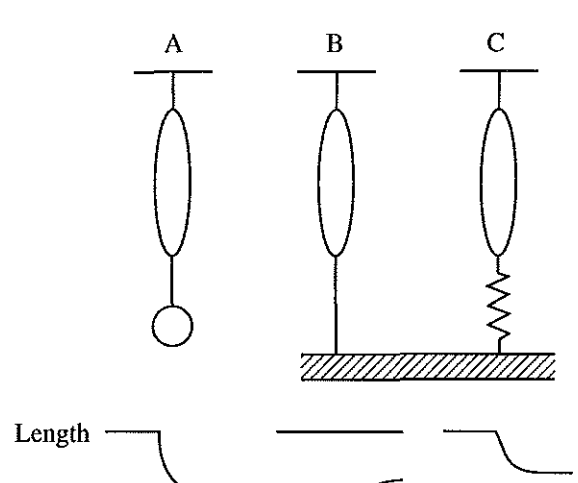

Figure 8. Recording of tension and length changes of smooth muscle obtained under isotonic (A), isometric (B) and auxotonic (C) conditions.

pressure and he added glucose (0.1%) to improve the survival time of the heart. Tyrode added phosphate to improve buffering and magnesium to maintain contractility of the smooth muscle preparation [139]. The disadvantage of Tyrode's solution is its tendency to become alkaline and to precipitate calcium carbonate. In the solution of Krebs and Henseleit, a higher concentration of bicarbonate is used, similar to that found in plasma [78]. The solution should be gassed with carbogen (95% O_2, 5% CO_2) to achieve a pH of 7.4. For studies on tissue respiration, Krebs replaced part of the sodium chloride with sodium salts of fumaric, pyruvic, lactic, or glutaric acid as additional substrates apart from glucose [79]. The K-H solution is most commonly used.

In studies of isolated perfused organs, a colloid should be added to the physiological salt solution in order to prevent edema. Several colloids have been used including dextrans [55], polyvinylpyrrolidone (PVP) [121] and bovine serum albumin [126]. Most investigators use albumin, however, disadvantages of albumin are its tendency to lower the pH, to bind calcium ions, and to froth when aerated [13]. Although most physiological salt solutions are gassed with carbogen (95% O_2, 5% CO_2), perfused mammalian organs may need more oxygen than can be provided in this way. Oxygen transport can be increased by using erythrocytes or fluorocarbons [53 58 128]. Fluorocarbons are inert organic substances in which the

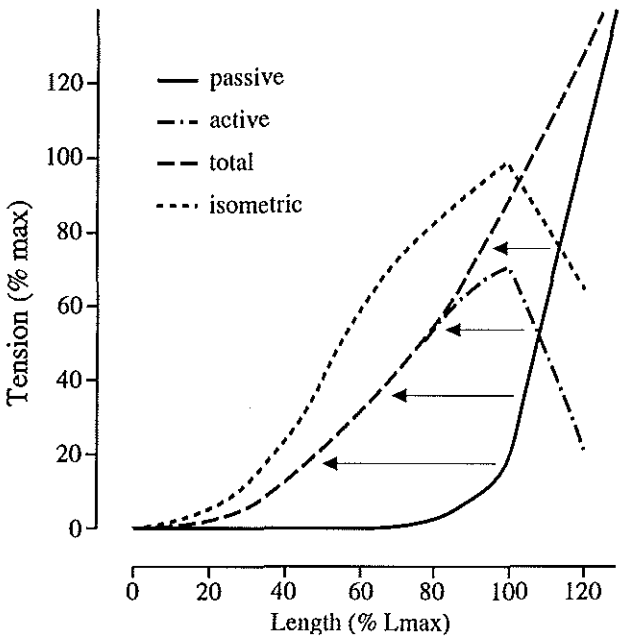

Figure 9. Length-tension relationships of airway smooth muscle. The active tension curve is obtained by subtracting passive tension from total tension. The isometric curve is also shown. The arrows indicate isotonic smooth muscle shortening at different lengths. Modified after reference 132.

hydrogen atoms are replaced by fluorine. They have the capacity to carry more oxygen than can be carried in human whole blood [13]. In the perfused ventilated lung, however, addition of an oxygen carrier may not be necessary because during ventilation of the preparation with supranormal O_2 tension, tissue hypoxia does not seem to be a problem [81].

4.5 RECORDING OF RESPONSES

Isometric, isotonic and auxotonic recording

Mechanical muscle activity can be measured under isotonic, isometric and auxotonic conditions. Under isotonic conditions, changes in length are recorded in a muscle to which a constant predetermined load is applied (figure 8a). Isometric recordings can be made by measuring changes in force of contraction in a muscle preparation which has a constant predetermined length (figure 8b). When changes in length and changes in force are measured simultaneously in a

muscle preparation where the applied load increases while the muscle shortens, this is called auxotonic (auxanein (Gr.) = to increase; figure 8c). In the early days of research of smooth muscle contractility in vitro, isotonic recording techniques were standard: The preparation was connected to the short arm of a lever, the long arm of which recorded changes in muscle length on a slowly moving kymograph. When isometric transducers became available length-tension relationships in canine tracheal smooth muscle were examined (figure 9) [132]. The preparations were stepwise stretched, and the resulting passive load and the total contractile force after electrical field stimulation (EFS) were recorded. The active load causing isotonic shortening can be derived by subtraction of resting load from total force generated. The active force development in airway preparations increases with length until an optimal length (L_0 or L_{max}) is reached. The passive tension at L_{max} is only 5% to 10% of the active tension. The horizontal difference between the active and the total tension (arrows in figure 9) represents the smooth muscle shortening under isotonic conditions. When the muscle is stretched beyond L_{max}, the maximum active tension will decrease. At all muscle lengths, force development in isometric experiments is higher, indicating that in isotonic contraction the smooth muscle does not contract fully [132]. This incomplete contraction may be due to thickening of the muscle hindering diffusion of the neurotransmitter and folding of contractile elements impairing optimal functioning [94 132]. At functional residual capacity (FRC) in vivo, the tracheal smooth muscle may be stretched to around L_{max} [99]. The experiments of Stephens and Van Niekerk imply that, for optimal results, isolated airway preparations should be stretched to approximately L_{max} by applying a weight (isotonic measurements) or increasing baseline length (isometric measurements) [132].

In the rabbit bronchus, Armour and coworkers found a correlation between the maximal isometric force generation in response to carbachol and the amount of airway smooth muscle present in the airway preparation [4]. In contrast, the maximal isotonic shortening was not related to the smooth muscle content of the preparation, and it was concluded that isometric measurements are preferable because they represent changes in smooth muscle contraction in response to an agonist more accurate [4]. In human small airways, however, only small differences were found between both methods, perhaps because the experiments were performed under near optimal tension and length conditions [26]. Although both recording techniques allow accurate and reproducible measurements of force generation, airway smooth muscle contraction in vivo is neither isometric nor isotonic, but auxotonic: during narrowing the load against which the smooth

muscle shortens increases due to an elastic load provided by the surrounding structures [99 131]. This situation has been simulated in a study performed by Ishida and colleagues who measured the effect of elastic loads on smooth muscle shortening in pig isolated airways [68]. It was shown that, at small loads, contractile responses were more or less isotonic, whereas, at large loads, minimal shortening was found indicating a isometric response [68]. Thus the size of the elastic load on the airway provided by the airway wall and the surrounding tissue determines the degree to which a contraction is isometric or isotonic.

Pressure or flow recording

The responses of perfused airway tube preparations are measured by recording changes in flow rate or perfusion pressure, depending on whether experiments are done under constant pressure or constant flow conditions, respectively. The configuration of the airway is left intact and the mode of contraction is more physiological than that in an airway strip preparation. However, in the constant flow model, the transmural pressure increases during contraction and this will influence the load on the muscle in an elastic (auxotonic) way. In the constant pressure model, airway closure may occur at higher doses of a contracting agonist [97], and this precludes accurate determination of the pharmacological sensitivity (EC_{50}) of the preparation. In human peripheral airways perfused at a constant pressure, we were able to avoid airway closure by stretching the airway preparations to 140% of their initial length [64].

Other methods to record airway responses

Other techniques to record airway responses have been developed and are briefly described below. These methods, however, are not commonly used, and their value remains to be established.

High-resolution ultrasonic imaging. In order to visualize airways smooth muscle contraction in vitro, Iizuka and colleagues introduced an ultrasonic catheter in porcine and human isolated bronchi [67]. The ultrasound technique produced a three-layer image of the bronchial wall corresponding to the mucosa, cartilage, and adventitia. The muscle could not be distinguished from the mucosa. Dose-dependent responses to acetylcholine could be obtained and it was found that human bronchus contracts elliptically, not circularly [67]. Because the diameter of the transducer is 1.7 mm, the technique can be only used in airways of at least this size.

Sonomicrometry. Okazawa and coworkers measured length changes of canine trachealis muscles in vivo with sonomicrometry [106]. This technique uses the transit time of ultrasound traveling between two piëzoelectric crystals as a measure for the linear distance between these crystals. Small (1 mm) piëzoelectric transducers were placed in the posterior tracheal wall in parallel with the muscle fibers. Length changes during mechanical ventilation and pressure-volume curves could be obtained. This method may be applicable in isolated large airways as well.

High-resolution computed tomography. With this technique, airways of 1-2 mm diameter can be visualized [137], and this method has been used to study carbachol-induced changes in airway dimensions in excised canine lung lobes [93]. The degree of airway narrowing could be accurately quantified, and it was shown that airway narrowing after carbachol is greatest in intermediate-sized airways (internal diameter: 2-6 mm).

Photoelectric recording. Schabert and colleagues developed a photoelectric method to record changes in blood vessel diameter [124]. A beam of parallel infrared light is directed at right angles to the blood vessel. The light passing the side of the vessel is a measure of the outer vessel diameter and is detected by a photocell. A decrease in vessel diameter causes an increase in photocell current. In isolated airways, photoelectric methods have been only used to study respiratory ciliary activity [133 151].

4.6 BETWEEN-PATIENT AND WITHIN-PATIENT VARIABILITY

With the above-mentioned recording techniques, accurate and reproducible measurement of parameters such as tissue sensitivity (EC_{50}), maximal contraction, or relaxation and intrinsic (baseline) tone is possible in human airway strips, rings, and tubes [22 64 73]. However, for some purposes an in vitro model should be able to detect between-patient differences. De Jongste and coworkers showed that in airway strips despite large within-patient variability, significant between-patient differences could be shown for EC_{50} and maximal response [22]. In airway tubes, between-patient differences in EC_{50} and intrinsic tone accounted for more than 90% of the total variability [64].

The finding of smaller within-patients variability in airway tubes compared to airway strips may indicate that within-patient variability in airway strips are largely due to disturbance of the airway structure during the cutting of the strips.

4.7 INTRINSIC TONE

Isolated airway preparations may exhibit an intrinsic muscle tone. In guinea pig airways this tone appears to be dependent on prostanoids and not on intrinsic innervation [109]. In human airways, however, the role of prostanoids is unclear since both enhancement [69] and reduction [70] of intrinsic tone have been described after inhibition of cyclooxygenase. In addition, peptidoleukotrienes may be involved because inhibition of 5-lipoxygenase decreased intrinsic tone [70].
Mansour and Daniel expressed the responses of guinea pig tracheas on a scale between maximal relaxation and maximal tension in response to carbachol [91]. It appeared that the responses to exogenous arachidonate were dependent on the intrinsic tone of the airway preparation; when this intrinsic tone was low, contraction was found; when it was high, relaxation was found [91]. Their findings emphasize the importance of the expression of responses on a scale that displays the maximal active contractile range (MACR) in order to be able to compare responses of different airway preparations. Monitoring intrinsic tone is also relevant when EFS is used. With high intrinsic tone, EFS may predominantly give relaxations, whereas with low tone, contraction will result.
We routinely determine maximal contraction to exogenous cholinergic stimulation at the beginning of experiments, and maximal relaxation to ß-adrenoceptor stimulation and calcium free buffer after completion of the experiments. Alternatively, a supramaximal dose of theophylline, sodium nitroprusside, or papaverine can be used to obtain maximal relaxation.

4.8 REMOVAL OF THE EPITHELIUM

Classically, the modulatory role of the airway epithelium is evaluated in paired observations of intact and epithelium-denuded isolated airway preparations. The epithelium is commonly removed by 'gentle rubbing' with a wet cotton gauze, and its effectiveness is verified histologically [3 45 74]. In guinea pig tracheas the effectiveness of epithelium removal can be also verified functionally by adding arachidonic acid which causes smooth muscle contraction in epithelium-denuded tracheas, whereas intact tracheas respond with relaxation [105]. This procedure has not been tested in human airways. With mechanical rubbing, it is possible to remove over 95% of the epithelium leaving the basal membrane and the smooth muscle histologically intact. However, Franconi and colleagues showed that

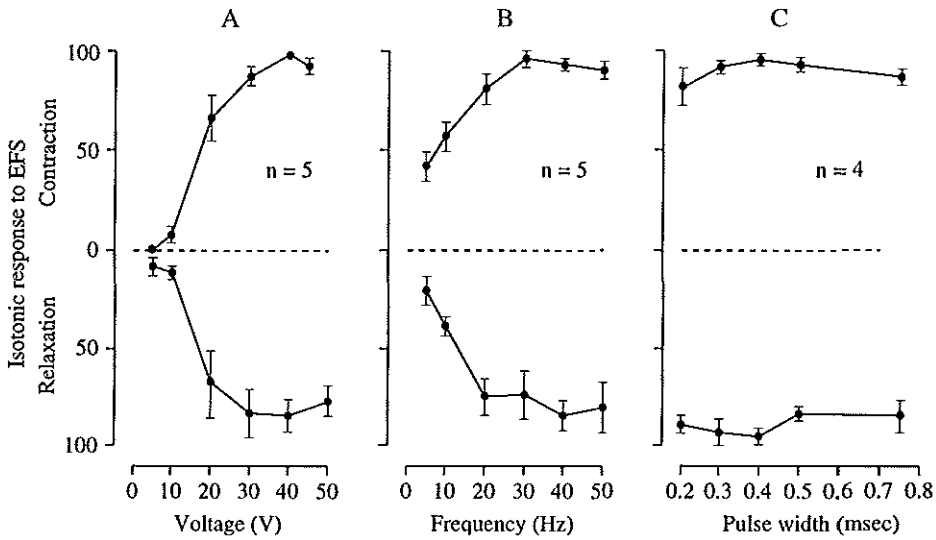

Figure 10. Effect of change in pulse voltage, -frequency and -width on the contraction and relaxation responses of human bronchial strips. (A) effect of voltage (0 to 50 V) with constant frequency (30 Hz) and pulse width (0.3 msec). (B) effect of frequency (0 to 50 Hz) with constant voltage (30 V) and pulse width (0.3 msec). (C) effect of pulse width (0.2 to 0.75 msec) with constant voltage (30 V) and frequency (30 Hz). Pulse trains of 30 sec. Contractions and relaxations are expressed as a percentage of the maximal response in a given strip. Mean values ± SEM of 4-5 experiments are shown. From reference 27, with permission.

mechanical rubbing of the epithelium may lead to release of granules containing tryptase from mast cells present in the lamina propria [47]. In dog airways, tryptase causes hyperresponsiveness, probably due to an effect on Ca^{2+}-channels [127]. This mechanism may explain at least part of the hyperresponsiveness reported in epithelium-denuded airways. Furthermore, epithelial damage and denudation in asthma is not caused by mechanical rubbing but, probably, by the release of basic proteins such major basic protein (MBP) and eosinophil peroxidase (EPO) and oxygen radicals released from inflammatory cells present in the inflamed airway [7 48 98]. Deposits of EPO were found in areas of mucosal injury in asthmatics [11]. Human MBP causes epithelial damage [101] and hyperresponsiveness in vitro [46] and in vivo [56]. Oxygen radicals increase the permeability of cultured epithelium [145] as well as epithelium in the guinea pig trachea [72], and the responsiveness of human airways is enhanced after damaging the epithelium with hydrogen peroxide [65]. In a model of asthma it may be more

appropriate to use these agressive substances rather than mechanical rubbing to induce epithelial damage. Apart from basic proteins and oxygen radicals, several other methods for damaging and removal of epithelium in vitro have been described. Franconi and cowokers removed epithelium from animal and human airways by perfusing the preparations during 0.5 to 2 hours with pronase (protease type XIV; 1 mg/ml) [47]. Whereas the epithelium was effectively removed, the integrity of mast cells in the lamina propria and the smooth muscle was not affected.
Interferon-γ, produced by intra-epithelial T-lymphocytes [35] enhances tight junction permeability in a human intestinal epithelial cell line [88] but has not been examined in airway epithelium. Perfusion of rat arteries with a hypotonic Tyrode solution [113] or the nonionic, nondenaturing detergent CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate) [136 153] resulted in a disruption of endothelial cells, and this method may be applicable for epithelial cell removal in airways as well. It appears, therefore, that there are alternatives for the mechanical removal of airway epithelium. These alternatives have the advantage that they mimic the damage that is found in asthma (basic proteins and oxygen radicals) or that they produce less artifacts (pronase).

4.9 ELECTRICAL STIMULATION

Basically, there are two methods of electrical stimulation of isolated organ preparations: contact stimulation using electrodes that are attached to the tissue and field stimulation via electrodes that are not in direct contact with the tissue. Also, one electrode may be in contact with the tissue while the other, often a ring, is not (hybrid stimulation). EFS is most commonly used to study neural responses in smooth muscle preparations including isolated airways. The technique is relatively simple; platinum- or silver-silver chloride sheet electrodes are suspended close to, but not in contact with, the tissue, in an organ bath containing K-H solution (figure 2). A stimulator generates rectangular pulses of short duration (0.1 to 1 msec) at a constant current. Voltage- and frequency-response curves can be obtained and the interval between stimuli and pulse width can be varied (figure 10).
EFS was introduced by Paton in 1955 who demonstrated that single electrical pulses (1 to 25 V, 0.5 msec) elicited brief twitches (1 sec) in the guinea pig isolated ileum [111]. Since the twitch was abolished by atropine, prolonged by

eserine, and was insensitive to hexamethonium, he concluded that postganglionic nerve fibers were excited by EFS. In later studies it was confirmed that with appropriate stimulation parameters, nerve fibers can be activated selectively without stimulating the smooth muscle directly [34 110].

In human airways, EFS causes a fast, nerve-mediated cholinergic contraction followed by a slow non-adrenergic, non-cholinergic inhibitory nerve-mediated (iNANC) relaxation [21 118 134]. In addition, De Jongste and coworkers found a rapid non-neural contraction and a sustained non-neural contraction resulting from synthesis of cyclooxygenase metabolites and leukotriene-like substances by fresh human airway tissue, respectively (figure 11) [25]. These nonneural contractions may interfere with neural responses and should be taken into account as a confounding factor. In guinea pig airways vasoactive intestinal peptide (VIP) and nitric oxide (NO) may be the neurotransmitters of the non-adrenergic inhibitory nerves [83 85], whereas in human bronchi the response may be due mainly to NO [9 36].

Although EFS is a useful tool to evoke neural responses in isolated airway tissues, the technique has an important side effect. During EFS with commonly used stimulation parameters activated oxygen molecules may be generated in carbogenated K-H buffer. These activated oxygen molecules have been shown to relax smooth muscle preparations directly [54] and may oxidize contractile drugs [66 150]. The inactivation of histamine by EFS may even occur at a frequency of 2 Hz (50 V, pulse duration 0.3 msec) [66].

4.10 CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

In the present overview, we discussed models that have been developed over the years to study the effects of drugs, inflammatory mediators, autonomic nerves, and epithelial cells on the responsiveness of airway smooth muscle in vitro. These models range from the simple bronchial strip preparation to the complex ventilated and perfused lung preparation. Although these models have substantially contributed to the progress in our understanding of the pathophysiology of asthma and COPD, the precise relationships between airway inflammation, bronchial hyperresponsiveness, and airway narrowing is still not clear. The relative contributions of inflammatory cells and their products, of autonomic nerves and of the tracheobronchial circulation to airway disease should be further investigated. With the currently available in vitro models, however, the role of the

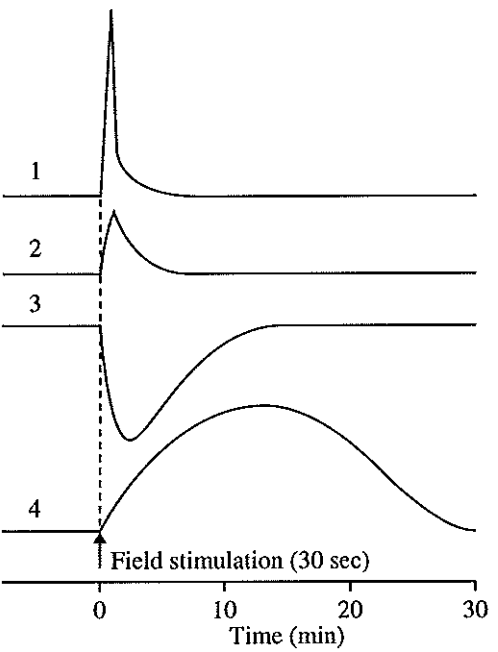

Figure 11. Schematic representation of time course, peak latency and amplitudes of the 4 phases that constitute the response of fresh human bronchus to electrical field stimulation (EFS) in vitro. Phases are numbered according to peak latency after EFS; 1: cholinergic nerve-mediated, rapid contraction. 3: non-adrenergic inhibitory nerve-mediated relaxation. 2 and 4: non-neural contraction due to the release of cyclo-oxygenase and lipoxygenase metabolites, respectively. From reference 25, with permission.

bronchial circulation in airway disease cannot be elucidated. Efforts should now be made to develop models in which the normal relationships between the ventilatory and the circulatory unit is preserved in vitro. The study of isolated perfused and ventilated lung tissue may be an important step in this direction, and it might be possible to develop such a model for the human lung.

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Chapter 5

The Perfused Human Bronchiolar Tube: Characteristics of a New Model

5.1 Introduction

Airway reactivity in vitro is usually assessed using strips or rings of airway tissue immersed in organ baths. Due to spiralization and cutting in such preparations the airway has lost its normal structure and the agonist reaches both the mucosal and the serosal side of the preparation. Hence, the specific modulatory role of the epithelium on airway smooth muscle responsiveness cannot be assessed accurately. Furthermore, the magnitude of the contractile response of strips is dependent on the orientation of the smooth muscle, and this is an important confounder when absolute responses are measured.

Recently, in vitro methods that allow for independent stimulation from either the mucosal or the serosal surface have been described for animal airways. These airways were perfused with Krebs-Henseleit (K-H) buffer under conditions of constant flow [7 9 10] or constant pressure [6 11], and airway reactivity was measured as a change in pressure or flow respectively. Results obtained in perfused tube models in presence and absence of epithelium were significantly different from those with conventional preparations [7-11]. Perfused tube models have been described for pig and rodent airways [7-11 13].

In human asthma, the relative contribution of the different levels of the bronchial tree to bronchoconstriction is not known and it has recently been suggested that small airways contribute to airflow regulation to an important degree [4 12]. Hence, it is interesting to develop an in vitro model of human peripheral airways which offers the possibility to study the modulatory effects of human airway epithelium and to study small airway mechanics. This paper describes the characteristics of the isolated, perfused human bronchial segment.

Based on: Hulsmann AR, Raatgeep HR, Bonta IL, Stijnen T, Kerrebijn KF, De Jongste JC. The perfused human bronchiolar tube: characteristics of a new model. *J Pharmacol Toxicol Methods* 1992; 28:29-34.

5.2 MATERIALS AND METHODS

Tissue preparation

Human lung tissue was obtained from 17 patients (3 females), with a mean age of 62 y (range 39 to 79 y) who underwent a thoracotomy for bronchial carcinoma. None of the patients had asthma. Immediately after surgical resection, a macroscopically normal part of the resected tissue was immersed in K-H buffer at room temperature (composition in mM: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 5.55 glucose), carbogenated (95% O_2-5% CO_2) to produce a pH of 7.35, a PCO_2 of 4.7 kPa, and a PO_2 of 71.8 kPa. The tissue remained in fresh carbogenated buffer throughout the dissection procedure and during the experiments. Tubular segments of peripheral airways (generation 9-11; internal diameter 1.0-1.4 mm; length 5-8 mm) were carefully dissected free from parenchyma and blood vessels under a 20x stereo microscope with iris scissors and forceps[3]. The side branches were ligated with thin surgical silk threads (6/0). Each end of the segment was cannulated with stainless-steel cannulas (external diameter 1.6 mm; internal diameter 1.2 mm). The resistance of the cannulas was low compared with the bronchial segment. The preparation was placed horizontally in a double jacketed 4 ml organ bath that contained carbogenated Krebs buffer at 37°C. The bronchial lumen was perfused with carbogenated K-H solution (inner perfusate) from a seperate reservoir (figure 1). The temperature, O_2 tension, and CO_2 tension in this reservoir were maintained at the same level as in the tissue bath.

The transmural pressure of the airway was maintained at 6 cm H_2O, which is similar to the normal transpulmonary pressure in vivo. The distal cannula was connected to an electromagnetic flowmeter (Nihon Kohden, model MFV-1200, Tokyo, Japan) after which the perfusate was carried to waste. The flow rate (ml/min) through the bronchial segment was displayed on a pen recorder (Kipp BD 40, The Netherlands). The use of human lung tissue for in vitro experiments was approved by the Ethical Committee of the Rotterdam University Hospital.

Drugs

Methacholine hydrobromide (Janssen Pharmaceuticals, Beerse, Belgium) and EDTA (Sigma Chemicals, St. Louis, MO, USA) were dissolved in 0.9% NaCl. L-Isoproterenol sulfate (Janssen) was dissolved in distilled water containing ascorbic acid (88 mg/l). Fresh drug solutions were prepared daily and kept on ice during the experiments.

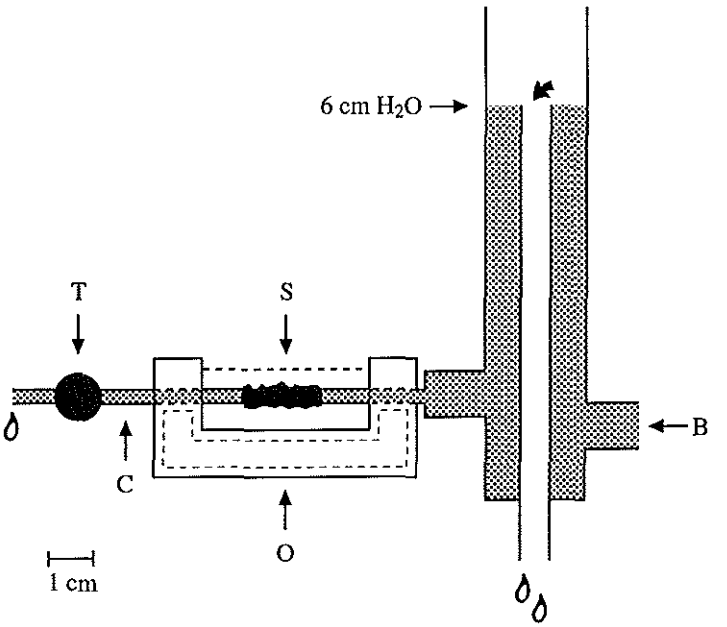

Figure 1. Schematic presentation of the measurement system. The bronchiolar segment (S) is cannulated with stainless steel cannulas (C) and mounted in a double jacketed organ bath (O) that contains carbogenated Krebs-Henseleit (K-H) solution at 37°C. The bronchial lumen is perfused with carbogenated K-H buffer (B). The transmural pressure is maintained at 6 cm H_2O. The transducer (T) of the electromagnetic flowmeter is connected to the distal cannula (C).

5.3 EXPERIMENTAL PROTOCOLS

Before the experiments, the preparations were allowed to equilibrate for 1 h. During that time, the organ bath fluid was replaced every 15 min and the preparation was continuously perfused with fresh K-H buffer. To assess contractile functions before each protocol, the preparations were contracted maximally with two consecutive doses of methacholine (10^{-5} and 10^{-4}M) added to the organ bath, followed by wash-out. The cholinergic sensitivity was measured as the -log EC_{50} of methacholine: the negative logarithm of the methacholine concentration that produces 50% of the maximal effect.

Pressure-flow relationship

To evaluate whether laminar flow was present at the pressures used, we examinated the pressure dependency of flow rates by perfusing 4 segments with a

stepwise increasing pressure (1 to 10 cm H_2O). The airways were studied at 100%, 140% and 180% of their initial length respectively.

Length-dependency of responses
Excised lung tissue is collapsed because of lack of the negative intrathoracic pressure, which, in vivo, counteracts the elastic recoil of the lung tissue. To mimic the in vivo situation stretching of the excised airway is necessary. We stepwise stretched seven segments of six patients from their initial length (i.e., excision length) to 180% of this length by 20% increments with a microscrew. Perfusion pressure was 6 cm H_2O. After each step the preparation was allowed to equilibrate and then stimulated from the serosal side with methacholine (10^{-5}M) once, followed by wash-out. After reaching 180%, the length of the preparation was returned to 100% and a final methacholine stimulation was given. For each length, baseline flow rate and maximal flowreduction after methacholine stimulation were measured.

Reproducibility of responses within preparations
Airway preparations (n=10) of 10 patients were stretched to 140% of their initial length which in the above-mentioned experiments had been established as the optimal length. Two consecutive cumulative concentration responce curves (CCRC) were made with an interval of 2 h by adding methacholine (10^{-8} to 10^{-3}M) to the organ bath, followed by wash-out. After both CCRCs were completed, full relaxation of the preparations was obtained by adding L-isoproterenol (10^{-4}M) and EDTA ($4x10^{-3}$M) to the organ bath. From each CCRC, the -log EC_{50} was determined.

Between-patients and within-patients/between segments variability
Lung tissue was obtained from seven patients. From each patient three different segments were used. Airway preparations were stretched to 140% of their initial length. A CCRC to methacholine administered to the serosal side of the preparation (10^{-8} to 10^{-3}M) was made, followed by washout. Next, preparations were fully relaxed by adding L-isoproterenol (10^{-4}M) and EDTA ($4x10^{-3}$M) to the organ bath. For each segment the -log EC_{50} and the spontaneous intrinsic contractile activity (SICA; baseline flow as a percentage of maximal flow in the presence of L-isoprenaline and EDTA) were obtained.

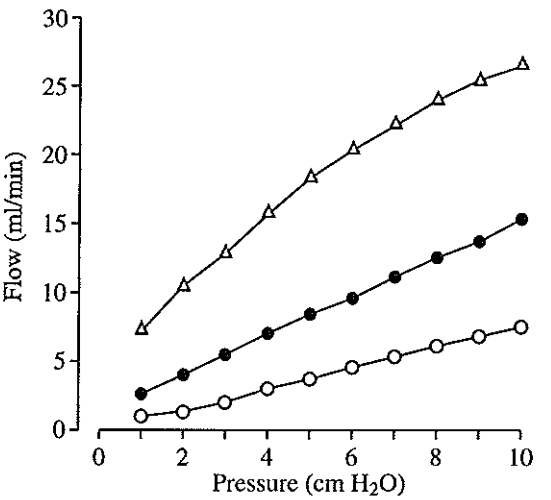

Figure 2. Mean pressure-flow relationship of 4 airway preparations. Horizontal axis displays transmural pressure, vertical axis represents the resulting flow rate at 100% (open circles), 140% (closed circles) and 180% (open triangles) of the initial length. For reasons of clarity, SEM bars were omitted. The SEM was <20% (n=4).

Data analysis

The -log EC_{50} was calculated using a BMDP software module for non-linear curve fitting [1]. The curves were fitted to a four parameter logistic function [2]. The -log EC_{50} of the two consecutive CCRCs were compared by Student's *t* tests for paired data (two-tailed, α=0.05). The contribution of the between-patients and within-patients/between segments variation to the total variation for -log EC_{50} and SICA was computed using a one-way analysis of variance (ANOVA). All data are expressed as means ± SEM.

5.4 RESULTS

Pressure-flow relationship

The flow rate proved to be pressure dependent at all airway lengths; however, only at 100% and 140% of the initial length there was a linear relationship between pressure and flow rate indicating laminar flow (figure 2). At 180% of the initial length, flow rate tended to level off at high pressures indicating turbulent flow.

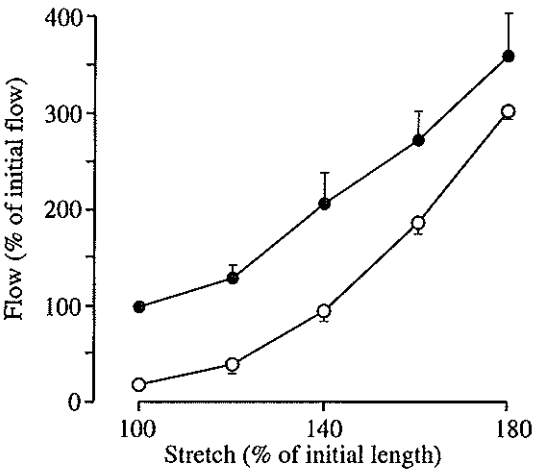

Figure 3. Baseline flow (closed symbols) and flow rate in the presence of 10^{-5} M methacholine (MCh) added to the organ bath (open symbols, n = 8 segments of 8 patients). The horizontal axis depicts stretching the preparation in 20% increments. The vertical axis represents changes in baseline flow and flowreduction after MCh. Vertical bars represent SEM.

Length-dependency of responses

Stepwise stretching the airway preparation to 180% of their initial length resulted in an increase of baseline flow by 356 ± 53% (figure 3). Serosal stimulation with methacholine (10^{-5}M) caused a length-dependent reduction of flow rate ranging from complete closure in 4 out of 7 unstretched preparations to only a minor flow reduction of 16 ± 9% after stretching to 180% (figure 3). After returning the length of the airways to their initial value (100%), flow reduction to methacholine returned to values that were not different from those initially obtained at that length (paired *t* test).

Reproducibility of responses within preparations

The initial buffer flow before stimulation with methacholine with a perfusion pressure of 6 cm H_2O and the preparations stretched to 140% of the initial length was approximately 7 ml/min. Serosal stimulation with methacholine (10^{-8} to 10^{-3}M) consistently reduced the flow in a concentration dependent way (figure 4). The maximal reduction of flow by methacholine was 74 ± 6% of the initial flow. A small but significant increase in flowrate was observed at the onset of the second CCRC. No significant difference in -log EC_{50} was found between the two consecutive CCRCs (figure 4).

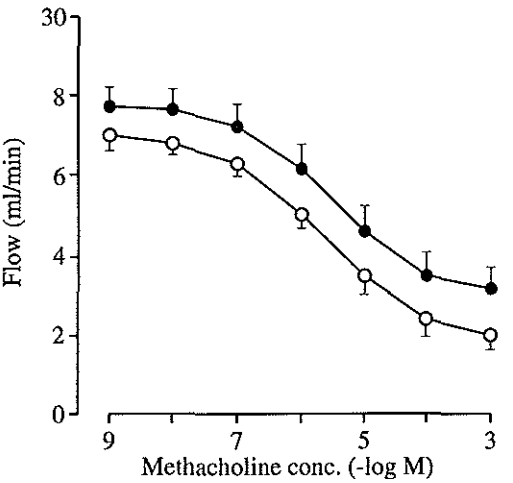

Figure 4. Consecutive CCRCs to methacholine (MCh) of human bronchiolar tubes (n = 10 segments of 10 patients), measured with an interval of 2 h. (Open circles: first CCRC; closed circles: second CCRC). The horizontal axis depicts the negative log of the Mch concentration in the organ bath, the vertical axis represents the contractile response to Mch as a reduction in flow. Vertical bars represent SEM.

Between-patients and between-segments/within-patients variability. The values of -log EC_{50} and SICA for each patient are summarised in table 1. It can be seen that the between-patients component for both -log EC_{50} and SICA is responsible for the major part of the total variation. The estimates of the between-patients and within-patients/between-segments variability for -log EC_{50} were 0.256 and 0.026, respectively. Thus the differences between the patients accounted for 91% of the total variability. For SICA these values were 113.6 and 520.9, respectively, thus 82% of the total variance in SICA was due to between-patients differences.

5.5 DISCUSSION

The purpose of the present study was to develop an in vitro model that allows for independent stimulation of the mucosal and serosal sides of intact, human peripheral airways. Our results show that human peripheral airways can be perfused under a constant pressure, and that the resulting flow is laminar. The flow rate and reduction of flow rate by methacholine administered to the serosal

Table 1. Methacholine responses of 7 patients

	-Log EC_{50}			SICA (%)		
	S1	S2	S3	S1	S2	S3
Patient 1	6.73	6.30	6.50	20.1	21.0	22.4
Patient 2	5.32	5.75	5.76	49.4	54.4	46.0
Patient 3	6.15	6.16	6.17	0.0	12.6	5.2
Patient 4	5.39	5.78	5.62	46.4	30.4	68.8
Patient 5	6.25	6.51	6.34	64.3	48.1	26.3
Patient 6	5.28	5.11	5.08	ND	ND	ND
Patient 7	6.37	6.39	6.33	ND	ND	ND

S = segment; ND = not determined

side of the preparation depend on the initial degree of stretching of the airway tubes. Repetitive serosal stimulation of the same airway preparation with methacholine (10^{-8} to 10^{-3}M) provides accurate and highly reproducible responses. Finally, a significant between-patients variability and a small nonsignificant between-preparations/within-patients variability for both -log EC_{50} and SICA was found.

The model is a more realistic approach to the in vivo situation than is the bronchiolar strip or ring preparation for the following reasons. First, the bronchoactive drug can be selectively applied to the mucosal side of the airway, thus mimicking the situation in bronchial provocation studies in vivo. This makes it possible to compare serosal and mucosal stimulation, and thereby investigate the modulatory role of the airway epithelium; exploring this aspect was beyond the scope of the present study. Second, the configuration of the smooth muscle bundles in the airways is preserved. This is important, because it removes an important source of variation. Indeed, the present results indicate a much smaller within-patient variation of airway responses than previous studies that used airway strips or segments[3,5]. Third, airway contraction is directly related to airway narrowing and not like in strips or rings of airway tissue measured as an isotonic shortening or isometric force development. Finally, in our model, preload is applied by keeping the luminal pressure at 6 cm H_2O, which is similar to the transpulmonary pressure at functional residual capacity (FRC).

At 100% and 140% of the initial length, flow rate was directly related to pressure. This indicates that there was a laminar flow, and Poiseuille's equation for

laminar flow, which states that resistance is proportional to the inverse of the fourth power of the radius, can be used at these lengths. At 180% the relationship between pressure and flow was not entirely linear. This can be explained by a tendency for turbulent flow which increases in direct proportion of the flow rate. Our observation that stepwise stretching of the airway preparation results in an increase of baseline flow (i.e., a decrease in resistance) is surprising because according to the Poiseuille equation for laminar flow, increasing the length of the preparation should result in an increase in resistance. The most probable explanation of this finding is that stretching the airway preparation removes mucosal folds and thereby decreases the resistance. Stepwise stretching the airway preparation decreased the flowreduction by methacholine 10^{-5}M (figure 3). To explain this finding, it is important to remember that in the smaller human airways, smooth muscle is oriented spirally and makes an angle of ±30° with the cross-sectional plane [4]. Stretching the airway preparation, increases the pitch of the helix of the smooth muscle bundles and thereby diminishes the constrictive forces which act to narrow the airway. Mitchell observed near-complete closure in pig bronchial cylinders perfused at constant pressure when stimulated with 10^{-5} M carbachol, that is, airway closure occurred before the pharmacological end point of the CCRC was reached [6]. We also observed near-complete closure in 4 out of 7 preparations by 10^{-5}M methacholine but only in the unstretched situation. Mitchell's results may be caused by the use of insufficiently stretched airway segments, or species differences may be present.

Because excised lung tissue is collapsed, we chose to do further experiments at 140% of the initial length because this assured a stable, reproducible signal and avoided airway closure at high agonist concentrations. Repetitive stimulation of the same airway preparation showed that reproducible measurements of -log EC_{50} could be obtained. However, an increase in flow was observed during the second CCRC for all concentrations of methacholine (figure 4). This difference may be explained by an increase in airway diameter in time due to the stretching of elastic components in the airway wall while smooth muscle contractility remained stable. Alternatively, it may be explained by the washout of mucus. Clearly, the sensitivity of the airways is not affected by repetitive stimulation.

Analysis of variance revealed significant between-patients variation and a small non-significant between-segments/within-patients variation for both -log EC_{50} and SICA. In bronchiolar strips, de Jongste and coworkers found a relatively large within-patients variability for both -log EC_{50} and the maximal tension, and it was assumed that this was due to the mechanical forces applied to the

airway tissue during the preparation procedure [3]. A somewhat better reproducibility was found in airway segments, where spiral cutting is avoided [5]. The use of bronchiolar tubes requires still less preparation and less disturbance of airway wall structure. This may explain the smaller within-patient variability that we observed.

We conclude that this in vitro model offers interesting possibilities for evaluating the modulatory effects of the airway mucosa on luminally applied stimuli. Measurements of the sensibility of perfused intact human peripheral airway tubes are both accurate and reproducible. In addition, the model provides the opportunity to study human small airway mechanical properties.

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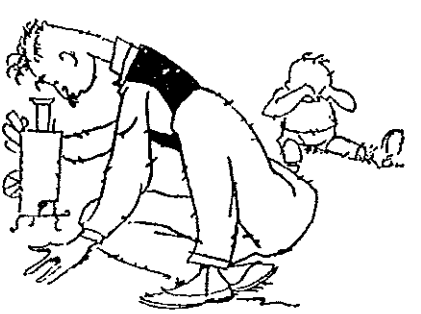
Part 3

Original studies



Chapter 6

Oxidative Epithelial Damage produces Hyperresponsiveness of Human Peripheral Airways

6.1 Introduction

Epithelial damage or loss is a characteristic finding in biopsies obtained from patients with asthma and is related to airway hyperresponsiveness [16 20]. The epithelium may modulate airway responsiveness because it releases relaxing factors [2 8], inactivates agonists [1 5], and, finally, acts as a physical barrier to inhaled stimuli [12 31]. In human airway strips, however, only a small 1.5- to 2.5-fold increase in sensitivity to contracting agonists was found after mechanical removal of the epithelium [2 27]. This may be due to shortcomings of the airway strip preparation since in rodent perfused tracheas where the stimulus could be selectively applied to the mucosal or the serosal side, a much larger modulatory role of the epithelium has been found [24 26 31]. Furthermore, epithelial cell injury in vivo is not caused by mechanical rubbing but, probably, by the release of basic proteins and oxygen radicals by inflammatory cells present in the lumen and mucosa of the airways [3 21].
Previously, we developed a perfusion model for human peripheral airway tubes [13] and in the present study we assessed the modulatory role of the epithelium on the responsiveness of tubes and strips of human peripheral airways by comparing responses of intact preparations with those of preparations with epithelial damage. In strips, the epithelium was damaged mechanically and in tubes the oxidant hydrogen peroxide (H_2O_2) was used to damage the epithelium. Finally, we investigated whether the effect of oxidative epithelial damage on histamine responses could be explained by a change in barrier function to luminally applied histamine.

Based on: Hulsmann AR, Raatgeep HR, Den Hollander JC, Stijnen T, Saxena PR, Kerrebijn KF, De Jongste JC. Oxidative epithelial damage produces hyperresponsiveness of human peripheral airways. *Am J Respir Crit Care Med* 1994; 149:519-25.

6.2 MATERIALS AND METHODS

Tissue preparation

Human lung tissue was obtained from 24 male and 3 female patients (mean age 64.1 y; range 31 to 75 y) who underwent thoracotomy for bronchial carcinoma. Their mean preoperative forced expiratory volume in 1 sec as a percentage of the inspiratory vital capacity was 63.4 ± 4.1%. None of the patients had characteristics of asthma. The premedication was the same for all patients: atropine, thiopentone, fentanyl, O_2/N_2O, halothane, and pancuronium. Immediately after surgical resection, a macroscopically normal part of the lung tissue was immersed in carbogenated (95% O_2, 5% CO_2) Krebs-Henseleit (K-H) buffer at room temperature (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 8.3). The tissue remained in fresh carbogenated buffer throughout the dissection procedure and during the experiments. The use of human lung tissue for these in vitro experiments was approved by the Ethical Committee of the Rotterdam University Hospital.

Airway strips

Airways with a diameter of 1 to 2.5 mm (generation 7 to 12) [33] were carefully dissected free from parenchyma and blood vessels. As described previously [18], segments were cut open to obtain bronchial strips and thin surgical silk threads (6/0) were tied to the ends of the strips. The tissues were studied at 37°C in double-jacketed 10 ml organ baths containing carbogenated K-H buffer. One thread was attached to a glass hook at the bottom of the bath and the other to the arm of a high precision isotonic angular position transducer (Penny and Giles type 3810/60, Christchurch, Dorset, UK) which was connected to a digital voltmeter (Fluke 73 multimeter, Everett, WA, USA) and a pen recorder (Kipp BD 40, Delft, The Netherlands).

Perfused airway tubes

For these studies we used a modification of a model that was recently developed at our laboratory [13]. In short, tubular segments of peripheral airways (generation 7 to 12; internal diameter 1 to 2.5 mm; length 5 to 8 mm) were carefully dissected and side branches were ligated with oculists silk threads (6/0). Each end of the segment was cannulated with a stainless steel cannula (external diameter 1.6 mm; internal diameter 1.2 mm). The preparation was placed horizontally in a double-jacketed 4 ml organ bath that contained carbogenated K-H buffer at

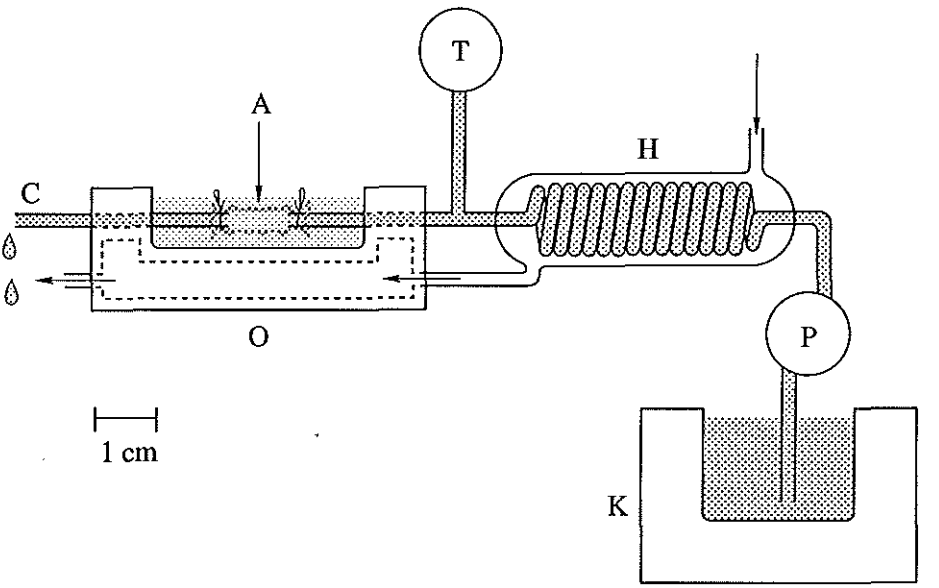


Figure 1. Schematic representation of the measurement system. The airway segment (S) is cannulated with stainless steel cannulas (C) and mounted in a double-jacketed organ bath (O) that contains carbogenated Krebs solution at 37°C. The bronchial lumen is perfused with carbogenated Krebs-Henseleit buffer (B) from a separate reservoir at a constant flow rate of 1 ml/min. Responsiveness is measured by recording changes in transmural pressure with a pressure transducer (T) that is connected to the inlet port of the airway. H = heat exchange.

37°C. The bronchial lumen was perfused at a constant flow rate of 1 ml/min with carbogenated K-H solution from a separate reservoir (inner perfusate, figure 1). The temperature, O_2 tension, and CO_2 tension in this reservoir were maintained at the same values as in the tissue bath. The responsiveness of the airway preparation was measured as a change in perfusion pressure with a piezoresistive type pressure transducer (Microswitch 176 PC14HG2; Honeywell, Freeport, IL, USA) connected to the inlet of the airway and was displayed on a pen recorder (Kipp BD 40, Delft, The Netherlands).

Pretreatment with hydrogen peroxide

Organ baths containing perfused airway tubes were cooled to 10°C (Frigomix 1496; Braun, Melsungen, Germany) to inactivate the endogenous radical scavenger catalase which degrades H_2O_2 [11]. Then the tubes were perfused at 1 ml/min

with K-H solution containing 100 mmol/l H_2O_2 for 10 min. This concentration was chosen because in guinea-pig tracheas exposure to this concentration of H_2O_2 for 20 min caused selective damage to the epithelium [17], and because we found in preliminary experiments that 100 mM, but not 10 mM or 1 mM, produced selective epithelial damage in human airways. Control tissues were perfused with K-H solution without H_2O_2 under the same experimental conditions. After treatment, the lumen was flushed with fresh K-H solution to remove H_2O_2. Finally, the preparations were rewarmed to 37°C and stabilized for 1 h before pharmacologic stimulation.

Assessment of barrier properties to histamine

Paired airway preparations from seven patients were used. One airway was pretreated with H_2O_2, the other served as a control. The airways were perfused with histamine (10^{-5} M, 1100 µg/l) and samples (0.5 ml) of the organ bath were taken at 0, 5, 15, 25, 35, and 45 minutes and stored at -70°C. Each sample was replaced by 0.5 ml of fresh K-H solution. The histamine concentration in these samples was measured with an automated fluorometric assay (Technicon, Tarrytown, NY, USA).

Airway histology and morphometry

After the experiments, the preparations were fixed in 10% buffered formaldehyde solution and embedded in paraffin wax. Transverse sections of 6-µm thickness were cut and stained with hematoxylin and eosin. The sections were examined under a light microscope to assess the presence or absence of epithelium and the extent and nature of oxidative damage to the epithelium of airways strips and perfused airways, respectively. To assess whether the modulation by the epithelium was dependent on airway size, additional experiments were performed. Airway tubes (17 tubes from 4 patients) were stimulated with histamine or methacholine from the serosal and the mucosal side in random order. After fixation, airway size was determined by measuring the internal perimeter (Pi) which is independent of the degree of bronchoconstriction and can therefore be used as a marker of airway size [15]. The internal perimeter was measured with a light microscope (Zeiss Axioplan; Carl Zeiss Gmbh, Jena, Germany) connected to an automated image analysis system (Ibas 2000; Kontron Bildanalyse, München, Germany).

Drugs

Methacholine hydrobromide (Janssen Pharmaceutica, Beerse, Belgium), hista-

mine dihydrochloride (Janssen), EDTA (Sigma Chemicals, St. Louis, MO, USA), and salbutamol hemisulphate (Sigma) were dissolved in 0.9% NaCl. L-Isoproterenol sulfate (Janssen) was dissolved in distilled water containing ascorbic acid (88 mg/l). Fresh drug solutions were prepared daily and kept on ice during the experiments.

6.3 EXPERIMENTAL PROTOCOLS

Airway strips

To assess contractile functions, the airway strips were stimulated once with methacholine (10^{-5} and 10^{-4}M) against an isotonic load of 250 mg. From each patient one pair of airways was used for each agonist. From one of each pair, the epithelium was removed by gentle rubbing with a wet gauze. Cumulative concentration response curves (CCRCs) to methacholine and histamine were made by adding the drugs to the organ bath (10^{-8} to 10^{-4}M, by log increments). CCRCs to salbutamol (10^{-9} to 10^{-4}M, by log increments) were made after precontraction with methacholine ($3x10^{-6}$M). The $ß_2$-adrenoceptor agonist salbutamol was chosen since this drug, unlike isoproterenol, is not metabolized by epithelial enzymes [28]. After washout, full relaxation of the preparations was obtained by adding L-isoproterenol (10^{-4}M) and EDTA ($4x10^{-3}$M).

Perfused airway tubes

From each patient two pairs of airways were studied for each agonist; one pair was treated with hydrogen peroxide, the other pair served as a control. Of each pair, one airway was stimulated from the mucosal side and the other from the serosal side. First, all preparations were stimulated once from the serosal side with methacholine (10^{-5}M and 10^{-4}M) to assess contractile function. Thereafter, CCRCs were made by adding the agonists either to the organ bath (serosal stimulation) or to the inner perfusate (mucosal stimulation). Finally, the preparations were maximally relaxed by isoproterenol and EDTA.

Data analysis

From each CCRC the following parameters were derived: *-log EC_{50}*: The negative logarithm of the agonist concentration that caused 50% of the maximal effect. *Smax*: Maximal contractile response of a preparation expressed as shortening (mm), or increase in perfusion pressure (cm H_2O), for the airway strips

and the perfused airways, respectively. *Rmax*: Maximal relaxation to salbutamol expressed as a percentage of the difference between methacholine ($3x10^{-6}M$) induced precontraction level and maximal relaxation by EDTA and isoproterenol at the end of an experiment. *Precontraction level*: Precontraction induced with methacholine ($3x10^{-6}M$) expressed as shortening (mm) or increase in perfusion pressure (cm H_2O) from maximal relaxation. *Baseline contractile state (BCS)* was expressed as a percentage of the difference between Smax (100% contraction to the methacholine or histamine) and maximal relaxation with EDTA and isoproterenol. The -log EC_{50} was calculated using the SPSS software module for nonlinear curve fitting. The curves were fitted to a four parameter logistic function [4]. Means of -log EC_{50}, BCS, Precontraction level, Smax and Rmax of the CCRCs were compared with paired Student's *t* tests (two-sided, α=0.05). The relationship between the differences in sensitivity between mucosal and serosal application of histamine and methacholine (i.e., modulation, expressed as Δ-log EC_{50}) and airway size (expressed as the internal perimeter in mm) was examined using analysis of covariance. Analysis of variance (ANOVA) was used to test the differences between the histamine concentration in organ baths of H_2O_2-treated tissues and their paired controls. Values of $p < 0.05$ were considered significant. All data are expressed as mean ± SEM.

6.4 RESULTS

Airway strips

All airway preparations responded dose-dependently to the agonists. Mean values for BCS, -log EC_{50}, Precontraction level, Smax, and Rmax for intact and epithelium-denuded airway strips (n=8) are given in table 1. There was a significantly higher BCS in epithelium-denuded preparations. Removal of the epithelium produced a small, nonsignificant 1.4-fold and 1.3-fold increase in mean sensitivity to histamine and methacholine, respectively. No difference in Smax was found between intact and epithelium-denuded airways for both histamine and methacholine. The precontraction level to methacholine ($3x10^{-6}M$) and the sensitivity to salbutamol were also not different in intact and epithelium-denuded airways. However, maximal relaxation to salbutamol was significantly smaller in epithelium-denuded airways (table 1).

Table 1. Baseline contractile state (BCS), sensitivity (-log EC_{50}), precontraction level (Precon) and maximal effect (Smax and Rmax) of agonists on human airway strips.

	Intact Strips (n=8)	Denuded Strips (n=8)
• Histamine		
BCS (%)	21.7 ± 5.8	40.3 ± 7.3*
-log EC_{50}	5.96 ± 0.16	6.12 ± 0.13
Smax (mm)	2.77 ± 0.52	2.43 ± 0.35
• Methacholine		
BCS (%)	22.5 ± 5.6	39.0 ± 8.6**
-log EC_{50}	5.69 ± 0.04	5.82 ± 0.08
Smax (mm)	2.53 ± 0.31	2.72 ± 0.29
• Salbutamol		
Precon (mm)	1.80 ± 0.21	1.82 ± 0.14
-log EC_{50}	6.57 ± 0.11	6.48 ± 0.10
Rmax (%)	62.5 ± 5.6	62.5 ± 5.6

* $p<0.01$ and ** $p<0.05$ compared to intact airways.

Perfused Airway Tubes

The mean values for BCS, -logEC_{50}, precontraction level, Smax, and Rmax for perfused airways are given in table 2. In intact airways no differences in BCS, Smax, or Rmax were found between serosal and mucosal stimulation for all agonists (n=8). Neither were there differences in BCS, sensitivity, precontraction level, Smax, and Rmax between the H_2O_2-treated preparations stimulated either from the serosal or the mucosal side and the untreated preparations stimulated from the serosal side, although the mean Smax and the precontraction level with methacholine ($3x10^{-6}$M) tended to be lower in H_2O_2-treated preparations (non-significant). The sensitivity to histamine and methacholine of airway tubes when they were stimulated from the mucosal side was 10.0- and 3.4-fold lower than when they were stimulated from the serosal side ($p<0.001$). In contrast, no difference in sensitivity to salbutamol was found between serosally and mucosally stimulated intact airways. After treatment with H_2O_2, the differences in -log EC_{50} between mucosal stimulation and serosal stimulation were abolished for the contractile agonists and similar for salbutamol.

The time required to reach a response plateau (steady-state contraction or -relaxation) after mucosal stimulation was similar to that after serosal stimulation for all agonists and did not change after H_2O_2 treatment (data not shown).

Table 2. Baseline contractile state (BCS), sensitivity (-log EC_{50}), precontraction level (Precon) and maximal effect (Smax or Rmax) of agonists applied to serosal or mucosal side of perfused human airways.

	Mucosal stimulation		Serosal stimulation	
	Intact tubes (n=8)	Treated tubes (n=6)	Intact tubes (n=8)	Treated tubes (n=6)
• Histamine				
BCS (%)	25.9 ± 8.2	16.8 ± 4.7	21.9 ± 4.4	21.9 ± 8.7
-log EC_{50}	4.87 ± 0.19*	5.64 ± 0.20	5.87 ± 0.23	5.60 ± 0.36
Smax (cmH_2O)	17.6 ± 2.2	14.4 ± 2.6	21.5 ± 2.4	15.0 ± 5.2
• Methacholine				
BCS (%)	11.6 ± 4.3	17.2 ± 4.3	18.9 ± 5.5	10.4 ± 3.6
-log EC_{50}	4.92 ± 0.14*	5.45 ± 0.17	5.45 ± 0.09	5.38 ± 0.08
Smax (cmH_2O)	19.8 ± 3.7	16.2 ± 2.6	21.9 ± 3.6	15.8 ± 4.2
• Salbutamol				
Precon (cmH_2O)	22.3 ± 3.1	15.2 ± 2.1	16.4 ± 2.6	12.9 ± 1.9
-log EC_{50}	6.19 ± 0.16	6.20 ± 0.21	6.29 ± 0.10	6.29 ± 0.17
Rmax (%)	72.5 ± 9.0	65.7 ± 9.2	69.6 ± 10.2	68.9 ± 10.0

*$p<0.001$, compared to stimulation from the serosal side of intact airways.

This suggests that the intact epithelium does not limit the rate at which the agonists reach the smooth muscle receptors.

Airway histology and morphometry

In mechanically rubbed strips the epithelium was effectively removed and the basement membrane and smooth muscle were intact. The epithelium of intact perfused airways was also largely undamaged (figure 2a), whereas in airway tubes treated with H_2O_2 the superficial epithelial cell layer was damaged or lost, leaving the basement membrane covered with basal cells (figure 2b).

In figure 3 the internal perimeter of 17 airway preparations from four patients is expressed against the difference in sensitivity (Δ-log EC_{50}, in log-units) between mucosal and serosal stimulation of these preparations. Covariance analysis showed significant positive correlations between the modulatory effect by the airways epithelium and airway size ($p<0.007$) for histamine and methacholine.

Measurements of histamine concentration

Measurements of the histamine concentrations in organ baths of seven intact

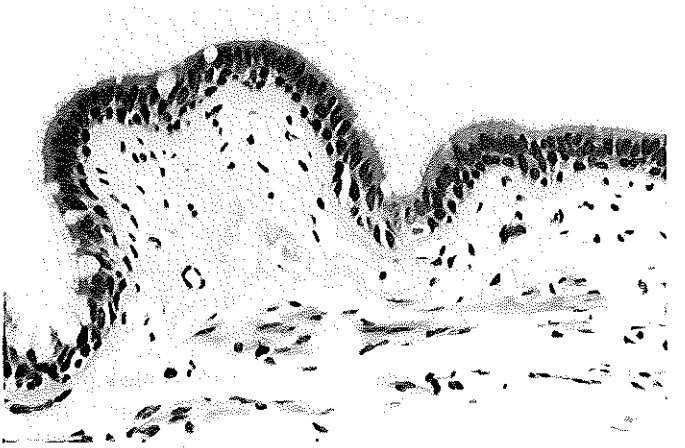


Figure 2a. Section of human airway showing an intact epithelium. Hematoxylin-eosin (H&E) stain; magnification: x400).

Figure 2b. Section of a H_2O_2-treated airway showing patchy loss of the superficial cell layers whereas the basal cell layer largely remains. Hematoxylin-eosin (H&E) stain; magnification: x400).

and seven H_2O_2-treated airways perfused with a histamine concentration of 10^{-5} M (1100 µg/l) are shown in figure 4. During 45 min of perfusion there was an increase in the histamine concentration in the organ baths of both intact and H_2O_2-treated airways but no plateau was reached. No difference was found in the histamine concentration from organ baths containing intact and H_2O_2-treated airways (p=0.98, ANOVA).

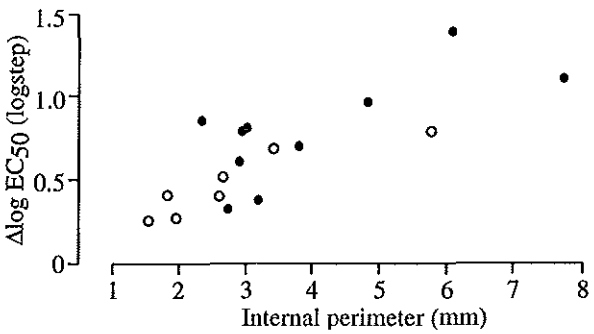


Figure 3. Correlation between the modulatory effect of the epithelium to the contractile agonists histamine (closed circles) and methacholine (open circles) and airway size (n=17). Horizontal axis depicts airway perimeter (mm), vertical axis represents the difference in sensitivity (Δ-log EC_{50}, in log-units) between serosal and luminal stimulation of an airway preparation.

6.5 DISCUSSION

Our results show that intact bronchial epithelium modulates the sensitivity of human perfused peripheral airways to the contracting agonists histamine and methacholine but not to the relaxing agonist salbutamol. The extent of the modulatory effect on contractile agonists increases with airway size and is completely abolished after oxidative damage to the epithelium. The barrier properties of the airways to the agonist histamine were unchanged after oxidative epithelial damage.

In earlier studies of human central airway strips, only a small modulatory role of airway epithelium to contractile reponses was found[2,18,27]; mechanical removal of the epithelium increased the sensitivity to histamine by 1.6-fold[2] and the sensitivity to acetylcholine by 2.6-fold[2] or 2.2-fold[27]. Our study of peripheral airway strips shows even smaller nonsignificant differences in sensitivity between intact and epithelium-denuded airways (table 1). Several mechanisms have been proposed to explain the modulatory role of the bronchial epithelium. The epithelium may release an epithelium-derived relaxing factor (EpDRF)[2,7,8], it may act as a diffusion barrier to pharmacologic agents[1,2,31], or it may inactivate them as has been shown not only for adenosine[1], and tachykinins[5] but also for histamine[19] and acetylcholine[25]. Inhibition of cholinesterase and of histamine N-methyltransferase increased the sensitivity of intact- but not of epithelium-denuded guinea-pig trachea rings to acetylcholine and histamine, respectively,

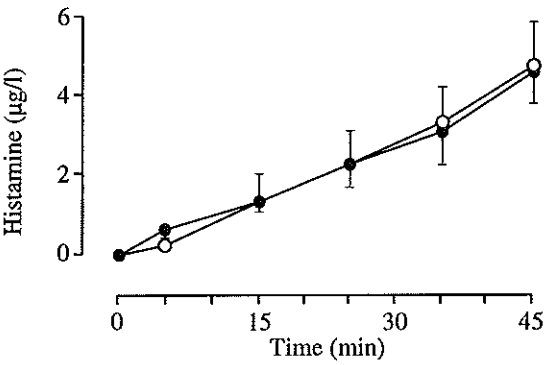


Figure 4. Histamine concentration in the organ baths of intact (closed circles) and hydrogen peroxide-treated (open circles) airways (n=7) during a 45-min period of perfusion with histamine 10^{-5} M (1100 µg/l). The horizontal axis depicts time (min), the vertical axis represents histamine concentration in the organ baths (µg/l).

suggesting a significant role of the epithelium in inactivation of these agonists [19 25]. It seems, therefore, that removal of these epithelial enzymes during treatment with H_2O_2 explains the finding of a modulatory role of the epithelium in the present study. However, this is unlikely for several reasons: Firstly, we did not find a modulatory effect of the epithelium in intact airway strips. Secondly, a modulatory role of the epithelium has also been found in airways perfused with the carbamic ester carbachol which is very resistant to cholinesterase [7 31]. Finally, it has been shown that after inhibition of cholinesterase with ecothiopate or physostigmine, epithelium removal still augments responses to acetylcholine [7 8 31].

In airway strips, the stimulus reaches the smooth muscle not only via the mucosal side but also via the serosal side and the cut surface. Therefore, the role of the epithelium as a barrier cannot be assessed, and this may be the reason why only small sensitivity shifts are found between intact and epithelium-denuded airway strips. Indeed, after sealing of the nonmucosal surfaces of airway strips with vaseline, Iriarte and coworkers [14] observed a 27-fold decrease in sensitivity to acetylcholine applied to the organ bath. Recently, in vitro models have been developed that enable independent stimulation of rodent isolated tracheas from either the serosal or the mucosal side [24 26 31]. Large differences in sensitivity (30-fold or more) between serosal and mucosal stimulation with contractile agonists were found in guinea-pig [24 31] and rat tracheas [26], which were abolished after mechanical rubbing of the epithelium. In the present study of human

perfused airways, we found a smaller difference in sensitivity between serosal and mucosal stimulation with contractile agonists: 10- and 3.4-fold for histamine and methacholine, respectively (tabel 2). However, these differences are much larger than the differences in sensitivity between intact and epithelium-denuded central airway strips reported in earlier studies [2 18 27].

In canine airway strips (generation 2 to 4) the potentiating effect of epithelium removal on the response to contractile agonists diminished with decreasing airway diameter and was absent in fourth order airways [32]. These investigators concluded that in canine airways there is a release of a relaxing factor that reduces the sensitivity to constricting agents in larger airways but not in smaller bronchi [32]. The present study confirms a positive correlation between airway size or generation and modulatory role of epithelium in human peripheral airways. Furthermore, our results suggest that in peripheral human airways there may be a release of a relaxing factor as well.

Considering the limitations of the airway strip preparation and our finding of a positive correlation between airway size and modulation to contractile agonists, it can be hypothesized that the actual modulatory role of the epithelium in human central airways is much larger than previously reported [2 18 27] and may be similar to that in rodent tracheas. We were not able, however, to examine human central airways since these are rarely available.

In most in vitro studies, the epithelium is damaged by mechanical rubbing. This may induce an artifact, since it has been shown that in isolated dog airways the mechanical stimulus of rubbing increases the release of tryptase from mast cells in the submucosa [9] and this may cause hyperresponsiveness of the smooth muscle preparation via an effect on Ca^{2+} channels [30]. This may explain the increased sensitivity in epithelium-denuded airway strips noted in our report and in other studies. In addition, it may explain the increase in BCS that we found in epithelium-denuded airway strips. It is not clear why, in epithelium-denuded airway strips, we found a smaller relaxation to salbutamol than in intact strips and in airway tubes. The smaller relaxation in epithelium-denuded strips may be related to the rubbing procedure resulting in loss of relaxing properties of the smooth muscle, although histologically no evidence for damage to the smooth muscle was found. Our finding that salbutamol-induced relaxation in airway tubes is not modulated by the epithelium contrasts with a study of Yang and coworkers [36], who found a lower sensitivity to salbutamol when it was applied to the mucosal side of isolated guinea-pig tracheas. However, central airways were studied and differences between species may exist.

Epithelial cell injury in vivo is probably mainly due to the release of basic peptides from eosinophils and to the release of oxygen radicals from various inflammatory cells [3 21] but not by mechanical rubbing. Therefore, perfusing the preparation with H_2O_2 is likely to produce a more physiologic damage to the epithelium. The concentration of H_2O_2 that we used produces visible epithelial damage in guinea-pig trachea [17] and the studies in human airways at our laboratory have shown that this is also the case for human airways. After treatment with H_2O_2, BCS, sensitivity, precontraction level, Smax, and Rmax of treated tissues stimulated from the serosal side or from the luminal side were not significantly different from serosally stimulated intact tissues, although precontraction level and maximal contraction were consistently lower in H_2O_2-treated preparations. In guinea-pig tracheas exposed to similar concentrations of H_2O_2, maximal contractions after a single dose of methacholine were also decreased [23]. Although it is likely that H_2O_2 is reduced to H_2O and O_2 during its passage through the epithelium, the decrease in maximal contraction may be due to some H_2O_2-induced smooth muscle damage. The sensitivity (-log EC_{50}) to the serosally applied agonists, however, remains unchanged in both H_2O_2-pretreated guinea-pig tracheas [29] and human airways (this study) and this suggests intact receptor function. Therefore, the hyperresponsiveness to luminally applied histamine and methacholine that we observed after H_2O_2 treatment is likely to be due to oxidative damage to the airways epithelium and not to a direct effect of H_2O_2 on smooth muscle responsiveness.

In cultured (monolayer) epithelium, exposure to H_2O_2 increased the paracellular permeability [34]. At physiologic pH, histamine is in a monovalent cation form [10] and will therefore cross the epithelium primarily via the paracellular route. On the basis of the experiments by Welsh and coworkers [34], one might expect an increase in permeability of the epithelium in airways treated with H_2O_2. However, the epithelium in situ is not a monolayer and the basal membrane of airway epithelium may also form an important additional barrier to large and hydrophilic molecules [35].

In H_2O_2-treated airways we found loss of superficial cells (columnar cells) leaving basal cell rows intact. These basal cell rows are connected to the basal membrane and neighboring cells through junctional (desmosomes, hemi-desmosomes) and non-junctional adhesive mechanisms [6 21]. Because columnar cells do not have hemidesmosomes, they cannot form junctional attachments with the basal lamina [6] and this may explain why only superficial cell layers are lost after H_2O_2 treatment in our model. In patients with asthma, disruption of the epithelium

also occurs in a suprabasal plane [22]. Therefore, the H_2O_2-induced epithelial damage in our model is a realistic approach of epithelial damage in asthma.
After 45 min of perfusion of intact and H_2O_2-treated airways with histamine 10^{-5} M (1100 µg/l), the histamine concentration in the organ baths was only 0.42% and 0.44% of the concentration of the perfusate for intact and treated airways, respectively, and there was no difference between histamine concentration in organ baths of intact and H_2O_2-treated airways. Thus, although the epithelium forms a barrier to histamine, the abolition of the modulatory role of the epithelium after oxidative damage cannot be explained by a change in barrier properties. Another argument against a barrier function as the single explanation of the modulatory role of the epithelium is our finding that the time to steady-state contraction or steady-state relaxation was similar in luminal and serosal application and, moreover, did not change after H_2O_2 treatment. Our results contrast with the findings of Jeppsson and associates [17] who found an increased efflux of ^{3}H-terbutaline in H_2O_2-treated guinea-pig trachea. However, differences in pharmacologic and physical properties between histamine and terbutaline may be involved. Mitzner measured the transfer of histamine in guinea-pig airways with and without epithelium (W. Mitzner, personal communication). 'Diffusivity' (D) to histamine was defined as: $D = (F/\Delta c)T/A$; where F is the rate of transfer, Δc is the concentration difference between airway lumen and the organ bath, T is the estimated airway wall thickness, and A is the surface area of the perfused airway. He found a diffusivity for histamine of $3x10^{-8}$ cm^2/s and $7x10^{-6}$ cm^2/s for intact and epithelium-denuded tracheas, respectively (W. Mitzner, personal communication). Assuming a surface area of 0.3 cm^2 and an airway wall thickness of 0.01 cm, we estimated a diffusivity of approximately $3x10^{-7}$ cm^2/s in both intact and H_2O_2-treated airways. Thus, intact human peripheral airways may be more permeable to histamine than intact guinea-pig tracheas. Since the permeability of human intact peripheral airways seems high already, an increase in diffusivity after H_2O_2-induced epithelial damage may be difficult to detect. Alternatively, the basal cell layer that remains after treatment with H_2O_2 may still form an important barrier to histamine.
We conclude that the epithelium modulates the sensitivity of human isolated peripheral airways to the contractile agonists histamine and methacholine but not to the relaxing agonist salbutamol. The modulatory effect to the contractile agonists increases with airway size and is abolished after oxidative damage to the epithelium. It cannot be assessed accurately in airway strips because of shortcomings of this preparation. Although the epithelium forms a physical barrier

to agonists, the modulatory role of the epithelium cannot be explained by a change in barrier function of the epithelium because the responsiveness of salbutamol did not change and because the permeability to histamine did not increase after damage to the epithelium. Our results show that oxidative epithelial damage, similar to that found in asthma, may contribute to the enhanced responsiveness to histamine and methacholine that characterizes this disease.

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Chapter 7

Permeability of Human Isolated Airways increases after Hydrogen Peroxide and Poly-L-arginine

7.1 Introduction

The airway epithelium forms the interface between the respiratory system and the external environment and consists of ciliated and nonciliated cells tightly attached to each other [31]. In bronchial asthma, areas of airway epithelium become denuded of columnar epithelial cells and the degree of epithelial damage correlates with the level of bronchial responsiveness both in vivo [25 32] and in vitro [33]. Eosinophils may contribute to the epithelial damage by producing reactive oxygen species and cationic proteins such as major basic protein (MBP) and eosinophil cationic protein (ECP) [1 7 15 17 35]. In addition, the bronchial epithelium of asthmatic patients may be more fragile than that of normal subjects [31]. Several mechanisms have been proposed to explain the relationship between the loss of epithelial integrity and airway hyperresponsiveness. These include reduced production of epithelium-derived relaxing factor [2 13], exposure of intraepithelial peptidergic nerves with reflex bronchoconstriction [3] and decreased metabolization of contractile agonists [10]. Alternatively, airway permeability to bronchoactive agonists may be increased after epithelial damage [20 37]. Indeed, airway permeability to hydrophilic drugs is increased in asthmatic subjects in vivo [23] and this may explain why inhaled but not infused histamine causes increased bronchoconstriction in these patients [4].
Because of the supposed key role of eosinophil products in the pathogenesis of airway hyperresponsiveness, we investigated the effect of the oxidant hydrogen peroxide (H_2O_2) and the cationic protein poly-L-arginine, a synthetic analogue similar in charge and molecular weight to MBP [9 38], on the permeability and

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morphology of nonasthmatic, human peripheral airways. We also examined the relationship between airway permeability and airway size, since airway permeability in vivo increases with decreasing airway size [5].

7.2 MATERIALS AND METHODS

Tissue preparation

Human lung tissue was obtained from 24 male and 3 female patients (mean age 64.1 y; range 31 to 75 y) who underwent thoracotomy for bronchial carcinoma. None of the patients had characteristics of asthma. The medication was the same for all patients: atropine, thiopentone, fentanyl, O_2/N_2O, halothane, and pancuronium. Immediately after surgical resection, a macroscopically normal part of the lung tissue was immersed in carbogenated (95% O_2, 5% CO_2) Krebs-Henseleit (K-H) buffer at room temperature (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 8.3). The tissue remained in fresh carbogenated buffer throughout the dissection procedure and during the experiments. For these studies we used a modification of a model that was recently developed at our laboratory [22]. In short, tubular segments of peripheral airways (generation 7 to 12; internal diameter 1.0 to 3.0 mm; length 10 to 20 mm) were carefully dissected and side branches were ligated with oculists silk threads. Each end of the segment was cannulated with a stainless steel cannula (external diameter 1.6 mm; internal diameter 1.2 mm). Airways with an internal diameter that was smaller than the external diameter of the cannula could be easily stretched to fit around the cannula without causing any damage to the preparation. The airway was placed horizontally in a double-jacketed 4 ml organ bath that contained carbogenated K-H buffer at 37° C. The length of the airway preparation was measured with Vernier calipers. The bronchial lumen was perfused at a constant flow rate of 10 ml/h with carbogenated K-H solution from a separate reservoir (inner perfusate). The temperature, O_2 tension, and CO_2 tension in this reservoir were maintained at the same values as in the organ bath. The use of human lung tissue for these in vitro experiments was approved by the Ethical Committee of the Rotterdam University Hospital.

Assessment of airway permeability

After equilibration of the airway tube preparation, the luminal K-H buffer was replaced with K-H buffer containing either ^{111}Indium-labelled diethylene triamine

pentaacetic acid (^{111}In-DTPA; 10^{-6}M), a hydrophilic tracer that crosses the epithelium via paracellular pathways [27], or ^{14}C-antipyrine (^{14}C-AP; 10^{-6}M), a lipophilic molecule that passes through the epithelial cells [18]. The organ bath fluid was changed every 15 min and the radioactivity was measured in 4 ml samples of luminal perfusate and organ bath fluid. ^{111}In-DTPA radioactivity was measured in cpm using a gamma counter (Packard Minaxi Autogamma 5000, Packard Instrument Company, Downers Grove, IL, USA). ^{14}C-AP radioactivity was measured in dpm with a beta counter (Packard Tri-Carb liquid scintillation analyzer 1500). Because of the short half life of ^{111}In-DTPA (2.8 d) samples containing ^{111}In-DTPA were measured in the same order and with the same time interval (15 min) as they were obtained. Therefore, no correction for rate of decay was required.

The permeability (P; cm/s) of the airways to the radiolabeled tracer molecules during each 15 min period was calculated as described by Hanafi and coworkers [18]:

$$P=- (dQ/dt)/S\Delta c$$

where dQ/dt is the rate of uptake of ^{111}In-DTPA or ^{14}C-AP by the organ bath fluid in cpm/s, S is the luminal surface of the airway (in cm^2) calculated from the airway perimeter (measured after fixation) and length (recorded at baseline), and ΔC is the concentration difference of tracer (in cpm/ml) between perfusate and organ-bath fluid.

Airway histology and morphometry

After the experiments, the preparations were fixed in 10% buffered formaldehyde solution and embedded in paraffin. Three transverse sections of 5 μm thickness were cut from the middle part of each airway preparation and stained with hematoxylin and eosin (H&E). The sections were examined with a light microscope (Zeiss Axioplan; Carl Zeiss Gmbh, Jena, Germany) connected to an automated image analysis system (Ibas 2000; Kontron Bildanalyse, München, Germany). Airway size was determined by measuring the internal perimeter (Pi), which is independent of the degree of bronchoconstriction and can therefore be used as a marker of airway size [24]. In addition, the percentage of the basement membrane covered with intact epithelium was measured. The measurements were made by an experienced pathologist who was unaware of the previous treatment (H_2O_2, poly-L-arginine, or none) of the airway preparation.

The mean values of the Pi and the percentage of the basement membrane covered with epithelium of the three sections of each airway preparation were determined and used in further calculations.
Three airways (from three different patients) treated with hydrogen peroxide (100 mmol/l) were examined by transmission electronmicroscopy. After fixation in 2.5% glutaraldehyde and 0.8% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3), tissues were postfixed in cacodylate-buffered 1% osmium tetroxide (OsO_4), dehydrated in ethyl alcohol and embedded in epoxy resin. Thin sections (40 nm) were stained with lead citrate and examined with a Zeiss EM902 electron microscope.

Drugs
^{111}In-DTPA was purchased from Merck-Frost Radiopharmaceuticals (Quebec, Canada); ^{14}C-AP and poly-L-arginine were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

7.3 EXPERIMENTAL PROTOCOLS

Effect of luminal exposure of the airways to hydrogen peroxide
From each patient (n=10), five airways were studied in parallel. Three of these airways were perfused with K-H solution containing ^{111}In-DTPA (10^{-6}M), the other two airways were perfused with K-H solution containing ^{14}C-AP (10^{-6}M). Baseline permeability to the tracer molecules was measured every 15 min for 75 min by sampling the organ bath fluid. At t=75 min, H_2O_2 (10 or 100 mmol/l) or vehicle (control airway) was added to the ^{111}In-DTPA-containing perfusate and H_2O_2 (100 mmol/l) or vehicle (control airway) to the ^{14}C-AP-containing perfusate over a period of 15 min. These concentrations of H_2O_2 were chosen because in previous studies, 100 mmol/l but not 10 mmol/l H_2O_2 produced selective damage to the epithelium and an increase in airway responsiveness in both guinea pig and human airways [21 26]. Thereafter, permeability to the tracer molecules was measured by sampling the organ bath fluid every 15 min for another 2.5 h.

Effect of luminal exposure to poly-L-arginine
From each patient (n=8), four airways were studied in parallel. From t=0 to t=2 h, the perfusate of the airways contained ^{111}In-DTPA (10^{-6}M). Baseline permea-

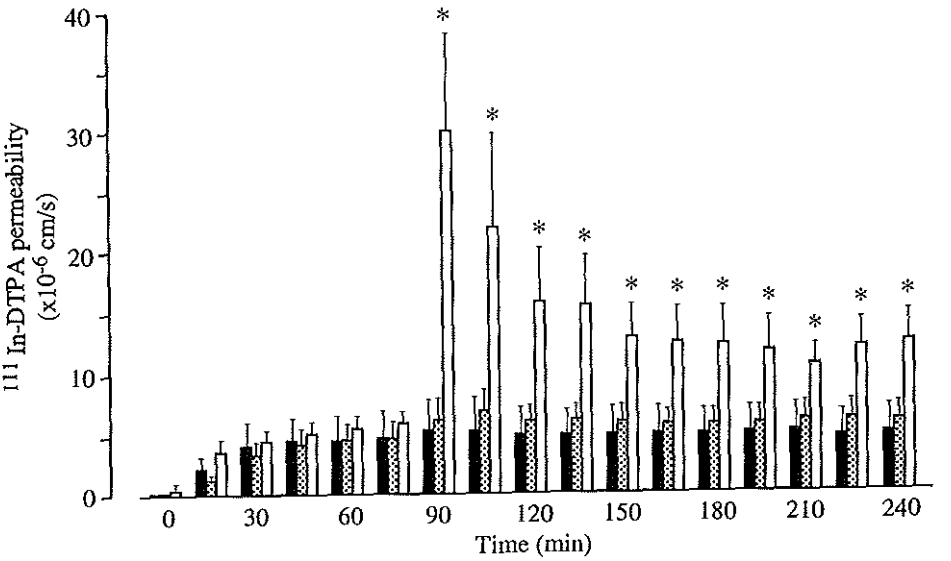

Figure 1. Time courses of the effect of luminal H_2O_2 on permeability of human isolated airways to ^{111}In-DTPA (n=10) exposed to 10 mmol/l H_2O_2 at T=75 min for 15 min (gray bars); exposed to 100 mmol/l H_2O_2 at T=75 min for 15 min (open bars). Closed bars represent controls not exposed to H_2O_2. $^*p< 0.05$ compared with controls.

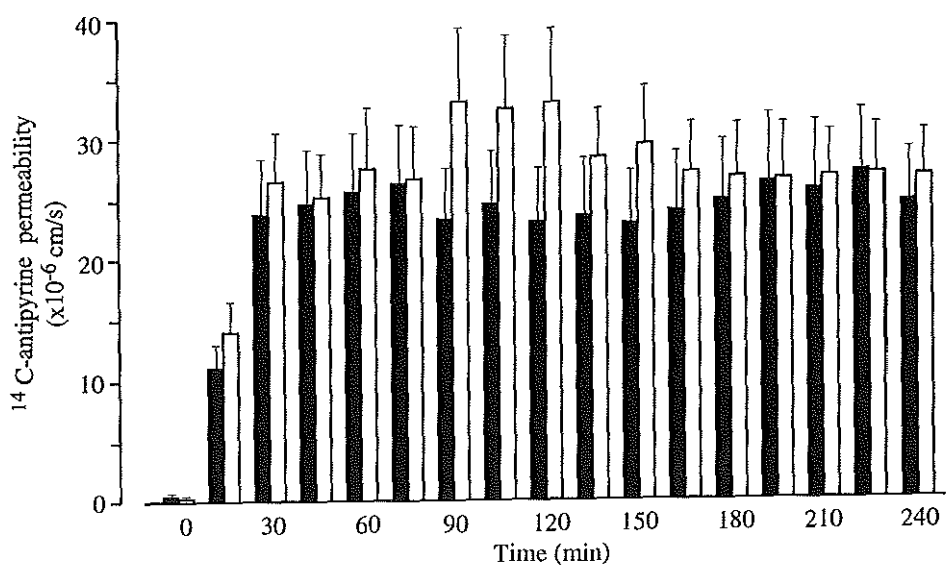

Figure 2. Time courses of the effect of luminal H_2O_2 on permeability of human isolated airways to ^{14}C-antipyrine (n=10) exposed to 100 mmol/l H_2O_2 at T=75 min for 15 min (open bars). Closed bars represent controls not exposed to H_2O_2.

bility to the tracer molecule was measured every 15 min for 2 h, and the mean permeability during the second hour was calculated. After these 2 h, the perfusate of two airways was changed to K-H buffer containing poly-L-arginine (100 µmol/l), the other two airways were perfused with K-H buffer without poly-L-arginine (controls). The concentration of 100 µmol/l poly-L-arginine was used because this concentration increased transmucosal flux of albumin in bovine bronchial mucosa [19]. From t=5 to 7 h, the permeability of one airway exposed to poly-L-arginine (for 3 h) and a control airway was measured by changing the perfusate to K-H buffer containing ^{111}In-DTPA . The mean permeability of these airways between t=6 h and t=7 h was calculated and compared, and the airways were fixed in buffered formaldehyde. From t=18 to 20 h, the permeability of the other airway exposed to poly-L-arginine (for 16 h) and of the remaining control airway was measured by changing the perfusate to K-H buffer containing ^{111}In-DTPA. The mean permeability between t=19 h and t=20 h was calculated and compared, and the airways were fixed in buffered formaldehyde.

Relationship between airway permeability and airway size

Thirty-two airway preparations from three patients were perfused for 2 h with K-H buffer containing ^{111}In-DTPA. Permeability was measured every 15 min. The mean permeability during the second hour was related to airway size, as determined by the internal perimeter (Pi) of the airway, and the correlation coefficient was calculated.

Data analysis

All data are expressed as mean ± SEM. Means of airway permeability in differently treated preparations at corresponding 15 min periods (in the experiments with H_2O_2) and the mean permeability during the second hour (in the experiments with poly-L-arginine) were compared with paired Student's *t* tests (two-sided, α=0.05). The correlation between baseline permeability and airway size (expressed as the internal perimeter in mm) was examined by calculating Spearman's rank correlation coefficient (r_s). Values of $p < 0.05$ were considered significant.

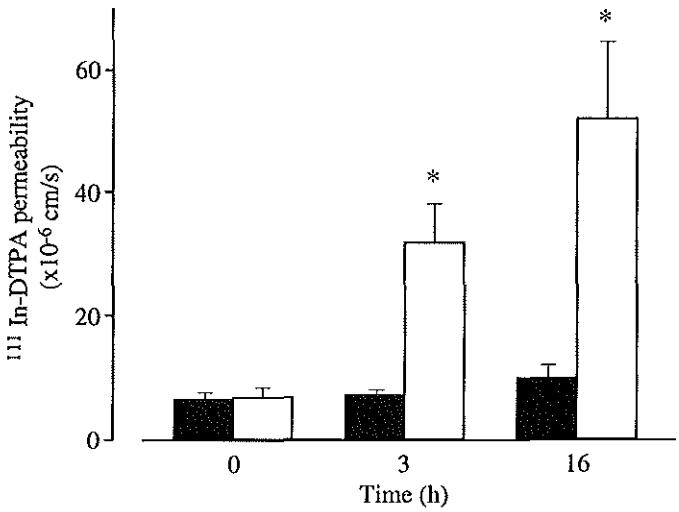

Figure 3. Time courses of the effect of luminal poly-L-arginine on permeability of human isolated airways to ^{111}In-DTPA (n=8) after exposure to poly-L-arginine 100 µmol/l (open bars). Closed bars represent controls not exposed to poly-L-arginine.
* $p<0.02$ compared with controls.

7.4 RESULTS

Effect of luminal exposure to hydrogen peroxide on airway permeability
The results of the studies are shown in figures 1 and 2. Within 30 min of perfusion a stable baseline permeability was obtained to both ^{111}In-DTPA and ^{14}C-AP. Baseline permeability (at t=1 to 2 h) of the airways to ^{14}C-AP was about five times greater than baseline permeability to ^{111}In-DTPA. Fifteen minutes of luminal exposure of the airways to 10 mmol/l H_2O_2 or vehicle did not change permeability to ^{111}In-DTPA. Luminal exposure of the airways to 100 mmol/l H_2O_2 produced a significant six-fold increase in permeability to ^{111}In-DTPA, whereas no increase in permeability to ^{14}C-AP was found. After removal of H_2O_2, airway permeability decreased and remained at a 2.5-fold greater level than in controls which was statistically significant.

Effect of luminal exposure to poly-L-arginine on airway permeability
Luminal exposure of the airways to poly-L-arginine for 3 h and 16 h produced a significant 4.5- and 7-fold increases in permeability to ^{111}In-DTPA, respectively, as compared with controls (figure 3).

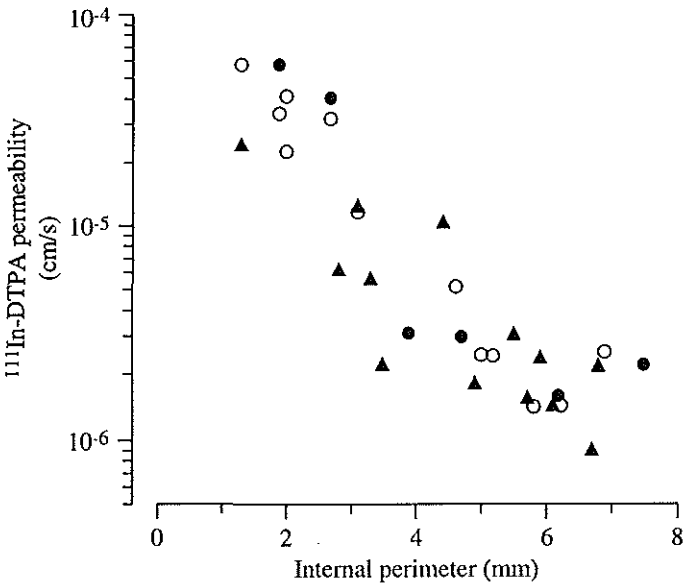

Figure 4. Correlation between ^{111}In-DTPA permeability and airway size of human isolated airways (n=32 airways from 3 patients). Horizontal axis depicts airway internal perimeter (mm); vertical axis represents airway permeability to ^{111}In-DTPA (x10^{-6} cm/s). Different symbols represent airways obtained from three different patients. Spearman's rank correlation coefficient r_s=-0.75, p<0.01.

Relationship between airway permeability and airway size

In figure 4 the Pi of 32 airway preparations from three patients is expressed against the permeability of these airways to ^{111}In-DTPA. The logarithm of the permeability to ^{111}In-DTPA correlated negatively with the Pi of the airways (r_s=-0.75, p<0.01).

Histopathologic effects of luminal exposure to hydrogen peroxide

Light-microscopic examination showed that 87.9 ± 2.6% of the basement membrane of the control airways was covered with intact epithelium. In airways exposed to 10 mmol/l H_2O_2, 86.9 ± 3.0% of the epithelium was intact (ns). After exposure to 100 mmol/l H_2O_2, 72.5 ± 6.1% of the basement membrane was covered with intact epithelium (p<0.05 compared to controls). In the damaged regions of the airways, focal preservation of the basal cells was seen. Electronmicroscopic examination of 3 airways exposed to 100 mmol/l H_2O_2 revealed opening of the tight junctions and widening of the intercellular space in areas that seemed intact on light-microscopic examination (figures 5a and 5b).

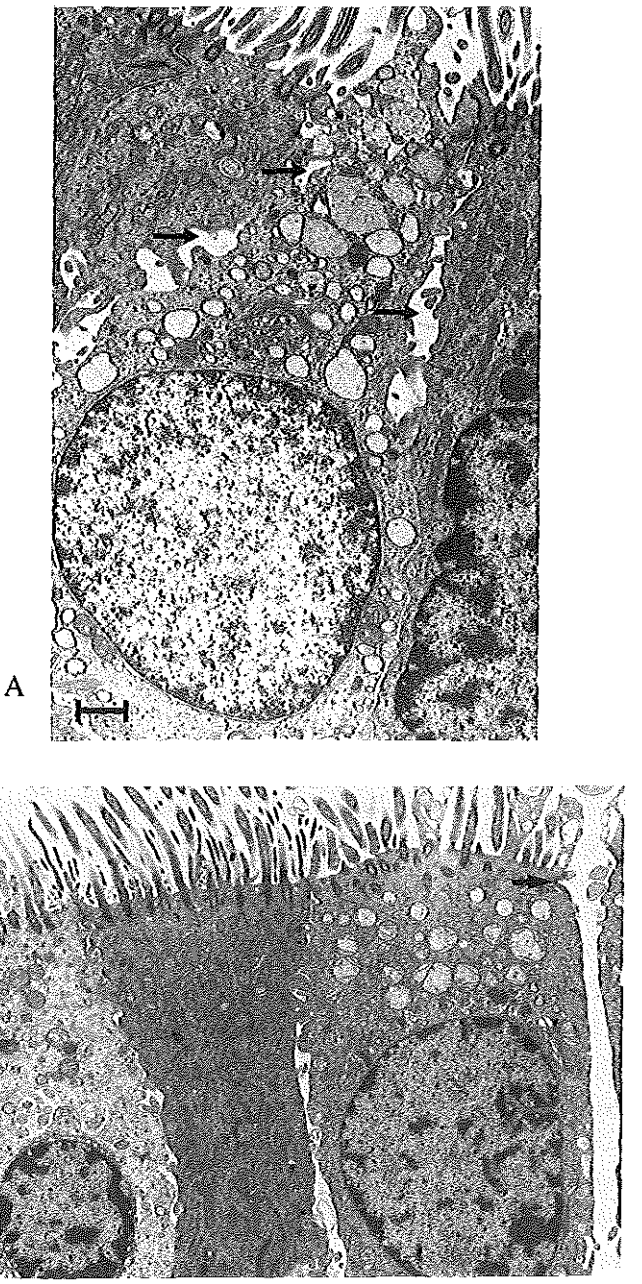

Figure 5. Electronmicroscopic photomicrographs of human bronchial epithelium (n=3) after exposure to 100 mmol/l H_2O_2 during 15 min. (A) widening of intercellular spaces (arrows), tight junctions intact (B) opened tight junction (arrow) and a widened intercellular space, bar = 1 µm.

Histopathologic effects of luminal exposure to poly-L-arginine
In the light-microscopic examinations, 78.8 ± 7.4% of the basement membrane of control airways (perfused for 18 h) was covered with intact epithelium (figure 6a). After 3 h and 16 h of exposure to poly-L-arginine, 45.8 ± 13.3 and 23.1 ± 6.4% of the basement membrane was covered with intact epithelium, respectively. In the damaged areas, focal preservation of basal cells was found in airways exposed to poly-L-arginine for 3 h (figure 6b), whereas in airways exposed to poly-L-arginine for 16 h basal cells were partly lost (figure 6c).

7.5 DISCUSSION

Our results show that both H_2O_2 and the synthetic cationic protein poly-L-arginine increased the permeability of human peripheral airways to the hydrophilic tracer molecule ^{111}In-DTPA. Permeability to the lipophilic tracer molecule ^{14}C-AP was not increased after exposure to H_2O_2, indicating that the increase in permeability is caused by opening of paracellular pathways. Light- and electronmicroscopic examination of airway epithelium exposed to hydrogen peroxide and poly-L-arginine showed epithelial damage with loss of ciliated cells and relative preservation of basal cells. Finally, we demonstrated that permeability of human peripheral airways increases with decreasing airway size.
Increased transfer of hydrophilic tracer molecules during and after exposure to oxidants has also been described in epithelial monolayers, isolated airways, and in vivo in experimental animals [6 26 34 39]. In most studies, oxidant-induced increases in permeability were accompanied by histopathologic changes in the epithelium. Welsh and coworkers demonstrated widening of intercellular spaces in cultured kidney epithelial cells [39], and Bhalla and colleagues found destabilisation of the cytoskeleton in rat airway epithelium following exposure to the oxidant gas ozone (O_3) [6]. In the present study of human airway epithelium exposed to H_2O_2, we observed focal loss of ciliated epithelial cells, opening of tight junctions, and widening of intercellular spaces. Similar histopathologic findings were described for bronchial biopsies from asthmatic subjects [12 32]. Bhalla and coworkers could not demonstrate opening of tight junctions after exposure to O_3, and suggested that disruptions of tight junctions may occur in a discontinuous, patchy manner and may not be included in the thin sections taken for electronmicroscopy [6].

The mechanism of action of oxidants is unknown. Extracellular H_2O_2 penetrates cell membranes and may deplete cellular adenosine triphosphate (ATP) levels which may lead to alteration of the cytoskeleton and the opening of tight junctions [30]. However, an oxidant-induced increase in epithelial permeability has also been observed without cytotoxicity supporting the hypothesis that oxidative stress increases paracellular permeability by activating intracellular signaling systems [34 36]. In the present study, the increase in airway permeability was partly reversible after removal of H_2O_2. This was also found in epithelial monolayers after discontinuation of the oxidative stress [6 34 39]. Welsh and coworkers showed that both H_2O_2-induced functional and structural changes were reversible [39]. Cells that separated during exposure to H_2O_2 fused again after removal of H_2O_2. These observations suggest that cell-to-cell adhesion is a dynamic process with a high capacity for repair, even after exposure to high concentrations of oxidants. Previously, we reported that exposure of airway epithelium to H_2O_2 (100 mmol/l) for 15 min increased the responsiveness of human peripheral airways to luminally applied histamine [21]. The epithelial permeability to histamine of airways exposed to H_2O_2, however, was not different from controls. In the present study, performed under similar experimental conditions, airway permeability to the radioactive tracer molecule. ^{111}In-DTPA increased after exposure of the epithelium to H_2O_2. In our previous study, however, we measured cumulative concentrations of histamine in the organ baths, which may be less sensitive than measuring permeability to a radioactive tracer at 15 min intervals.

Eosinophils release cationic proteins such as MBP and eosinophil cationic protein (ECP), which may play important roles in the pathophysiology of asthma [1 7 15 17 31]. Numbers of eosinophils and levels of ECP were increased in broncho-alveolar lavage fluid (BALF) from asthmatic subjects and were correlated with the severity of the disease [7]. Exposure of animal airways to cationic proteins causes increased airway responsiveness to histamine and methacholine in vivo [17 38] and in vitro [9]. In the present study, we demonstrated that luminal exposure of human airways to the synthetic cationic protein poly-L-arginine for 3 h caused extensive epithelial damage. Superficial columnar epithelial cells were damaged or lost, whereas basal cells, which are tightly anchored to the basement membrane through hemidesmosomes [31], were still present. After 16 h of exposure to poly-L-arginine, most of the basal cells were also lost, leaving large parts of the basement membrane uncovered. Similar patterns of mucosal injury were found in previous studies of the effects of cationic proteins on guinea-pig, bovine, and human airway epithelium [1 14 19]. We found that luminal exposure of the airways to poly-

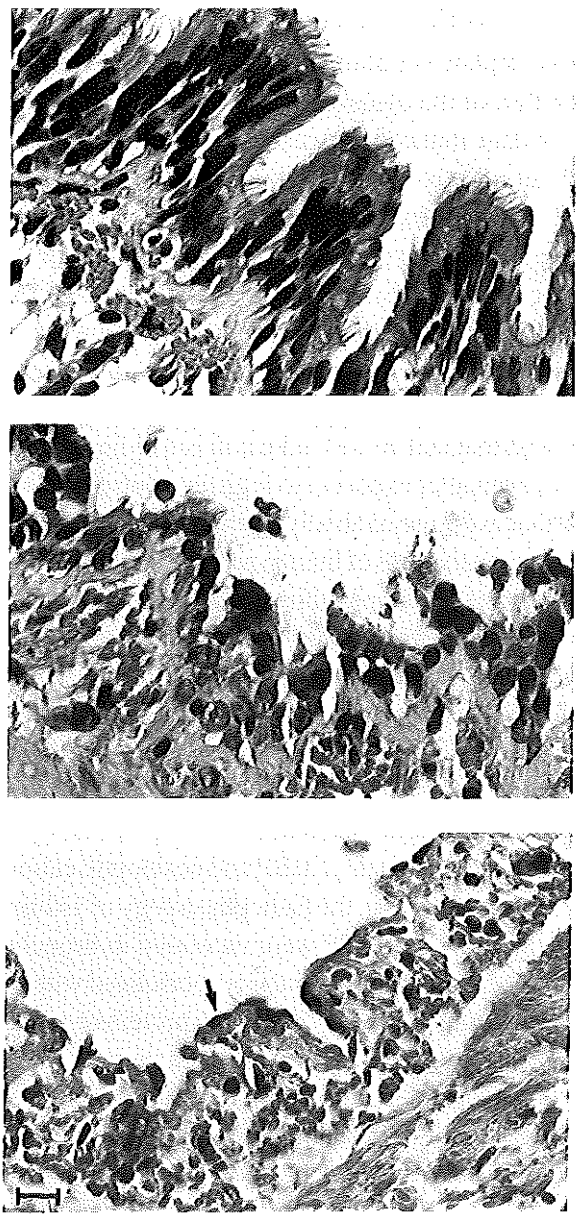

Figure 6. Light-microscopic photomicrographs of human bronchial epithelium (n=3) exposed to poly-L-arginine. (A) control airway after 18 h of exposure. The epithelium and cilia appear intact; (B) airway exposed to 100 µmol/l poly-L-arginine for 3 h. Destruction and exfoliation of epithelial cells; (C) airway exposed to 100 µmol/l poly-L-arginine for 16 hours. Ciliated epithelial cells are lost, only a few basal cells (arrow) are left. Hematoxylin-eosin (H&E) staining; bar = 10 µm.

L-arginine for 3 h and 16 h produced a 4.5- and 7-fold increase in permeability to ^{111}In-DTPA, respectively. Herbert and colleagues showed that a similar concentration of poly-L-arginine produced a doubling of the paracellular flux of albumin in bovine bronchial mucosa [19]. The concentration of poly-L-arginine used in their and our study was much higher than the concentration of MBP found in the sputum and BALF of patients with asthma [7 11 14]. Concentrations in sputum and BAL F, however, are influenced by unknown dilutional factors. It is possible, therefore, that local concentrations of cationic proteins in asthmatic airways are much higher than in sputum and BALF. In addition, the in vivo duration of exposure to such substances may be much longer. Furthermore, the airway epithelium in vivo may be exposed to a combination of cationic proteins and H_2O_2 and other oxidants which may produce more epithelial damage than H_2O_2 or cationic proteins alone [1]. Finally, the epithelium of asthmatic patients may be more susceptible to injury than the airways of nonasthmatic patient used for our studies [31]. Thus, even low concentrations of oxidants and cationic proteins may be responsible for epithelial damage in asthmatic patients.

The epithelial damage after exposure to poly-L-arginine in our study closely resembles the histopathologic findings reported by many investigators in asthmatic patients. Although epithelial damage is often regarded as a hallmark of asthma, several authors have reported the absence of such damage in asthmatic patients [28 29]. These observations, however, do not exclude a role of oxidants and cationic proteins in the pathophysiology of asthma: In laboratory animals, both oxidants and cationic proteins produce airway hyperresponsiveness without evidence of epithelial damage [34 36 38].

It is not clear how cationic proteins cause airway hyperresponsiveness and epithelial damage. Charge interactions between cationic proteins and airway epithelium have been suggested. This hypothesis is supported by the observation that the effects of poly-L-arginine in isolated guinea pig trachea were inhibited when albumin or heparin was added to the organ bath [9]. In a recent study in rats, Coyle and coworkers showed that cationic protein-induced airway hyper-responsiveness could be inhibited by the bradykinin receptor antagonist NPC 17713 [8]. Because bradykinin itself did not increase airway responsiveness, Coyle and colleagues suggested that an additional factor is involved, for instance an increase of epithelial permeability [8].

Lung permeability has been studied in vivo with aerosols of ^{99m}Tc-DTPA. After inhalation of the tracer molecule, the clearance of radioactivity from the lung can be measured with a gamma camera [5 23]. With this technique, a reduced

clearance of ^{99m}Tc-DTPA from central airways has been described in both animal and human lung, probably reflecting a low permeability of central airways [5 16]. However, these in vivo studies are confounded because the exposed surface is not known, and because of mucociliary clearance of ^{99m}Tc-DTPA [16 23]. Permeability to ^{99m}Tc-DTPA of the ferret trachea was about 10 times lower ($5x10^{-7}$ cm/s) than the permeability of the largest airways we studied [18]. In the present study, we found that airway permeability increased with decreasing airway size. This observation and the findings by Hanafi and coworkers [18], support the in vivo observation that central human airways have a lower permeability than peripheral airways.

In conclusion, we showed that human peripheral airways are more permeable to lipophilic than to hydrophilic molecules. The permeability to the hydrophilic ^{111}In-DTPA increased after exposure to the oxidant H_2O_2 or the cationic protein poly-L-arginine, which suggests the opening of paracellular pathways. In contrast, H_2O_2 did not change the permeability to the lipophilic ^{14}C-AP. Microscopic examination after exposure to H_2O_2 and poly-L-arginine revealed epithelial damage that resembled histopathologic findings in asthma and showed opening of tight junctions. Finally, we found that human airway permeability and airway size are negatively correlated. A more permeable epithelium no longer maintains a barrier to inhaled water-soluble antigens or spasmogens, such as histamine and methacholine, and this may at least partly explain the increased airway responsiveness to these agents in asthma. Enhanced entry of inhaled hydrophilic agents into the airway tissue after epithelial damage should be considered as a therapeutic target when new antiasthmatic drugs are designed.

7.6 REFERENCES

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Chapter 8

Epithelium Removal and Peptidase Inhibition enhance Relaxation to Vasoactive Intestinal Peptide

8.1 Introduction

In human airways, vasoactive intestinal peptide (VIP), a 28 amino acid peptide, is mainly colocalized to cholinergic fibers near the airway smooth muscle and submucosal glands [11]. VIP may mediate the non-adrenergic inhibitory response [14], which is considered as the only neuronal bronchodilator mechanism in human airways [18 23].

Early studies on human isolated central bronchi showed a small [2 17], or a large [16 19] bronchodilating effect of VIP. In contrast, VIP did not relax human isolated bronchioles [16]. Later studies suggested that the action of VIP on human airway tissue was limited by the enzymatic degradation of VIP by mast cell chymase and tryptase [6 22] or by epithelial neutral endopeptidase (NEP) [5].

Indeed, Tam and colleagues showed that a cocktail of inhibitors of chymase, tryptase, and NEP potentiates the effect of VIP on isolated central human airways [21]. This study, however, gives no insight in the role of the airway epithelium in the modulation of VIP responses. In addition, it remains unclear whether or not peripheral airways are less responsive to VIP than central airways.

Therefore, in the present study the responsiveness to VIP of isolated central and peripheral human airways, with or without epithelium, was directly compared in the absence or presence of peptidase inhibitors.

8.2 Materials and Methods

Tissue preparation

Human lung tissue was obtained from 6 male and 2 female patients (mean age 58.6 y; range 33 to 75 y) who underwent thoracotomy for bronchial carcinoma.

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Their mean preoperative forced expiratory volume in 1 sec as a percentage of the inspiratory vital capacity was 63.7 ± 2.8%. None of the patients had characteristics of asthma, 7 were current smokers. Immediately after surgical resection, a macroscopically normal part of the lung tissue was immersed in carbogenated (95% O_2-5% CO_2) Krebs-Henseleit (K-H) buffer at room temperature (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 5.55). The tissue remained in fresh carbogenated buffer throughout the dissection procedure and during the experiments. Tubular segments of cartilaginous bronchi (generation 3 to 5) and bronchioles with a diameter of 0.8 to 1.0 mm (generation 11 to 13) were carefully dissected free from parenchyma and blood vessels [3]. As described previously [10], the segments were cut transversely to obtain bronchial strips, and thin surgical silk threads (6/0) were tied to the ends of the strips. The tissues were studied at 37°C in double-jacketed 10 ml organ baths containing carbogenated K-H buffer. The baths were siliconized regularly to prevent adhesion of the peptides to the glass wall. One thread was attached to a glass hook at the bottom of the bath and the other to the arm of a high precision isotonic angular position transducer (Type 3810/60; Penny and Giles Ltd., Christchurch, UK) which was connected to a digital voltmeter (Fluke 73 multimeter, Everett, WA, USA) and a pen recorder (BD 40; Kipp, Delft, The Netherlands). The epithelium was removed by gentle rubbing of the luminal surface with a wet gauze. The efficacy of the epithelial removal was verified histologically. The use of human lung tissue for in vitro experiments was approved by the Ethical Committee of the Rotterdam University Hospital.

Drugs

Methacholine hydrobromide (Janssen Pharmaceutica, Beerse, Belgium), histamine hydrochloride (Janssen), VIP (Cambridge Research Biochemicals Inc., Wilmington, DE, USA), timolol maleate (Merck, Sharp & Dhome Research Laboratories, Rahway, NJ, USA) and EDTA (Sigma Chemical Company, St. Louis, MO, USA) were dissolved in 0.9% NaCl solution. The peptidase inhibitors phosphoramidon (Peninsula Laboratories, Inc., Belmont, CA, USA), leupeptin (Sigma), aprotinin (Sigma), soybean trypsin inhibitor (Sigma), captopril (Sigma), and bestatin (CRB) were prepared in 0.9% NaCl solution. Indomethacin (Bufa, Uitgeest, The Netherlands) and FPL 55712 (Fisons plc, Loughborough, Leicestershire, UK) were prepared in methanol. L-Isoproterenol sulfate (Janssen) was dissolved in distilled water containing ascorbic acid (88 mg/l). Fresh drug solutions were prepared daily and kept on ice during the experiments.

8.3 EXPERIMENTAL PROTOCOLS

To assess contractile functions and to ensure a stable function for the rest of the day, the preparations were contracted twice with methacholine (10^{-5} and 10^{-4}M) against an isotonic load of 500 mg for central airways and 250 mg for peripheral airways [3]. Following washout, all airway preparations were incubated with the following drugs: the ß-receptor antagonist timolol (10^{-6}M), the muscarinic receptor antagonist atropine ($1.2x10^{-6}$M), the cyclooxygenase inhibitor indomethacin ($6x10^{-6}$M) and the leukotriene C_4/D_4 receptor antagonist FPL 55712 ($11.5x10^{-6}$M). From each patient 2 pairs of central airways and 2 pairs of peripheral airways were used. From one of each pair, the epithelium was removed. After 10 minutes the peptidase inhibitors phosphoramidon (2.5 µg/ml), leupeptin (20 µg/ml), aprotinin (20 µg/ml), captopril (20 µg/ml), soybean trypsin inhibitor (20 µg/ml), and bestatin (2.8 µg/ml) were added to one pair of central airways and to one pair of peripheral airways. Another 10 minutes later, all preparations were precontracted to a submaximal level with histamine ($5x10^{-6}$M). After obtaining a stable contraction plateau, a cumulative concentration response curve (CCRC) to VIP (10^{-10} to 10^{-7}M, by half-log increments) was constructed. After washout, full relaxation of the preparations was obtained by adding L-isoproterenol (10^{-4}M) and EDTA ($4x10^{-3}$M). This parameter was calculated by linear interpolation between the two concentrations on either side of this 10% threshold. To minimize bias in the results, the calculations were based on the signal of the transducers as recorded by a digital voltmeter rather than on measurement of the response on the recording paper. The values of the level of precontraction and the net response were analyzed with a repeated-measurement analysis of variance (MANOVA), using the BMDP 5V program (BMDP statistical software, Inc., Los Angeles, CA, USA). The addition of a cocktail of peptidase inhibitors, the removal of the epithelium, and the airway size (central or peripheral) were taken as factors and the different patients were taken as random factors. The values of -log TC were compared by Student's two-tailed *t* tests for paired observations. Values $p < 0.05$ were considered significant. All data are expressed as means ± standard error of the mean (SEM).

Data analysis

The contractile state of the preparations was expressed on a scale which defines the maximal active contractile range (MACR) [13]. The second response to methacholine 10^{-4}M, obtained at the beginning of each experiment, was defined

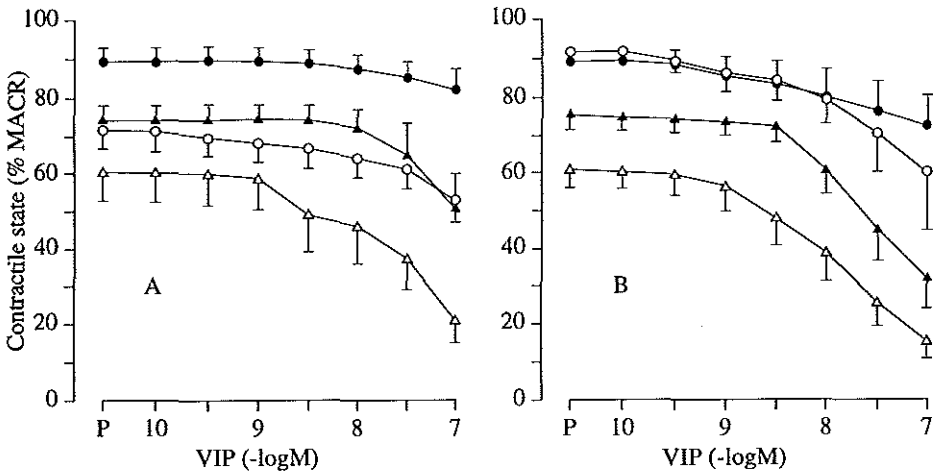

Figure 1. Mean CCRC to VIP of human central (A) and peripheral (B) airway strips precontracted with histamine (n=8). Closed symbols represent intact airways, open symbols depict epithelium-denuded airways. Triangles indicate that peptidase inhibitors are added. The horizontal axis depicts the negative log of the VIP concentration in the organ bath; the vertical axis represents the contractile state of the airway preparation as a percentage of the maximal active contractile range (%MACR). P = precontraction level. Vertical bars represent SEM.

as 100% shortening. Maximal relaxation after 10^{-4}M isoproterenol and $4x10^{-3}$M EDTA, determined at the end of each experiment, was defined as 0% shortening. The difference between the level of precontraction by $5x10^{-6}$M histamine and the contractile state after 10^{-7}M VIP is the net response (% MACR, table 1). Because of the high cost of VIP, it was not possible to construct complete CCRC's. In addition, minor responses to VIP in control preparations precluded accurate calculation of the negative logarithm of the effective concentration (-log EC_{50}) values. Therefore, the sensitivity to VIP was expressed as the negative logarithm of the VIP concentration that elicited a 10% reversal of the histamine-induced tone, and this was called the threshold concentration (TC).

8.4 RESULTS

Precontraction level

Removal of the epithelium significantly reduced the histamine induced precontraction level in central airways (89.4 ± 3.6% versus 71.6 ± 4.9% MACR,

Table 1. Net responses to VIP 10^{-7}M (%MACR)

	Central airways (n=8)	Peripheral airways (n=8)
Intact	7.1 ± 7.8	17.1 ± 6.4
Without epithelium	18.7 ± 3.4*	32.6 ± 11.6
With peptidase inhibitors	24.0 ± 7.8*	42.7 ± 4.3*
Without epithelium with peptidase inhibitors	39.0 ± 6.4*,†	45.5 ± 4.3*,†

Net responses to VIP 10^{-7}M of human central and peripheral airways precontracted with histamine with and without epithelium in the presence or absence of peptidase inhibitors. Data represent the reduction of histamine ($5x10^{-6}$M)-induced precontraction expressed as a percentage of the maximal active contractile range (% MACR). *$p<0.05$ compared to intact preparations. † $p>0.05$ for interaction meaning that an additive effect of epithelium removal and peptidase inhibitors is present.

figure 1a) but not in peripheral airways (figure 1b). In intact central and peripheral airways, the presence of the peptidase inhibitors also significantly reduced the precontraction level (89.3 ± 3.6% versus 74.3 ± 3.6% and 88.9 ± 3.6% versus 74.8 ± 3.8% MACR, respectively). The combination of epithelium removal and peptidase inhibitors caused a further decrease in precontraction, resulting in a similar precontraction level for central and peripheral airways (60.6 ± 8.0% and 60.9 ± 4.8% MACR, respectively).

Net response to VIP 10^{-7}M

VIP (10^{-10} to 10^{-7}M) induced a concentration-dependent relaxation in all preparations, except for the preparations with intact epithelium and without the peptidase inhibitors, in which only the highest VIP concentrations produced minor relaxations (figures 1a and 1b). Both removal of the epithelium and the presence of peptidase inhibitors increased the net response to VIP (table 1), although in peripheral airways the effect of removal of the epithelium did not reach statistical significance (p=0.11). MANOVA showed no interaction between the effects of epithelium removal and peptidase inhibitors on the net response in both central and peripheral airways. Thus, removal of the epithelium in the presence of peptidase inhibitors produced a significant additive increase of the net response to VIP in both central and peripheral airways.

Table 2. Threshold concentration (TC) values (-log M) of VIP-induced relaxations.

	Central airways (n=8)	Peripheral airways (n=8)
Intact airways	6.96 ± 0.22	7.27 ± 0.08
Without epithelium	8.00 ± 0.28*	7.73 ± 0.29†
With peptidase inhibitors	7.58 ± 0.16*	7.98 ± 0.22*,**
Without epithelium, with peptidase inhibitors	8.27 ± 0.29*	8.61 ± 0.20*,**

Threshold concentration (TC) values (-log M) of VIP-induced relaxations of human central and peripheral airways precontracted with histamine ($5x10^{-6}$M) with and without epithelium in the presence or absence of peptidase inhibitors (means ± SEM). * $p<0.05$, † $p=0.07$, compared with intact airways. ** $p<0.05$ compared with central airways.

Sensitivity to VIP

The -log TC values of central and peripheral airways with and without epithelium and in the absence or presence of peptidase inhibitors are listed in table 2. Removal of the epithelium or the presence of peptidase inhibitors increased the sensitivity to VIP in both central and peripheral airways, although in peripheral airways the effect of removing the epithelium only approached statistical significance (p=0.07, table 2). The addition of peptidase inhibitors to epithelium-denuded airways produced an additional increase in sensitivity, resulting in a more than 20-fold increase in sensitivity compared to intact airways without peptidase inhibitors. Compared with central airways, the -log TC for VIP was significantly higher in peripheral airways with and without epithelium, but only when the peptidase inhibitors were present (table 2).

Effect of precontraction level on the responsiveness to VIP

Since there were significant differences in precontraction level between intact preparations (controls) and peptidase-treated airways with or without epithelium, we investigated whether the responsiveness to VIP varied with the precontraction level. In seperate experiments intact peripheral airways were precontracted with $5x10^{-8}$M (n=5) or $5x10^{-6}$M (n=5) histamine (precontraction levels of 52.9 ± 2.6% and 81.2 ± 2.9% MACR, respectively). The net responses were 7.6 ± 2.7% and 8.5 ± 3.9% (p=0.55), the -log TC's were 7.31 ± 0.33 and 7.28 ± 0.27 (p=0.92) for airways precontracted with $5x10^{-8}$M and $5x10^{-6}$M

histamine, respectively. These results indicate that, between 52.9% and 81.2% on the MACR scale, precontraction levels have no effect on the sensitivity and net responses to VIP.

8.5 DISCUSSION

Our results show that exogenous VIP causes only minor relaxations of human isolated precontracted central and peripheral airways irrespective of the precontraction level. When the epithelium is removed or peptidase inhibitors are added, VIP effectively relaxes both central and peripheral airways. Removal of the epithelium and presence of the peptidase inhibitors had additive effects on the net response to 10^{-7}M VIP. The sensitivity, expressed as the -log TC, increased in both central and peripheral airways more than 20-fold when the epithelium was removed and peptidase inhibitors were added. Finally, we found that in the presence of peptidase inhibitors peripheral airways were slightly more sensitive to exogenous VIP than central airways.

The precontraction level in epithelium-denuded central airways was significantly lower than in intact airways (figure 1a). This difference may represent an artifact due to damage of the bronchial smooth muscle by the rubbing procedure. This seems unlikely, however, because in peripheral airways, which are much more vulnerable, no difference between precontraction level in rubbed and unrubbed preparations was seen. In our study, the presence of peptidase inhibitors resulted in a decreased precontraction response to histamine in both central and peripheral airways (figure 1a and 1b). In dog bronchi, exogenous tryptase enhances histamine-induced contraction by an effect on Ca^{2+} channels [20]. In contrast, inhibition of endogenous tryptase by peptidase inhibitors may explain the reduced contractile response to histamine in our experiments as a result of the impaired influx of extracellular Ca^{2+}. That in a previous study by Tam and colleagues [21] precontraction to carbamylcholine was not affected by the presence of peptidase inhibitors may be due to the fact that acetylcholine contracts airway smooth muscle by mobilizing intracellular Ca^{2+} stores [8].

Our results concerning the net responses to VIP confirm those of others [17 21], who found that exogenous VIP causes only minor relaxations of isolated human bronchi after precontraction with histamine or carbamylcholine. In the study of Tam and coworkers [21], peptidase inhibitors significantly potentiated the bronchodilator responses of central human airways to VIP, which is similar to our

findings. Our results contrast with those of Palmer and colleagues, however, who found a potent VIP-induced relaxation of intact human bronchi [16]. They reported a -log EC_{50} of 7.77 for VIP and a -log EC_{50} of 6.11 for isoproterenol in central airways, indicating that VIP was 50 times more potent than isoproterenol. The values of -log EC_{50} for isoproterenol in their study were considerabely lower than in other studies [4 17], however, and incomplete CCRC's to VIP were made, which precludes accurate EC_{50} determinations. Therefore, in intact central airways, VIP may not be as potent as has been suggested. In the study of Palmer and colleagues [16], intact bronchioles showed no response to exogenous VIP. They support their findings by referring to binding studies using ^{125}I-VIP in which density of labeling over smooth muscle in the bronchus was three times higher than in bronchioles [1]. Labeling with ^{125}I-VIP is merely a measure of uptake, however, and does not predict the physiological events that follow receptor binding. Furthermore, in a study of Leys and coworkers [12], no specific binding of ^{125}I-VIP to bronchial smooth muscle was seen in airways of all sizes, including extrapulmonary bronchus. These authors argue that VIP receptor density on airway smooth muscle may be too low for autoradiographic visualization [12]. Therefore, a relaxing effect of VIP on peripheral airways cannot be excluded on the basis of binding studies. The present study shows that VIP relaxes bronchioles, but only in the presence of peptidase inhibitors. Thus, it seems that VIP-receptor density on smooth muscle of peripheral airways is not too low to mediate relaxations but that the lack of relaxing effect on peripheral airways in previous studies may have been due to the breakdown of VIP by endogenous peptidases before it could act on the smooth muscle cell.

We found that both removal of the epithelium and the addition of peptidase inhibitors augment the sensitivity to VIP of both central and peripheral human airways. This is in accordance with previous studies in guinea-pig trachea, in which epithelium removal [5] or peptidase inhibitors [5 24] produced a leftward shift of the CCRC to exogenous VIP. In the study of Farmer and Togo [5], a similar increase in sensitivity to VIP was seen with phosphoramidon or thiorphan (NEP inhibitors) and epithelium removal, indicating that epithelial NEP is the principal source of degradation of VIP in the guinea-pig trachea [5]. In the present study, we found an additive effect of removal of epithelium and the presence of peptidase inhibitors in both central and peripheral airways. Two possible mechanisms may contribute to this additive effect. The most obvious explanation is that in human airways VIP is inactivated not only at epithelial sites where NEP is localized [9] but also by peptidases derived from subepithelial sources. Subepithelial pepti-

dases may be produced by mast cells (chymase and tryptase), macrophages (acid proteases and endopeptidases), or endothelium, which generates angiotensin converting enzyme, or they may be situated on the airway smooth muscle itself (NEP), as was suggested previously [15]. In addition, the epithelium in human airways may act as a diffusion barrier for VIP, and removal of this barrier could increase the sensitivity to VIP. It should be noted that the influence of epithelial peptidases in the present experimental setting may be underestimated since mechanical removal of epithelium may lead to peptidase release from subepithelial mast cells [7].

Under physiological conditions, the VIP-degrading properties of epithelial NEP may not be important since endogenous VIP will not reach the epithelial surface where NEP is situated. Peptidases derived from the lamina propria, however, may be able to degrade endogenous VIP produced by non-adrenergic, non-cholinergic (NANC) nerves in the vicinity of airway smooth muscle. Therefore, these peptidases may modulate NANC neural control of airway tone in both central and peripheral airways. In an inflammatory disease like asthma, an increase in VIP-degrading peptidases released by inflammatory cells in the airways may lead to increased bronchial obstruction and, perhaps, contribute to bronchial hyperresponsiveness. It can be questioned, however, whether VIP plays an important modulatory role in peripheral airways in vivo since in small airways few VIP-like immunoreactive nerves were found [11].

We conclude that exogenous VIP relaxes human precontracted central and peripheral airways only to a minor degree unless the epithelium is removed or peptidase inhibitors are present. Peptidase inhibitors and removal of the epithelium have additive effects on VIP-induced relaxation, indicating that in human airways epithelial and subepithelial peptidases are both important in the inactivation of VIP.

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Chapter 9

Bradykinin-induced Contraction of Human Peripheral Airways mediated by both Bradykinin B_2 and Thromboxane Prostanoid Receptors

9.1 Introduction

Bradykinin (Bk), a nine amino acid peptide formed from the plasma precursor kininogen, may be an important mediator in inflammatory diseases such as asthma [2]. Elevated kinin concentrations have been found in the bronchoalveolar fluid of asthmatic subjects after allergen challenge [5], and inhaled Bk induces bronchoconstriction in asthmatic but not in normal individuals [15 27 33]. In isolated central airways, however, only small contractile or potent relaxant effects of Bk were found [3 4 14 15 33]. After removal of the epithelium, Bk-induced smooth muscle relaxation changed to contraction, indicating a protective role of the epithelium. This may involve the production of relaxant prostanoids or degradation of Bk by epithelial neutral endopeptidase (NEP) [3 4 14].
Because epithelial damage is found in biopsies of asthmatic subjects, it is tempting to hypothesize that the loss of epithelium in asthma makes the airways prone to Bk-induced bronchoconstriction. Studies in asthmatic patients, however, show that inhalation of ipratropium bromide [15], sodium cromoglycate [12 15], and the tachykinin receptor antagonist FK-224 [18] protects against Bk-induced bronchoconstriction. This suggests activation of cholinergic and peptidergic nerves by Bk. The involvement of prostanoids in Bk-induced bronchoconstriction in the human is controversial. In one study, an oral dose of the cyclooxygenase inhibitor flurbiprofen significantly inhibited Bk-induced bronchoconstriction [26], whereas other investigators did not observe a protective effect of an oral dose of aspirin [15]. The aim of the present study was, therefore, to investigate the effects of epithelium removal, inhibition of the enzymes NEP and cyclooxygenase, and blockade of local axon reflexes with tetrodotoxin (TTX) on Bk-induced responses of

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human peripheral airways, which have been shown to contain many more Bk receptors than central airways [23]. Furthermore, we measured the Bk-induced release of the cyclooxygenase products prostaglandin E_2 (PGE_2), prostaglandin I_2 (PGI_2) and thromboxane A_2 (TxA_2). Because in the guinea pig trachea the direction of Bk-induced responses are dependent on airway tone [4], we also studied the effect of Bk on airways precontracted with methacholine. Finally, we examined the effects of the selective B_2 receptor antagonist Hoe 140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8] bradykinin) [16] and the thromboxane (TP)-receptor blocking drug GR32191 (Vapiprost) [22] to elucidate whether these receptors are important in Bk-induced responses of human isolated airways.

9.2 MATERIALS AND METHODS

Tissue preparation

Human lung tissue was obtained from 23 male and 6 female patients (mean age 65.8 y; range 46 to 77 y) who underwent thoracotomy for bronchial carcinoma. None of the patients had characteristics of asthma. The premedication was the same for all patients: atropine, thiopental, fentanyl, O_2/N_2O, halothane, and pancuronium. Immediately after surgical resection, a macroscopically normal part of the lung tissue was immersed in carbogenated (95% O_2 and 5% CO_2) Krebs-Henseleit buffer at room temperature (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 5.55). The tissue remained in fresh carbogenated buffer throughout the dissection procedure and during the experiments. Bronchioles with an inner diameter of 0.8 to 2.0 mm (generation 8 to 13) were carefully dissected and cut spirally to obtain a bronchial strip preparation. The tissues were studied at 37°C in double-jacketed 10 ml siliconized organ baths containing carbogenated Krebs-Henseleit buffer. Thin surgical silk threads (6/0) were tied to the ends of the strips. One thread was attached to a glass hook at the bottom of the organ bath and the other to the arm of a high-precision isotonic angular position transducer (Type 3810/60; Penny and Giles Ltd., Christchurch, UK) which was connected to a digital voltmeter (Multimeter 73; Fluke, Everett, WA, USA) and a pen recorder (BD40; Kipp, Delft, The Netherlands). The preparations contracted against a load of 250 mg, which has been shown to be optimal for human bronchial strips [20].
When indicated, the epithelium was removed by gentle rubbing of the luminal surface with a wet gauze. To quantify the effect of rubbing the epithelium, all

strips were examined histologically and the percentage of the basement membrane covered with epithelium was estimated by an observer who was unaware of the previous treatment of the preparations. The integrity of the basement membrane and the underlying smooth muscle was also examined. The study was approved by the Ethical Committee of the Rotterdam University Hospital.

Drugs

Methacholine bromide (Janssen Pharmaceutica, Beerse, Belgium), EDTA (Sigma Chemical Co., St. Louis, MO, USA), and the peptidase inhibitor phosphoramidon (Peninsula Laboratories Inc., Belmont, CA, USA) were dissolved in 0.9% NaCl. Bradykinin (Bachem California, Torrance, CA, USA), Hoe 140 (gift from Dr K.J. Wirth, Hoechst AG, Frankfurt, Germany), GR32191 (gift from Dr B.M. Bain, Glaxo Group Research, Ltd., Greenford, Middlesex, UK), and TTX (Sigma) were dissolved in distilled water. Indomethacin (Bufa, Uitgeest, The Netherlands) was prepared in methanol. L-Isoproterenol sulfate (Janssen) was dissolved in distilled water containing ascorbic acid (88 mg/l). Standard PGE_2, 6-keto $PGI_{1\alpha}$, and TxB_2 (Sigma) and the ^{3}H-labeled PGE_2, 6-keto $PGI_{1\alpha}$, and TxB_2 (Amersham, Amersham, Buckinghamshire, UK) were dissolved in ethanol. Antibodies were purchased from Advance Magnetics, Inc. (Cambridge, MA, USA) and dissolved in radioimmunoassay buffer.

9.3 EXPERIMENTAL PROTOCOLS

Before all experiments, the preparations were contracted twice with methacholine (10^{-5} and 10^{-4}M) to assess contractile function and to ensure a stable function for the rest of the day [10]. Cumulative concentration-response curves (CCRCs) to bradykinin were made with a dose range of 10^{-10} to 10^{-5}M, by logarithmic increments. After completion of the experiments, the preparations were washed and fully relaxed by adding L-isoproterenol (10^{-4}M) and ethylenediaminetetraacetic acid (EDTA; $4x10^{-3}$M).

Effect of bradykinin on airways with spontaneous intrinsic tone

From each patient (n=8) four pairs of airways were studied. Each pair consisted of one airway with and one without epithelium.

The first, second, and third pairs were incubated with either phosphoramidon ($5x10^{-6}$M), or indomethacin ($5x10^{-7}$M) or a combination of phosphoramidon

($5x10^{-6}$M) and indomethacin ($5x10^{-7}$M), respectively. The fourth pair served as a control. After 20 min of incubation, a CCRC to Bk was made.

Effect of bradykinin on precontracted airways
From each patient (n=6) four pairs of airways were studied. Each pair consisted of one airway with and one without epithelium. The airways were precontracted to a submaximal level with methacholine ($3x10^{-6}$M). After a stable precontraction level was obtained, the first, second, and third pairs were incubated with either phosphoramidon ($5x10^{-6}$M), or indomethacin ($5x10^{-7}$M) or a combination of phosphoramidon ($5x10^{-6}$M) and indomethacin ($5x10^{-7}$M), respectively. The fourth pair served as a control. After 20 min of incubation, a CCRC to Bk was made.

Determination of B_2-receptor antagonist potency
Four intact airway preparations from each of six patients were used. Phosphoramidon ($5x10^{-6}$M) was added to all airway preparations. Three airways were incubated with the B_2 receptor antagonist Hoe 140 [16] at the concentrations 10^{-8}, 10^{-7}, and 10^{-6}M, respectively. The fourth airway served as a control. A CCRC to Bk was made 20 min after incubation with the antagonist.

Determination of thromboxane prostanoid receptor antagonist potency
Six intact airway preparations from each of eight patients were used. Phosphoramidon ($5x10^{-6}$M) was added to all airway preparations. Four airways were incubated with the TP-receptor antagonist GR32191 (Vapiprost) [22] at the concentrations $3x10^{-8}$, 10^{-7}, $3x10^{-7}$, and 10^{-6}M, respectively. The fifth airway was incubated with GR32191 ($3x10^{-7}$M) and indomethacin ($5x10^{-7}$M). The sixth airway served as a control. A CCRC to Bk was made 20 min after incubation with the antagonist.

Effect of blocking neural conductance on Bk-induced responses
These experiments were performed in the presence of phosphoramidon ($5x10^{-6}$M) to prevent degradation of potentially released tachykinins. Paired intact airways of eight patients were incubated with the nerve conductance blocker tetrodotoxin (TTX, 3 μg/ml) or with the vehicle for TTX (distilled water; control). After 20 min, a CCRC to Bk was made.

Release of prostanoids by airway preparations

Radioimmunoassay of PGE_2, 6-keto $PGF_{1\alpha}$ (the stable metabolite of PGI_2), and TxB_2 (the stable metabolite of TxA_2) was performed in the organ bath fluid (10 ml) derived from five of the experiments on intact and epithelium-denuded airways with spontaneous intrinsic tone. Baseline PGE_2, PGI_2 and TxA_2 release was determined by collecting the organ bath fluid after a 30-min period. In addition, the release of these prostanoids was measured in the organ bath fluid after making the CCRCs to Bk (which also took 30 min) in the absence and presence of indomethacin. Organ bath fluids were applied to Sep-Pak C_{18} cartridges (Waters Associates, Millford, MA, USA) and rinsed with 5 ml of water. The prostanoids were extracted with 2.5 ml methanol and stored at -20°C until they were analyzed as a single batch. After the experiments, the wet weight of the preparations was measured. The presence of PGE_2, 6-keto $PGF_{1\alpha}$ and TxB_2 was determined in 0.5 ml aliquots using tritiated radioimmunoassay kits. The separation of free and bound tracer was accomplished with dextran-coated charcoal. The sensitivity of the assays was approximately 2 pg per tube. The cross-reactivity of the PGE_2 assay with $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$, and thromboxane B_2 was <0.01, <0.01 and <0.1%, respectively. The cross-reactivity of the 6-keto $PGI_{1\alpha}$ assay with $PGF_{2\alpha}$, PGE_2, and thromboxane B_2 was <0.01, <1.0 and <0.1%, respectively. The cross-reactivity of the TxB_2 assay with $PGF_{2\alpha}$, PGE_2, and 6-keto $PGI_{1\alpha}$ was <0.01, <1.0 and <0.01%, respectively[35]. The amounts of PGE_2, 6-keto $PGI_{1\alpha}$ and TxB_2 were expressed as pg/mg wet tissue weight.

Data analysis

The CCRCs were analyzed to obtain *-log EC_{50}* (the negative logarithm of the agonist concentration that caused 50% of the maximal effect) and *Smax* (maximal contractile response to bradykinin expressed as a percentage of the maximal contraction to methacholine 10^{-4}M). *Baseline contractile state (BCS)* was expressed as a percentage of the difference between the maximal contraction to methacholine 10^{-4}M and the maximal relaxation in the presence of EDTA ($4x10^{-3}$M) and isoproterenol (10^{-4}M). The *Precontraction level* after $3x10^{-6}$M methacholine was expressed as a percentage of the maximal contraction to methacholine. The -log EC_{50} was calculated using a software module for non-linear curve fitting. The curves were fit to a four-parameter logistic function[9]. Means of -log EC_{50}, BCS, precontraction level and Smax of the CCRCs were compared with paired Student's *t* tests (two-sided, α=0.05). The concentrations of PGE_2, 6-keto $PGF_{1\alpha}$, and TxB_2 in the organ bath fluid of the different treat-

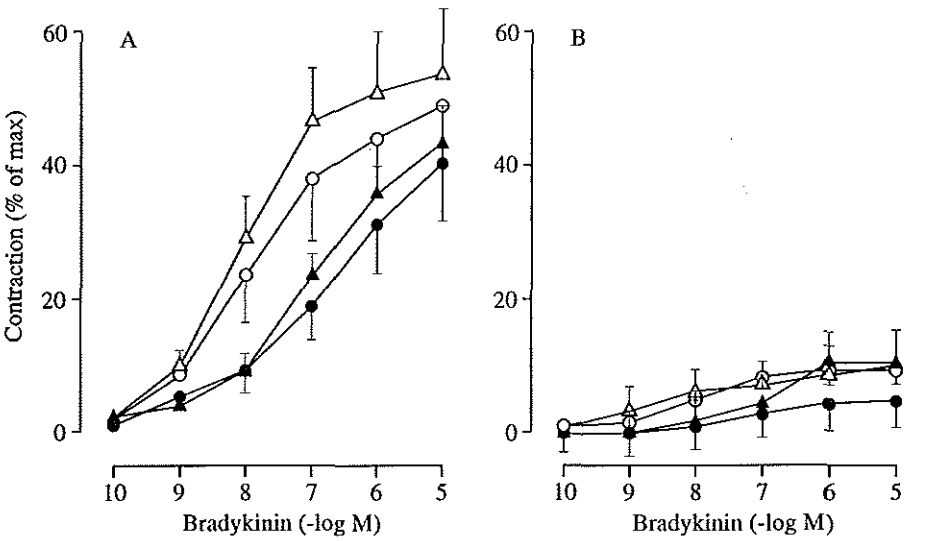

Figure 1. Mean CCRC to bradykinin (Bk) of human peripheral airways (n=8) in the absence (A) and presence (B) of indomethacin ($5x10^{-7}$M): closed symbols represent intact airways, open symbols depict epithelium-denuded airways, triangles indicate that phosphoramidon is added. Bk-induced contraction is expressed as a percentage of the maximal contraction to methacholine (10^{-4}M). Vertical bars represent SEM.

ment groups (control, and after stimulation with Bk in the presence or absence of indomethacin) were also compared by paired Student's *t* tests (two-sided, α=0.05). All data are expressed as mean ± standard error of the mean (SEM).

9.4 RESULTS

Histologic check of epithelium removal

In rubbed strips 6.2 ± 1.8% of the basement membrane was covered with epithelium, whereas in unrubbed strips this was 88.1 ± 2.5% ($p<0.0001$). After rubbing, the basement membrane was intact and no histologic evidence of smooth muscle damage was found.

Effect of bradykinin on airways with spontaneous intrinsic tone

Bk contracted seven of the eight intact airway strips without phosphoramidon and six of the eight strips with phosphoramidon; the remaining three strips (from three different patients) relaxed to Bk. All epithelium-denuded strips contracted to Bk (n=8, each with or without phosphoramidon).

Table 1. Sensitivity (-logEC_{50}), baseline contractile state (BCS), and maximal contraction to bradykinin of human peripheral airway strips (n=8).

	-log EC_{50}	Baseline contractile state (%)	Maximal contraction (%)
Intact	6.96 ± 0.25	38.9 ± 4.5	40.2 ± 8.3
Without epithelium	7.82 ± 0.25*	46.0 ± 5.1	43.8 ± 5.2
Intact, with phosphoramidon	7.14 ± 0.21	46.1 ± 4.1	49.0 ± 9.2
Without epithelium, with phosphoramidon	8.03 ± 0.13*†	48.3 ± 3.5	54.3 ± 9.2

* $p<0.05$ compared with intact airways,
† $p<0.05$ compared with epithelium-denuded airways.

The values of -log EC_{50}, BCS, and Smax of the airways that contracted to Bk are listed in table 1, and the CCRCs of these experiments are shown in figure 1a. Removal of the epithelium resulted in a significant 7-fold increase in the sensitivity to Bk. The addition of phosphoramidon tended to increase the sensitivity to Bk in both intact and epithelium-denuded airways although this change was not statistically significant. No difference was found in BCS and Smax between the different treatment groups.

Treatment of the airways with indomethacin largely inhibited the responses to Bk (figure 1b). The maximal contraction to 10^{-5}M Bk was 5.8, 11.1, 9.8, and 10.9% of the maximal contraction to methacholine for intact airways, intact airways incubated with phosphoramidon, epithelium-denuded airways, and epithelium-denuded airways incubated with phosphoramidon, respectively. Indomethacin did not alter the BCS of the airway preparations.

Effect of bradykinin on precontracted airways

After precontraction with methacholine, Bk relaxed the airways of three patients (from 73.7 ± 3.2 to 48.1 ± 5.8% and from 91.9 ± 3.4 to 69.7 ± 3.9% of Smax for intact and epithelium-denuded airways, respectively). In contrast, the airways of the other three patients contracted when Bk was added (from 74.5 ± 4.8 to 88.5 ± 4.8% and from 81.2 ± 2.1 to 101.4 ± 1.2% of Smax for intact and epithelium-denuded airways, respectively). The presence of phosphoramidon did not change the direction of the response (relaxation or contraction) within patients. Addition of indomethacin completely abolished both contractile and relaxation

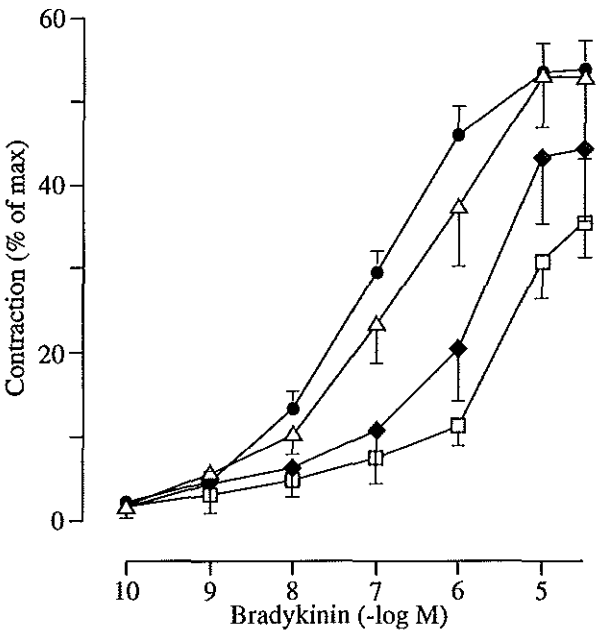

Figure 2. Mean CCRC to bradykinin (Bk) of human peripheral airways (n=6) in the presence of the selective B_2 receptor blocking drug Hoe 140 at the concentrations 10^{-8}M (open triangles), 10^{-7}M (closed diamonds) and 10^{-6}M (open squares). Closed circles represent control airways not incubated with Hoe 140. Bk-induced contractions are expressed as a percentage of the maximal contraction to methacholine (10^{-4}M).

responses to Bk in all airways, irrespective of the presence or absence of phosphoramidon. Because of the small number of observations (n=3, for each curve), no statistics were performed.

Determination of B_2 receptor antagonist potency

The effect of Hoe 140 (10^{-8}, 10^{-7} and 10^{-6}M) on Bk-induced contraction is shown in figure 2. Hoe 140 produced a significant rightward shift of the CCRCs to Bk (-log EC_{50} values: 7.28 ± 0.14, 6.75 ± 0.12, 6.31 ± 0.17, and 5.54 ± 0.13 for control, 10^{-8}, 10^{-7}, and 10^{-6}M Hoe 140, respectively). Furthermore, a decrease in Smax and a loss of parallelism was found.

Determination of thromboxane prostanoid receptor antagonist potency

In figure 3 the effect of GR32191 ($3x10^{-8}$, 10^{-7}, $3x10^{-7}$, and 10^{-6}M) on Bk-induced contraction is shown. In the presence of GR32191 airways relaxed to

Bk. Mean Bk-induced maximal relaxations tended to increase with increasing concentrations of GR32191. Relaxation to Bk in the presence of GR32191 was abolished when indomethacin was added.

Effect of blocking neural conductance on Bk-induced responses
The sensitivity and Smax to Bk in the presence and absence of TTX were not significantly different ($-\log EC_{50}$, 7.26 ± 0.20 and 7.32 ± 0.20; Smax, 38.4 ± 5.6 and $36.8 \pm 5.2\%$ of maximal contraction for TTX and controls, respectively, ns).

Release of prostanoids by airway preparations
The release of PGE_2, PGI_2, and TxA_2 by airway preparations before and after exposure to bradykinin in the presence or absence of indomethacin is shown in figure 4. Following stimulation with Bk, significantly more PGE_2, PGI_2, and TxA_2 was released from both intact and epithelium-denuded airways than in the control period. The release of the prostanoids following stimulation with Bk was largely inhibited in the presence of indomethacin, although in epithelium-denuded airways in the presence of indomethacin significantly more PGI_2 and TxA_2 were produced after stimulation with Bk than in the control period without Bk. After stimulation with Bk, the epithelium-denuded airways produced significantly more PGE_2 and TxA_2 and tended to produce more PGI_2 per mg wet weight than intact airways. In both intact and epithelium-denuded airways, the release of PGI_2 and PGE_2 was significantly greater than the release of TxA_2. In intact airways, the release of PGI_2 was significantly greater than the release of PGE_2.

9.5 DISCUSSION

Our experiments showed that Bk contracted intact and epithelium-denuded human peripheral airways with spontaneous intrinsic tone. Precontracted airways were either relaxed or further contracted. Removal of the epithelium increased the sensitivity to Bk, and the addition of phosphoramidon tended to increase the sensitivity to Bk in both intact and epithelium-denuded airways. Bk-induced responses and Bk-induced release of prostanoids were largely inhibited in the presence of indomethacin. The B_2-receptor antagonist Hoe 140 and the TP- receptor blocking drug GR32191 antagonized Bk-induced bronchoconstriction, suggesting B_2 receptor-mediated release of prostanoids that contracted airways via the TP-receptor.

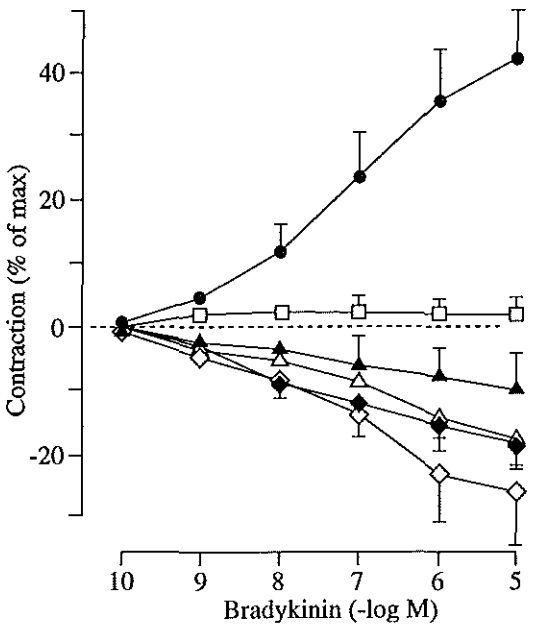

Figure 3. Mean CCRC to bradykinin (Bk) of human peripheral airways (n=8) in the presence of the thromboxane prostanoid (TP)-receptor blocking drug GR32191 at the concentrations 3 x 10^{-8}M (closed triangles), 10^{-7}M (open triangles), 3x10^{-7}M (closed diamonds) and 10^{-6} M (open diamonds). Open squares represent Bk-induced responses in the presence of 3x10^{-7}M of GR3219 and 5x10^{-7}M of indomethacin. Closed circles represent controls not incubated with GR32191 or indomethacin. Bk-induced contractions are expressed as a percentage of the maximal contraction to methacholine (10^{-4}M).

In earlier studies of human isolated central airways, only small contractile or relaxant effects of Bk were found [3 15 33]. The density of Bk receptors on peripheral airway smooth muscle is much higher than on smooth muscle of central airways, however, and therefore the effects of Bk may be greater on peripheral airways [23]. Our results show that Bk indeed has a potent contractile effect on human peripheral airways with spontaneous intrinsic tone and confirm studies by Molimard and colleagues [25]. Smooth muscle relaxation to Bk has been described in both human and guinea pig airways. This relaxation was converted into contraction after removal of the epithelium and after addition of indomethacin or phosphoramidon, suggesting that the epithelium protects the underlying smooth muscle by producing relaxant prostanoids or nitric oxide or by degrading Bk via NEP [3 4 14].

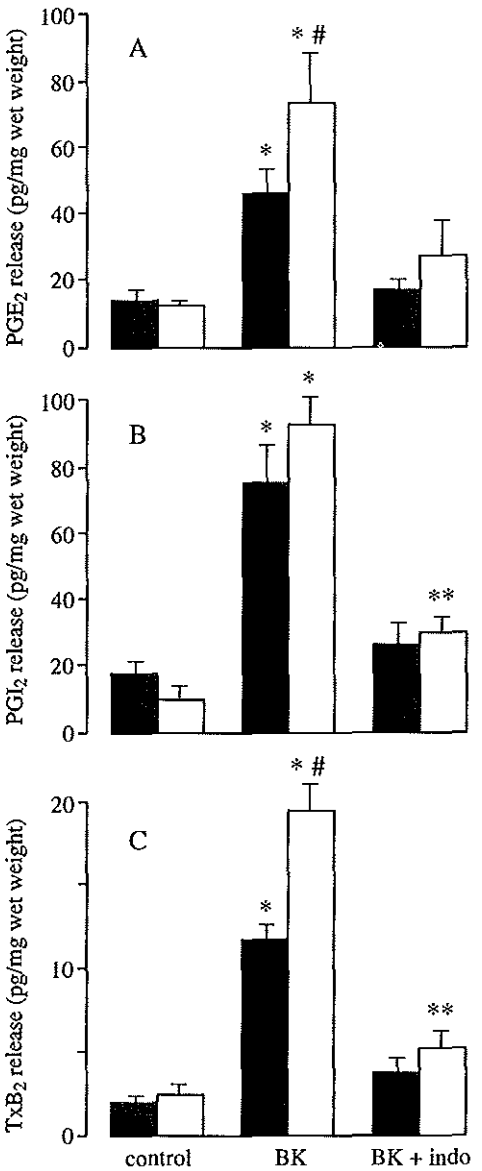

Figure 4. Release of the prostanoids PGE_2 (A), PGI_2 (B), and TxA_2 (C) by the airway preparations in 30 min with or without bradykinin stimulation (10^{-10} to 10^{-5}M) in the presence or absence of indomethacin (n=5). Closed bars represent intact airways, open bars represent epithelium-denuded airways. Indo = indomethacin is added ($5x10^{-7}$M). * $p<0.05$ compared with both the control period and after stimulation in the presence of indomethacin. # $P<0.05$ compared with intact airways. ** $p<0.05$ compared with the control period.

In the present study, indomethacin largely inhibited the response of human peripheral airways to Bk, but no change in direction of the response was seen. Measurement of the production of prostanoids supports our functional studies: the release of PGE_2, PGI_2, and TxA_2 was increased by Bk and was largely prevented by indomethacin. In the guinea pig trachea stimulated with Bk, PGE_2 release was much greater than PGI_2 release [4]. In the present study in human airways PGI_2 release was somewhat greater than PGE_2 release. TxA_2 release was approximately four times lower than PGE_2 and PGI_2 release. These results confirm an earlier study of the release of prostanoids by human bronchi, in which PGI_2 was also the predominant prostanoid released and TxA_2 release was much lower than PGE_2 and PGI_2 release [32]. It is remarkable that epithelium-denuded airway preparations released even more prostanoids. This contrasts with the results obtained by Bramley and associates [4], who observed a decrease in the production of prostanoids in epithelium-denuded guinea pig tracheas, and suggests that in human airways prostanoids are produced not only by the airway epithelium, as was shown earlier [30], but also by nonepithelial tissues.

In our experiments, addition of phosphoramidon tended to increase the sensitivity of the airways to Bk. Addition of phosphoramidon to intact airways, however, was not as effective as removal of the epithelium (figure 1a), perhaps because the epithelium acted as a barrier or because other Bk-degrading enzymes, such as angiotensin converting enzyme (ACE), may be present on the epithelium [13]. Airway reactivity to Bk in mildly asthmatic subjects remained unchanged after inhibition of ACE [11], however, suggesting that in human airways ACE is not important in the degradation of Bk. The tendency of phosphoramidon to increase the sensitivity to a bronchoactive peptide in epithelium-denuded airways was also found in an earlier study by our group [17] and suggests that NEP is also present below the epithelium. In the guinea pig trachea, the direction of Bk-induced responses depends not only on the presence of epithelium and the addition of indomethacin or phosphoramidon but also on the tone of the airway preparation.

Rubbed guinea pig tracheas precontracted with acetylcholine were relaxed by Bk [4]. In the present study, precontracted human peripheral airways either relaxed or contracted to Bk. The direction of the response was consistent within a single patient and was not changed by the removal of the epithelium or the addition of phosphoramidon. Indomethacin inhibited both the relaxing and the contractile effect. It therefore seems possible that the release of a patient-specific ratio of contractile and relaxing prostanoids determines whether precontracted airways

relax or contract to Bk in a particular patient. We found that GR32191 reversed Bk-induced contraction, indicating that the contractile effects of prostanoids released by Bk are mediated through the TP- receptor. Although TxA_2 is the most potent agonist for the TP-receptor [6], the prostanoids PGD_2, $PGF_{2\alpha}$, PGE_2, and PGI_2 also possess TP-receptor agonist activity [6-8] and may therefore contribute to bronchoconstriction. Not only did GR32191 inhibit Bk-induced contraction, but in the presence of higher concentrations of GR32191 airways relaxed to Bk. This relaxation was abolished by indomethacin, indicating that cyclooxygenase products are involved. Thus, it seems that functional antagonism between the TP receptor and relaxant prostanoid receptors determines the direction of the response to Bk. We did not investigate the identity of these relaxant receptors but studies in animals suggest that both the EP_2 and the IP receptor may be involved [7].

In the present study, increasing concentrations of Hoe 140 produced a decrease in Smax and loss of parallelism of Bk-induced contractions. This was also found by other investigators in both human [25] and guinea pig airways [28 34] and confirms that Bk-induced contractions are mediated through the B_2 receptor. From these experiments it was concluded that Hoe 140 is a noncompetitive antagonist, or alternatively, a B_3 receptor is involved [28 34]. In the present study, however, we showed that Bk acts as an indirect agonist, initiating the release of prostanoids. Therefore, it is still possible that Hoe 140 is a competitive antagonist because a competitive antagonist may produce a decrease in Smax and loss of parallelism to an indirect agonist [21].

In animal airways, Bk-induced responses involve the release of tachykinins [19 24 31]. In human peripheral airways, which contain many fewer tachykinin-containing nerves [24], no evidence for Bk-induced tachykinin release was found in a study by Molimard and coworkers [25], and this is confirmed by the present study. Tachykinins may also cause bronchoconstriction by stimulation of sensory nerve endings, resulting in a cholinergic reflex bronchoconstriction. The finding that in humans inhalation of a cholinergic antagonist before bronchoprovocation with Bk reduced the Bk-induced bronchoconstriction [15] supports such an indirect mechanism. Obviously, a central cholinergic reflex cannot be demonstrated in isolated airways.

We conclude that Bk contracts intact and epithelium-denuded human peripheral airways; precontracted airways are either relaxed or further contracted. The sensitivity to Bk increases after removal of epithelium, indicating a protective role of the epithelium against Bk-induced responses. The bronchoactive effects of

Bk are mediated through the B_2 receptor and involve the release of prostanoids that contract human airways via the TP receptor.
Bk may contribute to asthma by increasing microvascular leakage [29] and by inducing hypersecretion of mucus [1]. In addition, the present study showed that Bk also has a potent indirect contractile effect on human peripheral airways. We found that Bk is particularly effective in epithelium-denuded airways. Because epithelial damage is a characteristic of asthma and the Bk concentration is elevated in the bronchoalveolar fluid of asthmatic patients after allergen challenge [5], endogenous Bk may indeed contribute to bronchoconstriction in asthma.

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Chapter 10

Capsaicin induces Bronchoconstriction and Neurally-mediated Tachykinin Release in Human Isolated Airways

10.1 Introduction

The sensory neuropeptides or tachykinins substance P (SP) and neurokinin A (NKA) have been localized to nonmyelinated sensory nerve fibers (C-fibers) in human airways and may be the neurotransmitters of the excitatory non-adrenergic, non-cholinergic (eNANC) nervous system [2 17 20]. SP preferentially interacts with neurokinin$_1$ (NK_1) receptors and NKA with NK_2 receptors, both of which have been demonstrated in human airways [21].

Tachykinins induce airway smooth muscle contraction, mucus secretion, vascular hyperpermeability and dilation of tracheal and bronchial blood vessels, and stimulate and attract inflammatory cells in both animal and human airways [2 17 19 30]. Because of these effects, which are collectively known as 'neurogenic inflammation', tachykinins have been implicated in the pathophysiology of asthma [2 17 30]. Indeed, SP-immunoreactive nerves are more abundant in the airways from patients with asthma than in nonasthmatic subjects [25] and elevated SP concentrations have been found in the sputum and bronchoalveolar lavage fluid of patients with asthma [24 31].

Sensory nerve fibers can be activated by capsaicin, the pungent agent in chili peppers, and this may result in antidromic conduction with release of tachykinins [13 28 30]. This type of conduction of impulses without passing through a cell body is called an 'axon reflex' [30]. Although it has been hypothesized that axon reflexes with local release of tachykinins may play a role in asthma [3], this mechanism has not been demonstrated in human airways. In the present study, we investigated the effects of the nerve conductance blocker tetrodotoxin (TTX),

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the anti-asthmatic drug sodium cromoglycate (SCG) which may inhibit eNANC responses, and the selective neurokinin$_2$ (NK_2)-receptor antagonist SR48968 [9] on capsaicin-induced responses of human isolated airways. In addition, we measured the capsaicin-induced release of SP and NKA from human airways by radioimmunoassay, in the absence or presence of TTX, SCG and SR48968.

10.2 MATERIALS AND METHODS

Tissue preparation

Human lung tissue was obtained from 9 male and 2 female patients (mean age 63.8 y; range 43 to 76 y), who underwent thoracotomy for bronchial carcinoma. None of the patients had characteristics of asthma. The premedication was the same for all patients: atropine, thiopental, fentanyl, O_2/N_2O, halothane, and pancuronium. Immediately after surgical resection, a macroscopically normal part of the lung tissue was immersed in carbogenated (95% O_2 and 5% CO_2) Krebs-Henseleit buffer at 4°C (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 5.55). The tissue remained in cooled fresh carbogenated buffer throughout the dissection procedure. Cartilaginous bronchi with an inner diameter of 3 to 6 mm (generation 4 to 7) were carefully dissected and cut spirally to obtain a bronchial strip preparation. The tissues were studied at 37°C in double-jacketed 10 ml siliconized organ baths containing carbogenated Krebs-Henseleit buffer. Thin surgical silk threads (6/0) were tied to the ends of the strips. One thread was attached to a glass hook at the bottom of the organ bath and the other to the arm of a high-precision isotonic angular position transducer (Type 3810/60; Penny and Giles Ltd., Christchurch, UK), which was connected to a digital voltmeter (Multimeter 73; Fluke, Everett, WA, USA) and a pen recorder (BD40; Kipp, Delft, The Netherlands). The preparations contracted against a load of 500 mg. The study was approved by the Ethical Committee of the Rotterdam University Hospital.

Radioimmunoassay (RIA) of SP and NKA

The organ bath fluids (10 ml) were applied to reverse-phase C_{18} Sep-Pak cartridges (Waters Associates, Millford, MA, USA) and rinsed with 5 ml of distilled water. Tachykinins were extracted with 10 ml of 70% acetonitrile in 0.1% trifluoroacetic acid, lyophilized, and kept at -80°C until they were analyzed as a single batch. This protocol results in the recovery of 80-85% of SP and

NKA [22]. All the reagents were diluted in RIA buffer (0.1% BSA, 0.1% Triton X-100, 0.01% thimerosal and 19 mM monobasic and 81 mM dibasic sodium phosphate, pH=7.4). Lyophilized samples were reconstituted and standards SP ranging from 1 to 1000 pg per tube and standards NKA ranging from 1 to 128 pg per tube were prepared. Samples and standards 100 µl were pipetted in individual tubes and 100 µl of rabbit anti-SP or anti-NKA was added. After an overnight incubation at 4°C, 0.01 µCi ^{125}I-SP or ^{125}I-NKA (10,000 to 15,000 cpm) was added to each tube. After a second overnight incubation at 4°C, 100 µl of goat anti-rabbit γ-globulin and 100 µl of nonimmune rabbit serum were added for 2 hours at room temperature. Thereafter, the antigen-antibody complex was precipitated with 0.5 ml of 20% polyethylene glycol (MW 6000) and centrifuged at 1700 *g* for 20 min at 4 °C. The supernatant was decanted and the radioactivity in the pellet was measured in count.min^{-1} using a gamma counter (Packard Minaxi Autogamma 5000, Packard Instrument Company, Downers Grove, IL, USA).

The detection limit of the SP- and the NKA assay was approximately 2 pg and 1 pg per tube, respectively. The cross-reactivity of the SP antiserum with SP [3-11], SP [4-11], SP [5-11], neuropeptide K, neurokinin A and neurokinin B was 100, 100, 100, <0.01, <0.01 and 0%, respectively. The cross-reactivity of the NKA antiserum with neurokinin B, SP, physalaemin and neuropeptide Y was 80, 0.05, 0.02, and 0%, respectively. The RIA was not influenced by phosphoramidon and captopril. The amounts of SP and NKA in airway tissue are expressed as fmol/g wet weight.

Drugs

Methacholine bromide (Janssen Pharmaceutica, Beerse, Belgium), EDTA (Sigma Chemical Co., St. Louis, MO, USA), and the peptidase inhibitors phosphoramidon (Sigma) and captopril (Squibb & Sons, Inc., Princeton, NJ, USA), were dissolved in 0.9% NaCl. TTX (Sigma) and sodium cromoglycate (Sigma) were dissolved in distilled water. SR48968 (gift from Dr. X. Emonds-Alt, Sanofi Recherche, Montpellier, France) was dissolved in ethanol. L-isoproterenol sulfate (Janssen) was dissolved in distilled water containing ascorbic acid (88 mg/l). ^{125}I-SP and anti-SP rabbit serum were obtained from Amersham International plc, Amersham, Buckinghamshire, UK and standard SP from Bachem Pharmaceuticals, Torrance, CA, USA. ^{125}I-NKA, anti-NKA, standard NKA, normal rabbit serum and goat anti-rabbit γ-globulin antibodies were purchased from Peninsula Laboratories Inc., Belmont, CA, USA.

10.3 EXPERIMENTAL PROTOCOLS

Before the experiments, the preparations were contracted twice with methacholine (10^{-5} and 10^{-4}M) to assess contractile function and to ensure a stable function for the rest of the day[4]. After washout, all preparations were incubated with the neutral endopeptidase (NEP) inhibitor phosphoramidon (10^{-5}M) and the angiotensin converting enzyme (ACE) inhibitor captopril (10^{-5}M) to prevent degradation of potentially released tachykinins[5]. Then, airways were pretreated with either TTX (10^{-5}M), SCG (10^{-5}M), SR48968 (10^{-7}M), or vehicle. After 20 min of incubation, a cumulative concentration response curve (CCRC) to capsaicin (10^{-9} to 10^{-5}M, by logarithmic increments) was made. During each experiment, one airway served as a control that enabled us to determine baseline SP release without stimulation with capsaicin. After making the CCRC to capsaicin, the organ bath fluid was collected for the measurement of SP and NKA, released by the airway preparations. Finally, the airways were washed and fully relaxed by adding L-isoproterenol (10^{-4}M) and ethylene-diaminetetraacetic acid (EDTA; $4x10^{-3}$M) and wet tissue weight was determined.

Data analysis

The contractile responses to capsaicin are expressed as a percentage of the maximal contraction to methacholine 10^{-4}M. CCRCs for capsaicin-induced bronchoconstriction in the various treatment groups were compared with non-parametric ANOVA (Kruskal-Wallis test). The concentrations of SP and NKA in the organ bath fluid of the various treatment groups were compared using the Mann-Whitney *U*-test. All data are expressed as mean ± standard error of the mean (SEM) and values of $p < 0.05$ were considered significant.

10.4 RESULTS

Capsaicin (10^{-9} to 10^{-5}M) induced a concentration-dependent contraction in 29 of 34 airway preparations. The contractile state of three airways was not changed by capsaicin and two airways relaxed to capsaicin (both had been pretreated with SR48968).

Maximal contraction (Smax) to 10^{-5}M capsaicin was 9.4 ± 3.0% of the maximal contraction to methacholine 10^{-4}M (figure 1, n=7). TTX (10^{-5}M) and SCG (10^{-5}M) did not affect capsaicin-induced bronchoconstriction (Smax: 9.2 ± 3.3

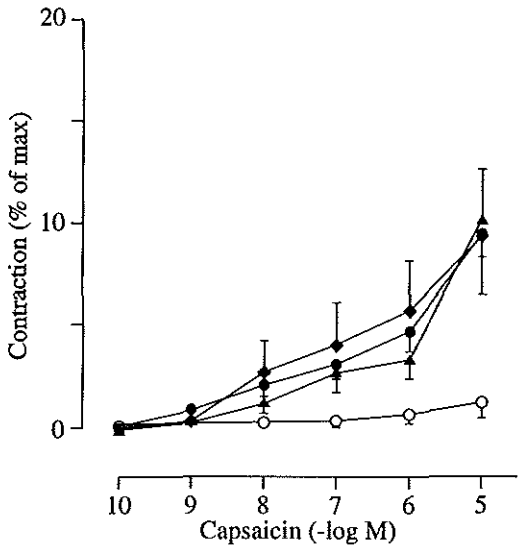

Figure 1. Mean CCRC to capsaicin of human airways (n=7) in the presence of vehicle (closed circles), in the presence of 10^{-5}M tetrodotoxin (closed squares) and in the presence of 10^{-5}M sodium cromoglycate (closed triangles). Open circles represent control airways not exposed to capsaicin. Capsaicin-induced responses are expressed as a percentage of maximal contraction to methacholine (10^{-4}M).

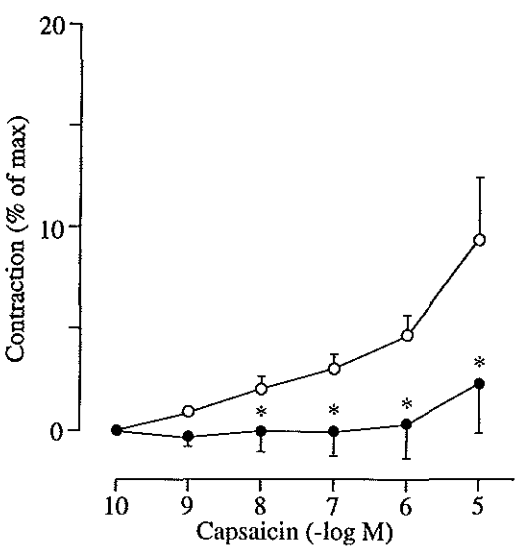

Figure 2. Mean CCRC to capsaicin of human airways (n=6) in the absence (open circles) or presence (closed circles) of the NK_2 receptor antagonist SR48968. Capsaicin-induced responses are expressed as a percentage of maximal contraction to methacholine (10^{-4}M). * $p<0.05$.

and 10.2 ± 1.9% of the maximal contraction to methacholine, respectively, figure 1, n=7). SR48968 significantly inhibited capsaicin-induced contraction; Smax was only 2.6 ± 2.5% of maximal contraction to methacholine (figure 2, n=6). The tone of control airways did not change during the experiment (figure 1). The amounts of SP and NKA released by the airway preparations are shown in figure 3. Capsaicin-induced release of both SP and NKA was significantly decreased by TTX, but not by SCG or SR48968. In the organ bath fluid of control airways, small amounts of SP could be detected, but no NKA.

10.5 DISCUSSION

In the present study we found that capsaicin caused concentration-dependent contractions and release of SP and NKA in human isolated airways. Capsaicin-induced bronchoconstriction could be inhibited by the selective NK_2-receptor antagonist SR48968, but not by TTX or SCG. Capsaicin-induced release of tachykinins could be suppressed by TTX, but not by SCG or SR48968.
Lundberg and colleagues [19] were the first to describe capsaicin-induced contractions in human airways and suggested that the contractions were caused by capsaicin-induced release of SP. In a later study, capsaicin contracted airway smooth muscle contraction in only half of the human airways tested [5]. Addition of the NEP inhibitor phosphoramidon, however, resulted in capsaicin-induced contraction of all airway preparations, suggesting that contractile effects of endogenous tachykinins were modulated by NEP [5]. In the present study, both phosphoramidon and the ACE-inhibitor captopril were present in the organ baths to prevent degradation of endogenously released tachykinins. Captopril was added because ACE degrades SP and this enzyme has been localized on endothelium of blood vessels in human airways [14]. In our experiments, most airway preparation contracted to capsaicin although maximal responses were small and variable. In addition, capsaicin-induced contractions were associated with an increase in tachykinin release by these airways, and this suggests activation of C-fibers by capsaicin, with release of tachykinins.
We found that the capsaicin-induced release of tachykinins could by inhibited by the nerve conductance blocker TTX. Capsaicin binds to a specific membrane receptor and causes depolarization of sensory nerve endings with release of tachykinins [8], a process which cannot be inhibited by TTX [27]. However, capsaicin may also cause antidromic impulse conduction to branches of the axon. This

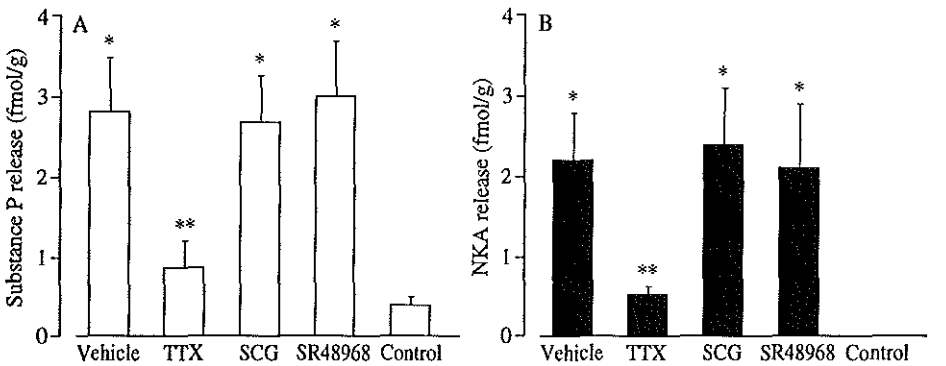

Figure 3. Capsaicin-induced release of the tachykinins substance P (A) and neurokinin A (B) by human airways incubated with vehicle, tetrodotoxin (TTX), sodium cromoglycate (SCG), or SR48968. Control columns represent airways not exposed to capsaicin. * $p<0.05$ compared with control airways, ** $p<0.05$ compared with airways pretreated with vehicle, SCG, or SR48968.

'axon reflex' is TTX-sensitive [18]. Because TTX inhibited capsaicin-induced release of tachykinins, our experiments suggest the generation of an axon reflex by capsaicin in human airways. In the guinea pig, evidence for axon reflex bronchoconstriction via afferent C-fibers was also found both in vitro and in vivo [12 34]. In a recent study of asthmatic patients, bradykinin-induced bronchoconstriction could be inhibited by the tachykinin receptor antagonist FK-224, suggesting local tachykinin release [11]. In the present study, capsaicin-induced bronchoconstriction could not be inhibited by TTX. We cannot explain this finding. Perhaps, the concentration of SP and NKA near the smooth muscle was too low to cause significant bronchoconstriction in the absence of TTX.

Sodium cromoglycate (SCG) has been shown to suppress bradykinin- and capsaicin-induced excitation of C-fibers in dogs [6]. In vivo studies in asthmatic patients showed that inhalation of SCG protected against bradykinin-induced bronchoconstriction suggesting that SCG prevents activation of C-fibers by bradykinin [10 32]. In rodent isolated tracheas, however, bradykinin-induced release of substance P and eNANC neural bronchoconstrictor responses to electrical field stimulation (EFS) were not or only partially inhibited by SCG [26 32]. In the present study we could not demonstrate an inhibitory effect of SCG effect on capsaicin-induced bronchoconstriction and release of tachykinins in human isolated airways. It is possible, however, that a higher concentration of SCG inhibits capsaicin-induced release of tachykinins because in the guinea pig

trachea 10^{-4} M of SCG decreased eNANC bronchoconstrictor responses by 9% [32]. It can be questioned, however, whether such concentrations are relevant to treatment in vivo.

In the guinea pig trachea, capsaicin or EFS-induced eNANC bronchoconstriction is mediated via the NK_2-receptor [29]. Tachykinin-induced contraction of the rat trachea, on the other hand, is caused by NK_1-receptor activation [15]. In the present study, we found that the selective nonpeptide NK_2-receptor antagonist SR48968 abolished capsaicin-induced bronchoconstriction. Previous studies of human airways using selective agonists and SR48968 have also shown that tachykinin-induced contractions are mediated by NK_2-receptor activation [1 23]. Moreover, although SP preferentially binds to the NK_1-receptor, SP-induced contraction of human isolated airways was also mediated by the NK_2-receptor [1]. These functional studies are supported by radioligand binding studies with ^{123}I-SP which localized NK_1-receptors to microvascular endothelium but not to airway smooth muscle of human airways [33]. Thus, although in our experiments capsaicin released larger amounts of SP than NKA, bronchoconstriction could mainly be due to NK_2-receptor activation only.

In conclusion, we found that capsaicin causes constriction of human airways and releases SP and NKA from these airways. The nerve conductance blocker TTX inhibited capsaicin-induced release of tachykinins suggesting that axon reflexes are involved. In vivo, these axon reflexes may not only cause bronchoconstriction, but also mucus secretion, vasodilation, vascular hyperpermeability and stimulation and chemotaxis of inflammatory cells, effects which are collectively known as neurogenic inflammation. Recently, the relevance of neurogenic inflammation for human airway disease has been questioned because of lack of evidence that axon reflexes leading to a release of tachykinins, actually takes place in human airways [16]. The findings in the present study suggests that release of neuropeptides via axon reflex mechanisms takes place in human bronchi, but the functional relevance of this release remains to be elucidated.

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Part 4

General Discussion and Summary

Chapter 11

General Discussion and Directions for Future Research

11.1 Introduction

As discussed in chapters 1 and 2, the level of airway responsiveness in asthmatic patients is related to the extent of epithelial damage in these patients. However, the relationship between epithelial damage and airway responsiveness is not clear. Several mechanisms have been proposed including reduced production of epithelium-derived relaxing factors, decreased metabolization of contractile agonists by epithelial peptidases, increased airway permeability providing easy access of bronchoactive mediators and exposure of intraepithelial sensory (peptidergic) nerves with reflex bronchoconstriction. The objectives of our studies were:

1. To assess the modulatory role of the airway epithelium on airway responsiveness, and to elucidate the mechanism of this epithelial modulation.
2. To investigate the presence and functional importance of peptidergic nerves and axon reflexes in human isolated airways.

This chapter encomprisis a critical evaluation of our methodology and findings.

11.2 Limitation of the Applied Methods

Modulation of airway responsiveness by the epithelium has been reported by many authors in many species, including man. In 1991, Busk and Vanhoutte reviewed the literature on epithelium-dependent responses of isolated airways and they cited 26 studies on the effects of contractile and 23 studies on the effects of relaxing agonists [11]. In most of these studies the differences in responsiveness between intact airways and airways without epithelium were too small to explain the airway increased responsiveness found in asthmatic patients. This may lead to the conclusion that the epithelium does not play an important modu-

latory role in the responsiveness of airway smooth muscle. Or, even more disappointing, that the results of many of these studies may be explained by bias because in vitro studies are not performed blinded [20]. The methodology used in most of the in vitro studies reviewed by Busk and Vanhoutte requires a critical reevaluation. Most authors used airway strip or ring preparations for their studies. The modulatory role of the epithelium was assessed by comparing intact airway preparations with preparations from which the epithelium had been mechanically removed. As explained in chapters 4, 5 and 6 of this thesis this method has important shortcomings: In these preparations a stimulus cannot be selectively applied to the mucosal side of the airway preparation. Because the stimulus can reach the airway smooth muscle via the serosal side and the cutting edges of the preparation, the modulatory role of the epithelium may be underestimated when airway strips or rings are used. Furthermore, mechanical rubbing used by all authors to denude airways from their epithelium may have side effects: Franconi and coworkers reported that mechanical rubbing of the epithelium may release proteases from mast cells present in the airway preparation [25]. Since mast cell proteases may increase airway responsiveness [90], it is possible that the hyperresponsiveness of epithelium-denuded airways is partly caused by the release of proteases during mechanical rubbing.

Recently, the shortcomings of the conventional airway strip and ring preparations were partly solved by the use of perfused airway tubes. These preparations provide the opportunity to apply stimuli selectively to the mucosal side of the airway preparation [37]. Large differences between serosal and mucosal stimulation with contractile and relaxing agonists have been reported in rodent [68 80], bovine [66 76], and human [38 75] airways. Thus, the experiments performed with perfused airways indeed suggest that the airway epithelium modulates airway responsiveness.

Although the perfused airway tube preparation offers the opportunity to study the modulatory role of the epithelium this model is still imperfect: The subepithelial or mucosal microvascular plexus is not perfused. This plexus, interspersed between the epithelium and the airway smooth muscle, receives a large amount of the bronchial blood flow [21 51 103]. Inhaled allergens and bronchoactive mediators and cytokines produced by the airway epithelium may be washed away and metabolized by this bloodstream before they reach the airway smooth muscle. In addition, many of the mediators and cytokines produced by the epithelium cause vasodilation and increase microvascular permeability which may contribute to bronchial obstruction in asthma [19 34 84]. Therefore, products released by

the airway epithelium may have a more important modulatory role on the tracheobronchial circulation than on airway smooth muscle responsiveness. However, this modulatory role cannot be assessed with the currently available in vitro models.

11.3 EPITHELIUM AS A DIFFUSION BARRIER

In chapters 1 and 2 of this thesis it was explained that impairment of barrier function may be an important factor causing airway hyperresponsiveness after epithelial damage: In animal studies a strong correlation between airway responsiveness and airway permeability was found both in vitro and in vivo [7 76 105]. In addition, several authors reported that the extent of epithelial damage in bronchial biopsies from asthmatic patients correlated with the level of airway responsiveness in these patients [42 74].

Airway responsiveness in vivo is usually assessed by bronchoprovocation with aerolized histamine or methacholine. After inhalation these drugs must pass the airway epithelium to reach the airway smooth muscle. In the experiments described in chapter 7 of this thesis we found that the airway epithelium forms a barrier against penetration of hydrophilic drugs. Since both histamine and methacholine are hydrophilic drugs, it is easy to understand that epithelial damage may lead to hyperresponsiveness to these drugs because the airway smooth muscle can be reached more easily. Indeed, the experiments described in chapters 6 and 7 show that epithelial damage produced by the oxidant hydrogen peroxide (H_2O_2) or the cationic protein poly-L-arginine (which is similar to MBP produced by eosinophils in asthmatic airways) resulted in an increase of both airway responsiveness and permeability to hydrophilic drugs. We also found that both airway responsiveness (chapter 6, figure 3) and airway permeability (chapter 7, figure 4) increased with decreasing airway size. These findings support a causal relationship between airway responsiveness and airway permeability.

Airway hyperresponsiveness induced by viral infections and allergens may also be partly explained by a change in barrier function of the epithelium: During acute viral respiratory tract infections, airway epithelial cells become necrotic. This will not only obstruct the airways by cellular debris and edema but also increase airway permeability [31]. Similarly, the proteolytic activity of the allergen Der p1 of the house dust mite may cause epithelial damage and increase airway permeability [33].

There are, however, arguments against epithelial damage as the main mechanism of bronchial hyperresponsiveness: In some studies of bronchial biopsies from asthmatic patients no structural damage to the airway epithelium was found although the patients were hyperresponsive to inhaled methacholine [50 54]. Similarly, exposure of laboratory animals to oxidants and cationic proteins caused airway hyperresponsiveness without apparent epithelial damage [16 98]. In this study, airway hyperresponsiveness was probably produced by the release of bronchoactive mediators such as prostanoids and bradykinin [16 83]. Furthermore, airway hyperresponsiveness after physical exercise or hyperventilation cannot easily be explained by a change in barrier function. Instead, hyperosmolarity due to evaporation of mucosal surface water and bronchial vasodilation and hyperemia of the tracheobronchial circulation during rewarming may play a role [63 64]. It can be hypothesized, however, that intact airway epithelium protects against exercise-induced asthma by preventing evaporation and cooling of the airways. Finally, the function of the epithelial barrier is not only to restrict access of bronchoactive substances or allergens from outside but also to prevent leakage of exudate/transudate from the tissues into the airways. Indeed, epithelial damage may lead to plasma exudation and this may contribute to airway hyperresponsiveness [81 82]. Thus, our studies show that epithelial damage increases both airway permeability and airway responsiveness. However, it may well be that other factors add to airway hyperresponsiveness in asthmatic patients.

11.4 EPITHELIAL DEGRADATION OF NEUROTRANSMITTERS AND CHEMICALS

As discussed in chapter 2 the membrane-bound peptidases neutral endopeptidase (NEP) and angiotensin converting enzyme (ACE), have been demonstrated on human airway epithelium [5 44 52]. Both NEP and ACE degrade a large variety of peptides including the tachykinins neurokinin A (NKA) and substance P (SP), kinins such as bradykinin (Bk), and the putative iNANC neurotransmitter vasoactive intestinal peptide (VIP) [6 69]. Epithelial damage or loss or inhibition of these peptidases may not only cause hyperresponsiveness of human airways to the abovementioned peptides [5 13 94] but also enhance 'neurogenic inflammation' [6 69].

In chapters 8 and 9 of this thesis, we described the effects of epithelium removal and inhibition of membrane-bound peptidases on airway responsiveness to Bk and VIP. Although peptidase inhibition increased airway responsiveness to both

peptides, removal of the epithelium produced a larger effect. This indicates that apart from epithelial peptidases other factors protect against an increase in airway responsiveness to peptide agonists, for instance an epithelial barrier that impedes the diffusion of Bk and VIP. Alternatively, other epithelial enzymes which were not inhibited in our experiments may be involved in degrading Bk and VIP. In rat lung microsomes, for instance, the enzymes aminopeptidase P and dipeptidylaminopeptidase IV have been demonstrated and these enzymes compete with angiotensin converting enzyme (ACE) in the degradation of Bk[77]. The matter is further complicated by the recent demonstration that in rat lungs chronic inhibition of either ACE or neutral endopeptidase (NEP) may result in induction of both enzymes [32]. These processes, however, may require several days and may, therefore, not confound our studies of isolated airways.
We also found that peptidase inhibitors increased airway responsiveness to both the contracting peptide Bk and the relaxing peptide VIP in epithelium-denuded airways. This suggests that these peptides are also degraded by non-epithelial peptidases. As discussed in chapter 9, these non-epithelial peptidases may be produced by mast cells, T-cells and macrophages, or they may be present on the endothelium or airway smooth muscle itself[6 89]. In vivo, asthmatic patients are hyperresponsive to inhaled tachykinins [14 17 45] although no reduction of NEP activity was found [14]. Considering our findings in vitro it can be hypothesized that epithelial damage may be a major factor causing hyperresponsiveness to tachykinins in asthmatic patients. The effect of NEP-inhibitors in vivo may be explained by sufficient residual epithelial and nonepithelial sources of NEP in these patients. Thus although epithelial peptidases modulate airway responsiveness to inhaled and, probably, endogenous peptides, airway responsiveness in asthmatic patients cannot be simply explained by a deficiency of epithelial peptidases.

11.5 GENERATION OF RELAXING SUBSTANCES BY THE EPITHELIUM

The discovery of endothelium-derived relaxing factor (EDRF, now identified as nitric oxide (NO)[67]) in the vasculature led to the hypothesis that a similar factor might be produced by the airway epithelium. In asthma, epithelium-derived relaxing factor (EpDRF) would not be produced in sufficient amounts to keep the airway smooth muscle in a relaxed state. Several putative candidates for EpDRF have been suggested including prostanoids[23 97] and, more recently, NO[61 71].

In the experiments described in chapter 9 we found that bradykinin-induced contractions of human airways were mediated via the release of prostanoids from human airways. After removal of the epithelium even greater amounts of prostanoids were produced by a nonepithelial source. In addition, we found that although greater amounts of the bronchodilatory prostanoids PGI_2 and PGE_2 were produced, human airway smooth muscle was contracted by a predominant effect of smaller amounts of thromboxane (TxA_2). A similar pattern of prostanoid release has been reported in hydrogen peroxide-induced bronchoconstriction of human airways [83]. Because of this predominant effect of TxA_2 and the production of prostanoids by nonepithelial sources, it is not likely that in human airways epithelium-derived prostanoids will act as EpDRF.

Recently, it has been reported that NO synthesis inhibitors increased histamine-induced constriction of the guinea pig trachea [61 71]. In epithelium-denuded airways, histamine-induced bronchoconstriction could not be enhanced by NO synthesis inhibitors suggesting that epithelium-derived NO relaxed the airway smooth muscle in this preparation. These in vitro studies, however, do not prove that epithelial NO is EpDRF: Firstly, NO synthesis inhibitors not only inhibit the production of nitric oxide but also the release of prostanoids [88]. Because in the guinea pig lung, prostanoids released by the epithelium relax the airway smooth muscle [8 97], experiments with NO synthesis inhibitors may be confounded by an effect of epithelial prostanoids. Secondly, it should be remembered that in blood vessels, the NO-producing endothelial cells directly face the vascular smooth muscle. NO will reach the vascular smooth muscle cells easily despite its short half life. In airways, the epithelial cells do not face the airway smooth muscle because the subepithelial vascular plexus is between the epithelial cells and airway smooth muscle. In vivo, NO produced by airway epithelial cells has to travel a large distance to reach airway smooth muscle, and, moreover, it has to pass a dense vascular plexus with a large blood flow [21 79 103]. Because NO eagerly binds to hemoglobin [67], and NO relaxes airway smooth muscle only at high concentrations [107], it is not likely that in vivo epithelial NO will reach airway smooth muscle in sufficient amounts to produce airway smooth muscle relaxation. Indeed, in both animals and humans inhaled NO has a potent dilatory effect on pulmonary vessels, whereas only a slight bronchodilating effect is found [22 35].

11.6 Modulation of Airway Responsiveness by Peptidergic Nerves

The sensory neuropeptides or tachykinins substance P (SP) and neurokinin A (NKA) have been localized to unmyelinated sensory nerve fibers (C-fibers or peptidergic nerves) in animal and human airways and may be the neurotransmitters of the excitatory non-cholinergic, non-adrenergic nervous system [2 46 56-59]. Excitation of these nerve fibers by chemical and physical stimuli may produce retrograde (antidromic) impulses that results in local release of tachykinins [3 40 53 87 91]. In rodent airways, tachykinins induce airway smooth muscle contraction, mucus secretion, vascular hyperpermeability and dilation of tracheal and bronchial blood vessels, and stimulate and attract inflammatory cells [2 40 53 59 87 91]. Because of these effects, which are collectively known as 'neurogenic inflammation' [41], tachykinins have been implicated in the pathophysiology of asthma [2 46 91]. Indeed, in the guinea pig endogenous release of tachykinins contribute to chemical- (Bk, histamine, capsaicin, toluene diisocyanate, and cigarette smoke) and allergen-induced hyperresponsiveness [4 36 40 53 87].

In the human lung, exogenous tachykinins cause bronchoconstriction [13 17 45 55], mucus hypersecretion [85], plasma extravasation [9], influx of inflammatory cells [9] and hyperresponsiveness to inhaled methacholine [15]. Compared to rodent airways, human airways contain less tachykinin-containing nerve fibers [56-59] and less tachykinin receptors [12 101]. Recently, however, an increased SP-immunoreactivity in bronchoalveolar fluid and sputum has been reported in asthmatic airways [70 95]. Furthermore, inhalation of a tachykinin receptor antagonist partly prevented Bk-induced bronchoconstriction [39]. Although these studies suggest that endogenous tachykinins are involved, they do not prove that in human airways these tachykinins are released by peptidergic nerves. In the experiments described in chapters 9 and 10, we investigated the effect of Bk and capsaicin on human isolated airways. In contrast to the experiments in guinea pig and dog airways [40 48], we could not demonstrate excitation of peptidergic nerves by Bk. Instead, in human airways Bk-induced bronchoconstriction appeared to be mediated through the release of prostaglandins.

In the experiments described in chapter 10, we used capsaicin to stimulate C-fibers in human isolated airways. We found that capsaicin caused small contractile responses in human isolated airways. This contrasts with the much larger effect of capsaicin in the guinea pig trachea [40 59 87]. However, the guinea pig may be the only species in which a potent capsaicin-induced bronchoconstriction can be found. In the rat trachea, for example, capsaicin induces small contractile

responses, similar to the contractions that we found in human airways [47]. The small contractile effect of capsaicin in human isolated airways also contrasts with the potent bronchoconstrictive effect of inhaled capsaicin in asthmatic patients [26 65]. This indicates that apart from local axon reflexes other bronchoconstrictive mechanisms are induced by capsaicin. Since peptidergic nerves have been demonstrated in the vicinity of vagal nerve fibers it is possible that tachykinins released by capsaicin elicit a cholinergic reflex bronchoconstriction in asthmatic subjects [26 65].

We found that capsaicin evokes the release of the tachykinins substance P (SP) and neurokinin A (NKA) from human airways. The release of the tachykinins could be inhibited by TTX, indicating that local axon reflexes may be involved. Although in our experiments with human airways these axon reflexes did not produce important bronchoconstriction they may produce other features of 'neurogenic inflammation' such as microvascular leakage, mucus hypersecretion, tracheobronchial vasodilatation, aspecific bronchial hyperresponsiveness, and attraction and activation of inflammatory cells. Both microvascular leakage and tracheobronchial vasodilatation may increase airway wall thickness which may contribute to airway obstruction [19 34 81 84 99]. In addition, the neurogenic inflammatory process may increase airflow obstruction by inducing the production of histamine by mast cells [30] and the proliferation of airway smooth muscle cells [73].

11.7 CONCLUSIONS

The results from the experiments described in this thesis show that in human airways:

1. The airway epithelium modulates airway responsiveness. Also, airway responsiveness and airway permeability increase with decreasing airway size and epithelial damage causes an increase of both airway responsiveness and permeability. Therefore, airway permeability may be an important factor determining airway responsiveness.
2. In human airways removal of the epithelium has a greater effect on airway responsiveness than inhibition of epithelial peptidases. In addition, peptidases are not only present on the epithelium but also on other tissues. Therefore, epithelial peptidases probably do not play a key role in the modulation of airway responsiveness.

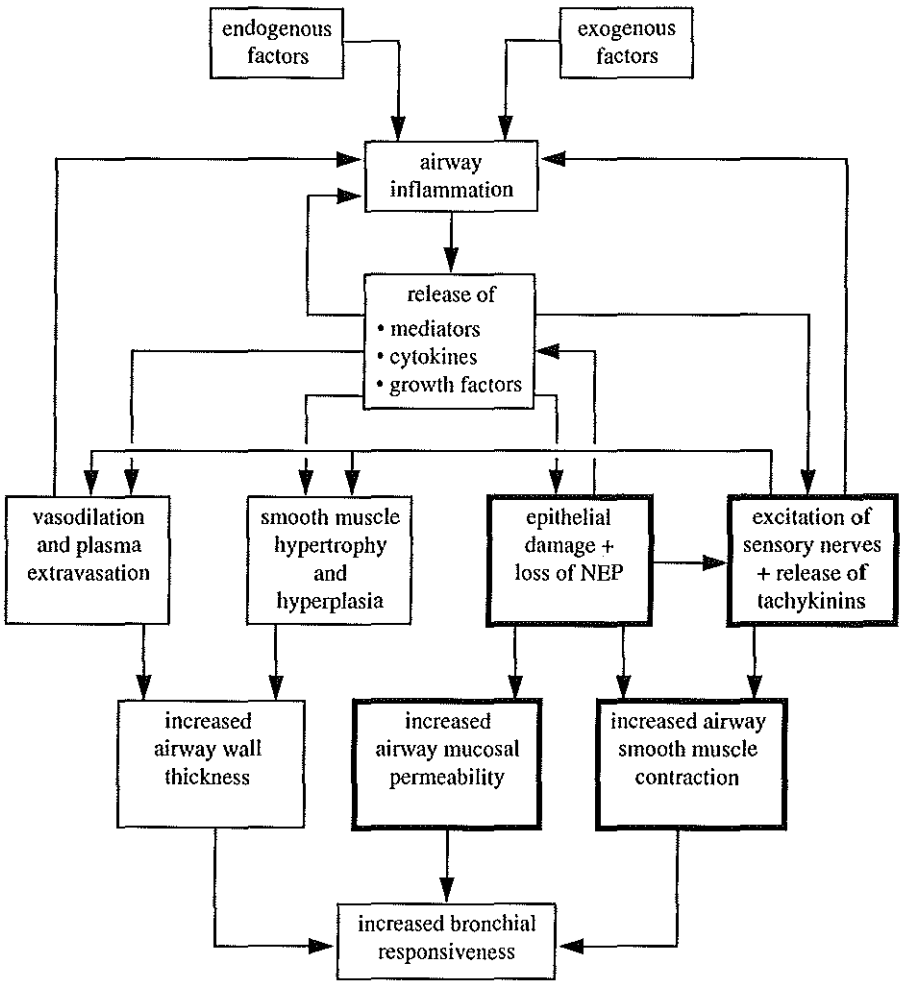

Figure 1. Schematic representation of several aspects of bronchial responsiveness. The accentuated boxes refer to the contribution of the experiments described in this thesis.

3. Excitation of sensory nerves causes a small contractile response and release of tachykinins in human airways. The release of tachykinins could be inhibited with the nerve conductance blocker TTX indicating that local axon reflexes are involved.

   The results of the studies described in this thesis are embedded in figure 1 which shows a concept of the mechanisms that may be relevant for the pathogenesis of bronchial hyperresponsiveness in asthma.

11.8 DIRECTIONS FOR FUTURE RESEARCH

Classically, airway smooth muscle contraction has been regarded as the primary cause of airflow limitation in patients with asthma. Smooth muscle contraction was thought to be caused by increase in smooth muscle responsiveness and/or abnormalities in the autonomic control of the airways. Later, it appeared that inflammatory cells in the airway wall play a key role in the pathophysiology of asthma. Mediators and cytokines released by these inflammatory cells cause epithelial shedding, smooth muscle contraction, enhanced mucus production, and increased vascular permeability and edema, processes which contribute to bronchial obstruction in asthma.

Recently, it was recognized that the tracheobronchial circulation may play an important role in the modulation of airway responsiveness. It is involved in the conditioning of inspired air, and the clearance and distribution of inflammatory mediators and migratory cells [21 100 103]. In addition, vasodilation and plasma leakage in the bronchial mucosa of asthmatic patients may increase airway wall thickness which may directly contribute to airway narrowing [21 34 81]. Despite many animal studies of the physiology of the tracheobronchial circulation, the physiology of the tracheobronchial circulation in humans and its contribution to the pathophysiology of asthma is poorly understood. Furthermore, the role of the tracheobronchial circulation in the absorption, distribution and delivery of inhaled or infused antiasthmatic drugs remains to be established. To investigate these questions, a ventilated and perfused human lung preparation should be developed. Isolated lung models have been used in laboratory animals to study lung mechanics [49], hypoxic vasoconstriction in the lung [43], pulmonary capillary transit time [1], uptake and metabolism of drugs and mediators by the lung [10], and the effects of infused inflammatory cells [86]. Such a model would enable us to study the effects of infused mediators and cytokines and perhaps even inflammatory cells derived from asthmatic patients, on lung function and airway responsiveness of lungs from nonasthmatic subjects. Release of mediators and cytokines could be measured in the effluent. Because central cholinergic reflexes are eradicated in such a model, local axon reflexes (via peptidergic nerves) in the airways and axon reflexes between airways and the pulmonary vasculature could be studied. Furthermore, the release of epithelium-derived relaxing factors, their uptake and clearance by the airway vasculature and their effect on airway smooth muscle tone could be studied in this model. Although human whole lungs are rarely available, the use of lung lobes may enable the development of

a ventilated, perfused human lung model. If possible, branches of the bronchial artery should also be cannulated and perfused with whole blood or synthetic oxygen carriers such as fluorocarbons [29] because K-H solution may not contain enough oxygen to supply the tissues.

More recently, there is a renewed interest in the role of airway smooth muscle in the pathophysiology of asthma: In the vasculature, hyperplasia and hypertrophy are prominent responses after mechanical injury. This process called 'remodelling' is regulated by a variety of growth factors, mediators, and cytokines derived from fibroblasts, platelets, endothelial cells, and smooth muscle cells themselves [27]. It has been hypothesized that similar remodelling may play a role in the repair phase of airway mucosal damage in asthma [92]. Airway smooth muscle hyperplasia and hypertrophy will increase airway wall thickness which may contribute to airway hyperresponsiveness in asthma [104]. Meanwhile, it has been shown that histamine, bradykinin, endothelin, thromboxane, leukotrienes and substance P stimulate airway smooth muscle proliferation in vitro [28 72 73 78]. Glucocorticoids, ß-adrenergic receptor agonists, and nitric oxide (NO), on the other hand, may inhibit proliferation of isolated airway smooth muscle [24 93 96 106]. Interestingly, levels of the abovementioned mediators are increased in the bronchoalveolar lavage fluid of asthmatics and decrease during treatment with glucocorticoids and ß-agonists [18 62 70 102]. At present, however, it is not clear whether airway smooth muscle hyperplasia and hypertrophia actually occur in asthma. In addition, the cells responsible for the production of growth factors have not been identified. Although the damaged epithelium may play a central role in airway remodelling, other cells, for instance inflammatory cells, may be involved. Furthermore, it can be hypothesized that non-adrenergic, non-cholinergic (NANC) neural mechanisms are involved: Proliferation of airway smooth muscle is stimulated by the eNANC transmitter substance P [73], and inhibited by the iNANC neurotransmitters NO and VIP [60 93]. If so, treatment with inhaled glucocorticoids early in the course of the disease may prevent the process of airway remodelling. Further research into the determinants and effects of airway remodelling may well provide important clues for understanding bronchial hyperresponsiveness, and give new insight that may lead to alternative treatment strategies.

In the past decades the fields of epidemiology, pathology, physiology and pharmacology advanced our knowledge of the pathophysiology of asthma. More recently, molecular biology was introduced and this branch of science provides

a promising future for asthma research. Although molecular biology may indeed contribute to our knowledge, one should recognize that asthma (or bronchial hyperresponsiveness) is a functional abnormality of the airways which has a heterogeneous etiology. Functional studies both in the laboratory and in clinical practice remain indispensable for putting the pieces together in the puzzle called ασθμα.

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Chapter 12

Summary; Samenvatting

12.1 Summary

In **chapter 1** asthma is defined as a chronic inflammatory disorder of the airways with widespread, variable airflow obstruction associated with an increase in airway responsiveness to a variety of stimuli. The clinical symptoms of asthma are recurrent exacerbations of cough, dyspnea, wheezing, and chest tightness, often provoked by exogenous factors. Histological examination of asthmatic airways shows damage or loss of the epithelium which may be caused by the release of oxidants and cationic proteins by inflammatory cells present in the airway wall. Although the extent of epithelial damage is related to the level of airway responsiveness, the relationship between epithelial damage and airway responsiveness is not clear. Several mechanisms have been proposed including reduced production of epithelium-derived relaxing factors, decreased metabolization of contractile agonists by epithelial peptidases, increased airway permeability providing easy access of bronchoactive mediators and exposure of intra-epithelial sensory (peptidergic) nerves with reflex bronchoconstriction. The aim of our studies was to investigate the modulatory role of the epithelium on the responsiveness of human isolated airways. We assessed the effect of epithelial damage produced by oxidants and cationic proteins on airway responsiveness and airway permeability. In addition, we assessed the role of epithelial peptidases in airway responsiveness to contracting and relaxing peptides. Finally, we tried to demonstrate a retrograde local axon reflex mechanism via sensory (peptidergic) nerves (the so-called 'axon reflex') in human isolated airways.

Chapter 2 describes the structure, physiology, and pathophysiology of the airway mucosa in asthma. Human airway epithelium consists of ciliated, secretory cells and basal cells closely attached to each other and to the basement membrane. The epithelium forms a barrier against leakage of solutes and penetration of inhaled material into the airways. Both, membrane-bound and cystolic enzymes (peptidases) degrade bronchoactive mediators and peptides and intra- and extracellular antioxidant systems inactivate reactive oxygen species. Human airway epithelial cells produce biologically active compounds such as arachidonic

acid metabolites, nitric oxide and cytokines that may modulate the function of neighbouring cells and tissues. The chronic inflammatory process in asthmatic airways leads to epithelial injury which is associated with an increase in airway responsiveness. The relationship between epithelial damage and airway hyperresponsiveness is not clear. The basement membrane consists of collagen and glycoproteins. It provides support to the epithelial cells and may constitute a barrier to macromolecules and noninflammatory cells. In asthma, the basement membrane zone is thickened by deposition of collagen III and V probably produced by myofibroblasts. The lamina propria contains a dense vascular plexus that consumes a considerable part of the total airway blood flow. The lamina propria provides nutrients to the epithelium and may have an important role in the conditioning of inspired air, and the clearance and distribution of inflammatory mediators. In pathologic conditions such as asthma, the mucosal microcirculation is the source of plasma exudate and inflammatory cells.

In **chapter 3** the autonomic innervation of human airways is described. The human tracheobronchial tree is innervated via efferent and afferent autonomic nerves that regulate airway smooth muscle tone, airway secretions, bronchial circulation, and microvascular permeability. The parasympathetic or cholinergic nervous system is the dominant autonomic pathway in human airways. Stimulation of cholinergic nerves causes bronchoconstriction, mucus secretion, and bronchial vasodilatation by stimulation of specific (muscarinic) receptors via release of the neurotransmitter acetylcholine. Although oxidants and viral infections may impair prejunctional (inhibitory) muscarinic receptors, resulting in increased postganglionic acetylcholine release, there is no direct evidence for an increase in basal cholinergic tone in asthma. The adrenergic or sympathetic nervous system may control the airways via sympathetic nerves which release noradrenaline and by circulating catecholamines released from the adrenal medulla. In contrast to the dense parasympathetic innervation, the sympathetic innervation of the human airways is sparse. However, sympathetic nerves supplying blood vessels may have an important role in the control of tracheobronchial blood flow. Despite the sparse sympathetic innervation, adrenergic receptors (mainly ß-adrenergic receptors) are abundant in human airways. Stimulation of ß-receptors results in relaxation of airway smooth muscle, and this is therefore used in the therapy of asthma. Although a reduced ß-adrenergic receptor function has been postulated in asthma, no evidence for this has been found thusfar. Recently, a functional third part of the autonomic nervous system called the non-adrenergic, non-cholinergic (NANC) nervous system has been demonstrated

in human airways. This nervous system has inhibitory (iNANC) and excitatory components (eNANC). Calcitonine gene related peptide (CGRP) and the tachykinins substance P and neurokinin A are the major transmitters of the eNANC nerves whereas vasoactive intestinal peptide (VIP) and nitric oxide (NO) may be neurotransmitters of the iNANC nervous system. Receptors for CGRP, the tachykinins and VIP have been demonstrated in human airways. NO diffuses through the the cell membrane and increases intracellular cGMP. Although both an increase in eNANC responses and a decrease in iNANC responses have been described in asthmatic patients, the functional relevance of NANC nerves in human airways remains to be elucidated.

Chapter 4 provides an overview of the models that have been developed to study airway function in vitro, emphasizing human airway preparations. The easily prepared airway strip and ring preparations are described first. The potential damage during preparation and the interference with airway structure are important disadvantages in these preparations. Lung parenchymal strips, described next, were designed to study responsiveness of small airways. However, parenchymal strips are anatomically complex, and responsiveness is determined by the relative amounts of airway and vascular smooth muscle. Airway tube preparations, in which luminal and serosal stimulation can be separated, enable us to study the modulatory role of the airway epithelium in vitro. In the isolated perfused lung preparation, the interaction between between the airways and vasculary systems can be studied. This model has yet not been used to study human lungs in vitro. Next, methodological aspects such as tissue handling and storage, recording of responses, removal of the epithelium and electrical field stimulation are discussed in some detail. The responses of isolated airway strips and rings are recorded under isometric or isotonic conditions. In perfused airway tubes responsiveness can be measured under auxotonic conditions as a change in perfusion pressure or flow. The effect of airway epithelium on airway smooth muscle responsiveness is commonly examined in epithelium-denuded airway preparations. Although mechanical removal of the epithelium is widely used, more physiological methods to mimic the epithelial damage found in asthma may be preferable and these methods are described in detail.

Finally, the methodology of electric field stimulation (EFS) is described. EFS stimulates airway smooth muscle indirectly via activation of autonomic nerves.

In **chapter 5** we describe an in vitro model developed at our laboratory, that allows for independent stimulation from either the serosal or the mucosal side of human peripheral airways. Segments of human peripheral airways were

perfused with a Krebs-Henseleit (K-H) solution at a constant pressure, and responsiveness was measured as a change in flow rate. Pressure/flow relationships indicated laminar flow over a wide pressure range, and a working pressure of 6 cm H_2O was chosen because this is a physiological transpulmonary pressure. When stepwise stretching the airway to 180% of its length, we noted an increase in baseline flow and a decrease in flowreduction after 10^{-5} M methacholine. At 140% of the length, accurate and reproducible measurements of the sensitivity (EC_{50}) to methacholine were obtained, and airway closure did not occur. A one-way analysis of variance (ANOVA) revealed that the between-patients differences accounted for 91% of the total variability for -log EC_{50}. We conclude that this in vitro model offers interesting possibilities to evaluate the modulatory effects of the human airway epithelium. In addition, the model provides the opportunity to study human small airway mechanical properties and secretory functions.

The perfused airway tube model was used in the experiments described in **chapter 6** in order to investigate the modulatory role of the airway epithelium. Damage to the epithelium was induced with luminally applied hydrogen peroxide (H_2O_2), and changes in responsiveness to the agonists histamine, methacholine, and salbutamol were measured during both luminal and serosal stimulation. To examine whether intact epithelium acts as a barrier to luminally applied histamine, the histamine concentration was measured in the organ baths containing luminally perfused airway tubes with intact or damaged epithelium. The sensitivity of airway tubes to both histamine and methacholine was significantly lower with mucosal stimulation than with serosal stimulation. No difference was found in the sensitivity to salbutamol between mucosally and serosally stimulated airways. The modulation of the sensitivity to contractile agonists by the epithelium increased with increasing airway size, and was abolished after treatment with H_2O_2. Light microscopic examination of H_2O_2-treated airway preparations revealed damage to the columnar ciliated epithelial cells, with preservation of basal cells. The penetration of histamine through the airway wall was similar in intact and H_2O_2-treated airways. From these results we concluded that oxidative damage to the airway epithelium may lead to hyperresponsiveness to inhaled stimuli. However, this may not be due to a change in barrier function of the damaged epithelium.

In the experiments described in **chapter 7**, we used the perfused airway tube model to study the effects of the oxidant H_2O_2 and the MBP analogue poly-L-arginine on permeability and morphology of human, non-asthmatic, peripheral

airways. In addition, we examined whether airway permeability depends on airway size. Human airway tubes were luminally perfused with Krebs-Henseleit buffer containing the hydrophilic tracer ^{111}In-DTPA, or the lipophilic tracer ^{14}C-antipyrine (^{14}C-AP). Permeability of the airways was calculated from the fluxes of the tracer molecules across the airway wall. After experiments, light- and electronmicroscopic examination of the airway epithelium was performed. Baseline permeability to ^{14}C-AP was 5 times greater than to ^{111}In-DTPA. Luminal exposure of the airways to H_2O_2 produced a significant 6-fold increase in permeability to ^{111}In-DTPA but not to ^{14}C-AP, indicating opening of paracellular pathways. These findings contrasted with the results of the experiments in Chapter 6 where luminal exposure of the airways to H_2O_2 did not increase airway permeability to histamine (a hydrophilic drug of similar size as ^{111}In-DTPA). However, in the experiments described in Chapter 6 we measured cumulative concentrations of histamine in the organ baths and this may be less sensitive than measurements of permeability to a radioactive tracer at 15 min intervals used in Chapter 7. Luminal exposure to poly-L-arginine for 3 h and 16 h produced a significant 4.5- and 7-fold increase in permeability to ^{111}In-DTPA, respectively. Histological examination of epithelium exposed to H_2O_2 or poly-L-arginine showed focal loss of superficial cells with preservation of basal cells. Baseline airway permeability increased significantly with decreasing airway size. We concluded that oxidant- and cationic protein-induced epithelial damage increase airway permeability and may at least partly explain the increased responsiveness to inhaled stimuli in asthma.

The metabolization of the bronchodilating peptide vasoactive intestinal peptide (VIP) by epithelial and subepithelial peptidases was studied in **chapter 8**. Intact or epithelium-denuded strips of central and peripheral airways were incubated with or without peptidase inhibitors. After precontraction with histamine cumulative concentration response curves to VIP were obtained. Both intact central and peripheral airways showed only minor relaxations to VIP irrespective of the precontraction level. Removal of the epithelium and addition of peptidase inhibitors additively increased the sensitivity (>20-fold) and maximal response to VIP in both central and peripheral airways. We concluded that VIP relaxes both central and peripheral human airways but only in the absence of epithelium and/or the presence of peptidase inhibitors, and that the epithelium does not seem to be the only source of peptidase activity.

In animal airways, it has been shown that the inflammatory peptide bradykinin (Bk) stimulates peptidergic nerves resulting in local axon reflexes with release

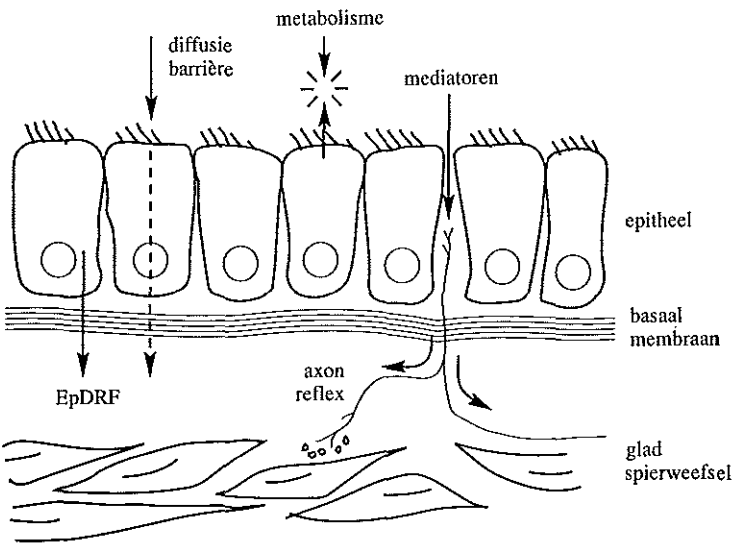
of tachykinins. The mechanism of Bk-induced airway narrowing in humans is not clear. In the experiments presented in **chapter 9**, we investigated the effects of epithelium removal, inhibition of the enzymes neutral endopeptidase (NEP) and cyclooxygenase, and blockade of nervous conductance with tetrodotoxin (TTX) on Bk-induced responses of human isolated peripheral airways. Responses to Bk were recorded from airways with spontaneous intrinsic tone and from airways precontracted with methacholine. Furthermore, we measured the Bk-induced release of the prostanoids PGE_2, PGI_2, and TxA_2 from airways with and without epithelium in the absence and presence of indomethacin. Finally, we examined the effect of the bradykinin B_2-receptor antagonist Hoe 140 and the thromboxane prostanoid (TP) receptor blocking drug GR32191 on Bk-induced responses. Bk contracted intact and epithelium-denuded airways with spontaneous intrinsic tone, whereas precontracted airways either relaxed or contracted to Bk. Removal of the epithelium increased the sensitivity to Bk 7-fold without changing the direction of the response. The NEP inhibitor phosphoramidon tended to increase the sensitivity to Bk (ns) and did not change the direction of the response. Both contractile and relaxation responses to Bk and the release of the prostanoids PGE_2, PGI_2 and TxA_2 by the airway tissues were largely inhibited by indomethacin, whereas TTX had no effect. PGE_2, PGI_2, and TxA_2 were released by both intact and epithelium-denuded airways. Bk-induced responses were antagonized by both Hoe 140 and GR32191. It was concluded that Bk is a potent constrictor of human peripheral airways, especially in the absence of epithelium. Involvement of local axon reflexes with release of tachykinins could not be demonstrated. The effect of Bk on human airway smooth muscle is, therefore, mediated via the B_2 receptor and involves the release of prostanoids that contract airways via the TP receptor.

Sensory nerve fibers (peptidergic nerves) can be activated by capsaicin and this may result in antidromic conduction (axon reflex) with release of tachykinins that cause bronchoconstriction and 'neurogenic inflammation'. In **chapter 10** we investigated the effects of neural conduction blocker tetrodotoxin (TTX), the anti-asthmatic drug sodium cromoglycate (SCG) and the neurokinin$_2$ (NK_2) receptor antagonist SR48968 on capsaicin-induced contractile responses of human isolated airways, and we measured release of tachykinins from these airways. Airway strip preparations were mounted in organ baths and isotonic concentration response curves to capsaicin were made. The organ bath fluid was collected and the release of SP and NKA was measured by radioimmunoassay. Maximal isotonic shortening (Smax) to 10^{-5}M capsaicin was

9.4 ± 3.0% of the maximal shortening to methacholine. TTX and SCG did not affect capsaicin-induced bronchocon-striction. SR48968 significantly decreased capsaicin-induced contraction to 2.6 ± 2.5% of maximal contraction. Capsaicin-induced release of both SP and NKA was significantly reduced by TTX but not by SCG or SR48968. In conclusion, we found capsaicin-induced bronchocon-striction and release of tachykinins in human airways. Our finding that the nervous conductance blocker TTX inhibited capsaicin-induced tachykinin release suggests that tachykinins were released by a local axon reflex mechanism and this is the first time that such a mechanism is demonstrated in human airways. In vivo, these axon reflexes may contribute to 'neurogenic inflammation'.

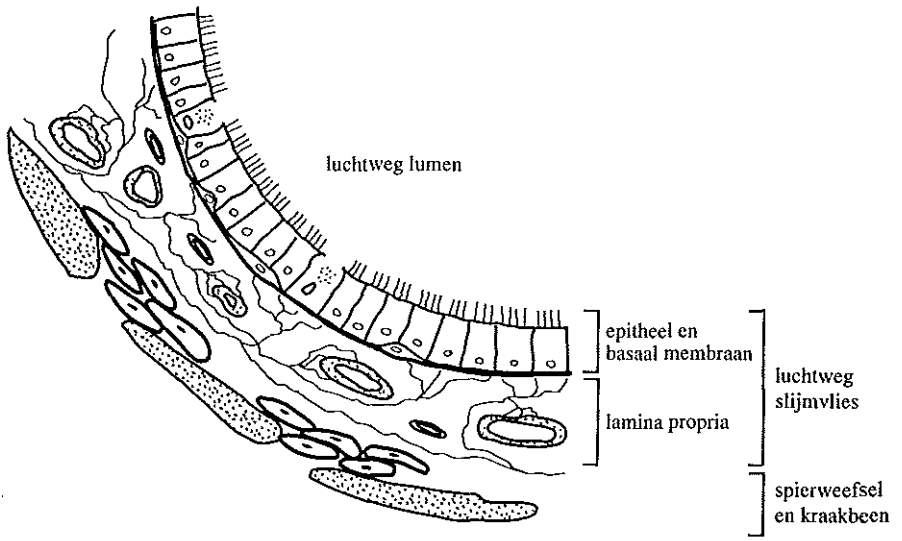
12.2 SAMENVATTING

In **hoofdstuk 1** wordt astma gedefinieerd als een chronische luchtwegontsteking met vernauwing en een toename van de prikkelbaarheid van de luchtwegen. De vernauwing van de luchtwegen ontstaat onder meer doordat spierweefsel in de luchtwegwand samentrekt en tevens door verdikking van de ontstoken luchtwegen. Astma is een veel voorkomende aandoening: ongeveer 10-15% van de bevolking lijdt aan de ziekte. Bovendien vertoont de frequentie van voorkomen een stijgende tendens. De verschijnselen van astma zijn: hoesten, piepende ademhaling, kortademigheid en een beklemd gevoel op de borst. De klachten zijn wisselend aanwezig en worden vaak veroorzaakt door prikkels uit de omgeving van de patiënt zoals allergenen, mist of koude lucht en door lichamelijke inspanning. Bij microscopisch onderzoek van de luchtwegen van astmapatiënten wordt schade aan het luchtwegslijmvlies, een toename van het aantal slijmproducerende cellen en ontstekingscellen en een verdikking van de spierlaag in de wand van de luchtweg gevonden. De beschadiging van het slijmvlies wordt waarschijnlijk vooral veroorzaakt door zuurstofradikalen en etsende eiwitten die gemaakt worden door ontstekingscellen in de luchtwegwand. De mate van prikkelbaarheid neemt evenredig toe met de mate van slijmvliesschade aan de luchtwegen. Het intacte slijmvlies heeft dus een beschermende werking tegen prikkelbaarheid van de luchtwegen. Het mechanisme van deze beschermende werking is echter onbekend. Verschillende theorieën zijn opgesteld die schema-tisch zijn weergegeven in figuur 1. Allereerst is het mogelijk dat het slijmvlies voortdurend een stof afscheidt die het achterliggende spierweefsel ontspant waardoor de luchtwegen worden verwijd. Bij schade aan het slijmvlies,



Figuur 1. Mogelijke oorzaken van de beschermende invloed van het luchtwegslijmvlies op luchtwegspierweefsel. EpDRF = epithelium-derived-relaxing-factor.

zoals gevonden wordt bij de astmapatient, zal de produktie van de stof verminderen waardoor de luchtwegen vernauwen. Een andere theorie is dat het slijmvlies van de luchtwegen prikkelende stoffen onschadelijk maakt waardoor deze het achterliggende spierweefsel niet kunnen bereiken. Een derde mogelijkheid is dat het slijmvlies ondoorlaatbaar is voor prikkelende stoffen. Beschadiging van het slijmvlies zal de doorlaatbaarheid verhogen waardoor prikkelende stoffen de spierlaag kunnen bereiken. Tenslotte is het mogelijk dat na beschadiging van het slijmvlies bepaalde zenuwuiteinden in de luchtwegwand worden blootgelegd. Deze zenuwuiteinden kunnen geprikkeld worden door ingeademde mediatoren en mediatoren gevormd door ontstekingscellen. De geprikkelde zenuwen zullen eiwitten afscheiden die het spierweefsel van de luchtweg doen samentrekken en de slijmproduktie en het ontstekingsproces in de luchtwegen stimuleren. Het doel van de in dit proefschrift beschreven experimenten was de beschermende rol van het luchtwegslijmvlies te onderzoeken in menselijke luchtwegen. Wij onderzochten het effect van slijmvliesschade door de zuurstofradikaal waterstofperoxyde en door etsende eiwitten op de prikkelbaarheid en doorlaatbaarheid van luchtwegen. Voorts werd de afbraak van prikkelende stoffen door het luchtwegslijmvlies bestudeerd. Tenslotte onderzochten wij of prikkeling van zenuwuiteinden in het slijmvlies bijdraagt aan luchtwegvernauwing.



Figuur 2. Schematische weergave van een doorsnede door de luchtwegwand in de menselijke long.

In **hoofdstuk 2** worden de struktuur en funktie van het luchtwegslijmvlies en de afwijkingen hiervan bij astma beschreven. Het slijmvlies bestaat uit verschillende lagen. Allereerst een laag cellen (epitheelcellen) die stevig aaneen gehecht zijn en de binnenkant van de luchtweg bekleden (figuur 2). Deze cellen zijn verankerd in een laag die de basaal membraan genoemd wordt. De epitheelcellen vormen een barrière tegen vochtlekkage vanuit de weefsels en binnendringen van prikkels van buiten. Voorts kunnen epitheelcellen bepaalde prikkelende stoffen en zuurstofradikalen afbreken en produceren zij stoffen die de funktie van naburige weefsels en cellen zoals spierweefsel, ontstekingscellen, bloedvaten en zenuwen beïnvloeden. Bij astmapatiënten zijn de epitheelcellen vaak beschadigd hetgeen gepaard gaat met een toename van de prikkelbaarheid van de luchtwegen. Onder de laag epitheelcellen zit een dicht netwerk van bloedvaten en -vaatjes. Via dit netwerk worden de epitheelcellen gevoed en worden afbraakprodukten en prikkelende stoffen afgevoerd. Tevens wordt de ingeademde lucht verwarmd en bevochtigd. In luchtwegen van astmatische personen worden veel meer ontstekingscellen gevonden die aangevoerd worden via het vaatnetwerk. De ontstekingscellen treden door de vaatwand heen naar het omliggende weefsel van de luchtwegwand waar zij het ontstekingsproces onderhouden.
Hoofdstuk 3 beschrijft het onwillekeurige zenuwstelsel van de menselijke long.

Dit zenuwstelsel reguleert de spierspanning, bloedsomloop, slijmproduktie en wellicht deels het ontstekingsproces in de long. Het parasympatische gedeelte van dit zenuwstelsel heeft een overheersende rol. Stimulatie van parasympatische zenuwen leidt tot vernauwing van luchtwegen door samentrekken van spierweefsel en overmatige afscheiding van slijm en verwijding van bloedvaten. Deze effecten ontstaan doordat uit parasympatische zenuwen de stof acetylcholine vrijkomt die aangrijpt op een specifieke plaats (de zogenaamde receptor) op lichaamcellen volgens het sleutel-en-slot principe.
Het sympatische gedeelte van het onwillekeurige zenuwstelsel heeft bij de mens in tegenstelling tot bij sommige diersoorten, geen invloed op het spierweefsel van de luchtwegen. De bloedtoevoer in de menselijke long wordt echter wel deels bestuurd door het sympatische zenuwstelsel en dit kan indirekt de luchtwegen beïnvloeden.
Onlangs werd een derde component van het onwillekeurige zenuwstelsel ontdekt in de luchtwegen van de mens. Dit zenuwstelsel, dat het non-adrenerge, noncholinerge (NANC) zenuwstelsel wordt genoemd, kan eiwitten produceren, genaamd tachykininen, die de luchtweg vernauwen en de slijmproduktie en het ontstekingsproces in de luchtwegen bevorderen. Bij astmapatienten zijn er mogelijk stoornissen in dit zenuwstelsel.
Hoofdstuk 4 geeft een overzicht van de technieken die gebruikt worden om longweefsel buiten het lichaam te onderzoeken. Bij deze technieken worden cellen of weefsels na verwijdering uit het lichaam onderzocht in een zogenaamd weefselbad. Dit glazen bakje is gevuld met een vloeistof die het weefsel enkele uren in leven houdt. Voordeel van onderzoek van een weefsel buiten het lichaam is dat allerlei lichaamsprocessen (bijvoorbeeld de bloedsomloop of zenuwimpulsen) die een waarneming kunnen verstoren worden uitgeschakeld.
In **Hoofdstuk 5** wordt een techniek beschreven, het zogenaamde luchtwegperfusiemodel, ontwikkeld in ons laboratorium, waarbij een prikkelende stof selektief aan de binnen- of buitenzijde van menselijke luchtwegen toegediend kan worden (zie figuur 1, hoofdstuk 5 en figuur 1, hoofdstuk 6). Zodoende is de rol van het slijmvlies, dat alleen aan de binnenkant van de luchtweg aanwezig is, te onderzoeken.
De in hoofdstuk 5 beschreven techniek werd in de experimenten beschreven in **hoofdstuk 6** gebruikt om de beschermende rol van het luchtwegslijmvlies ten aanzien van prikkelbaarheid van luchtwegen te onderzoeken. In de helft van de te onderzoeken luchtwegen werd het slijmvlies beschadigd door blootstelling aan waterstofperoxyde. De andere luchtwegen werden intact gelaten. Daarna

werd aan alle luchtwegen aan de binnenzijde een prikkelende stof die de luchtweg vernauwt (histamine of methacholine) of verwijdt (salbutamol, een medicijn gebruikt bij de behandeling van astma) toegediend. De prikkelbaarheid van de luchtwegen die blootgesteld waren aan waterstofperoxyde bleek sterk verhoogd (5- tot 10-voudig) voor de stoffen die de luchtweg vernauwen doch niet voor het verwijdende medicijn salbutamol. Voorts bleek dat het beschermende effect van het slijmvlies toeneemt met de luchtweggrootte. Microscopisch onderzoek van luchtwegen blootgesteld aan waterstofperoxyde toonde beschadiging van slijmvlies zoals ook bij astmapatiënten worden gezien.

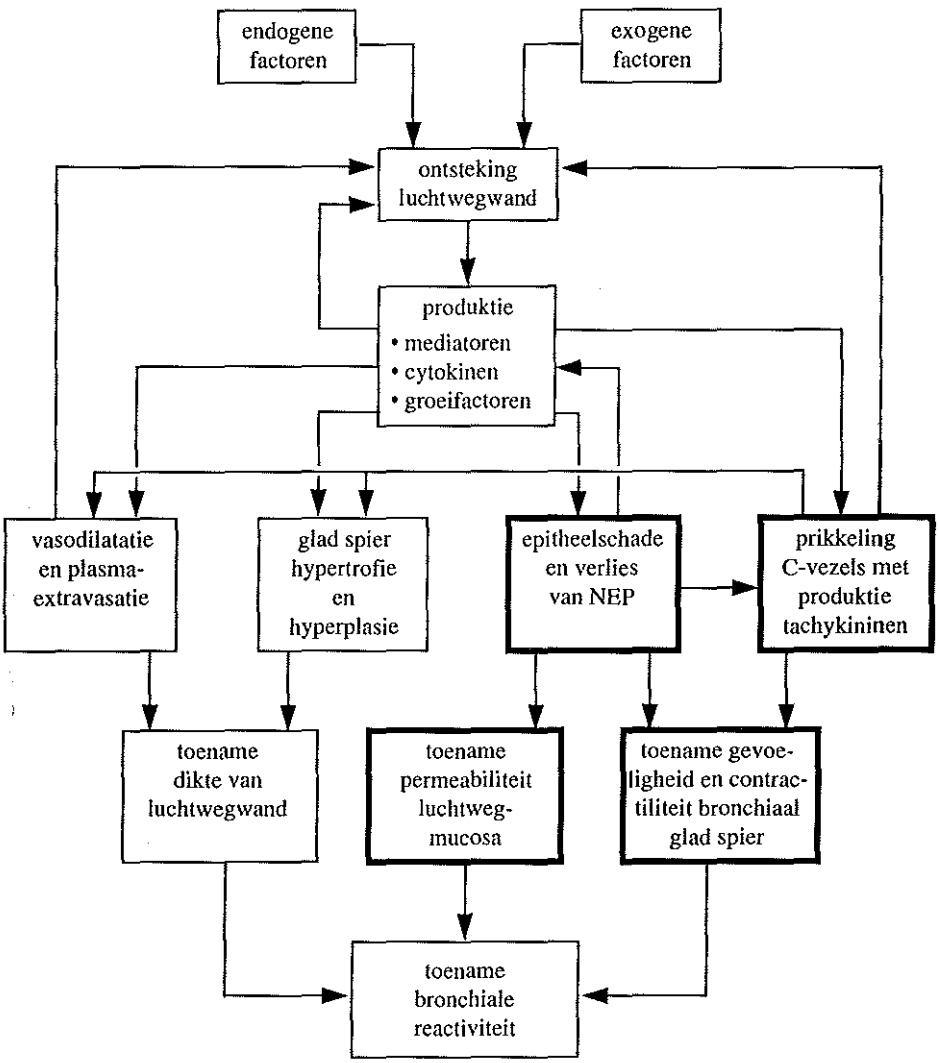
De in hoofdstuk 5 beschreven techniek werd ook gebruikt voor de experimenten beschreven in **hoofdstuk 7** waarin het effekt van waterstofperoxyde en een synthetisch etsend eiwit op de doorlaatbaarheid van menselijke luchtwegen werd onderzocht. Zowel waterstofperoxyde als etsende eiwitten worden in de luchtwegen van astmapatiënten in verhoogde mate geproduceerd door ontstekingscellen. De helft van de te onderzoeken luchtwegen werd aan de binnenkant (slijmvlieszijde) blootgesteld aan waterstofperoxyde het etsende eiwit. De andere luchtwegen werden niet blootgesteld. Daarna werd de doorlaatbaarheid van de luchtwegen voor een radioaktieve wateroplosbare stof en voor een radioaktieve vetoplosbare stof gemeten. De doorlaatbaarheid van de luchtwegen voor de vetoplosbare stof bleek in zowel blootgestelde als niet-blootgestelde luchtwegen hoog hetgeen niet verwonderlijk is daar bekend is dat vetoplosbare stoffen gemakkelijk weefselbarrières passeren. De doorlaatbaarheid voor de wateroplosbare stof nam 6- a 7-voudig toe na blootstelling aan waterstofperoxyde of het etsende eiwit. Daar wateroplosbare stoffen het slijmvlies alleen kunnen passeren tussen de epitheelcellen door (vetoplosbare stoffen gaan dwars door epitheelcellen heen) betekent deze bevinding dat waterstofperoxyde en etsende eiwitten openingen veroorzaken tussen de cellen. Met elektronen-microscopie konden wij dit inderdaad zichtbaar maken. Andere onderzoekers vonden deze openingen tussen epitheelcellen ook in de luchtwegen van astmapatiënten. Uit de experimenten beschreven in hoofdstukken 6 en 7 mogen we dan ook concluderen dat een toename van de doorlaatbaarheid, veroorzaakt door stoffen geproduceerd door ontstekingscellen in de luchtwegen zeker een bijdrage kan leveren aan de toename van prikkelbaarheid van de luchtwegen voor stoffen die luchtwegvernauwing geven.

In de experimenten beschreven in **hoofdstuk 8** werd de rol van het luchtwegslijmvlies bij de afbraak van een in de long geproduceerd eiwit dat de luchtwegen normaal gesproken verwijdt, onderzocht. Dit eiwit genaamd vasoactief intestinaal

peptide (VIP) wordt mogelijk onvoldoende aangemaakt in de luchtwegen van astmapatiënten waardoor luchtwegvernauwing optreedt. Het bleek dat VIP luchtwegen alleen verwijdt na verwijdering van het slijmvlies en/of nadat de enzymen die VIP afbreken geremd worden met bepaalde medicamenten. Voorts bleek dat de enzymen die VIP afbreken niet alleen in het slijmvlies voorkomen maar ook in andere weefsels. Na schade aan slijmvlies (zoals bij astmapatiënten gevonden wordt) zal VIP dus nog steeds afgebroken kunnen worden. Gezien het geringe luchtweg-verwijdende effect van VIP in onze experimenten lijkt het onwaarschijnlijk dat een tekort aan VIP de luchtwegvernauwing bij astmapatiënten kan verklaren. Voorts is het onwaarschijnlijk dat het slijmvlies een sleutelrol heeft bij de afbraak van prikkelende stoffen; ook andere weefsels blijken hiertoe in staat.

In de ontstoken luchtwegen van astmapatiënten wordt een grotere hoeveelheid bradykinine gevonden dan in luchtwegen van gezonde mensen. Bradykinine is een klein eiwit dat bij ontstekingsprocessen geproduceerd wordt door ontstekingscellen. Toediening van bradykinine per inhalatie aan astmapatiënten veroorzaakt een vernauwing van de luchtwegen gelijkend op een astma-aanval terwijl dit bij gezonde personen niet gebeurt. Het mechanisme van luchtwegvernauwing door bradykinine is onduidelijk. In de experimenten beschreven in **hoofdstuk 9** werd het mechanisme van luchtwegvernauwing door bradykinine onderzocht in luchtwegen met en zonder slijmvlies. Het bleek dat bradykinine zelf geen luchtwegvernauwing gaf maar dat het effect indirect plaatsvindt; bradykinine maakt stoffen vrij uit luchtwegen, vooral uit luchtwegen met beschadigd slijmvlies (zoals bij astmapatiënten), die het spierweefsel van de luchtwegen doen samentrekken. Een van deze stoffen, thromboxaan, bleek de oorzaak van de luchtwegvernauwing: Na blokkade van de aangrijpingsplaats (receptor) van thromboxaan bleken de luchtwegen zelfs te verwijden onder invloed van prostacycline, een stof die ook door bradykinine vrijgemaakt werd. Wellicht is de verhoogde afgifte van thromboxaan uit de luchtwegwand na slijmvliesbeschadiging de belangrijkste oorzaak van de luchtwegvernauwing door ingeademde bradykinine bij astmapatiënten.

In de experimenten beschreven in **hoofdstuk 10** hebben wij de zenuwen van het onlangs ontdekte non-adrenerge, non-cholinerge (NANC) gedeelte van het onwillekeurige zenuwstelsel kunstmatig geprikkeld met capsaïcine, een stof afkomstig uit spaanse pepers. Tijdens de toediening van capsaïcine werd gemeten of er luchtwegvernauwing optrad en werd gemeten of er inderdaad eiwitten (tachykininen) werden vrijgemaakt door deze zenuwen. Tevens werd onderzocht



Figuur 3. Schematische weergave van de mechanismen die mogelijk een rol spelen in de pathogenese van astma. De geaccentueerde kaders betreffen het in deze thesis beschreven onderzoek.

of de luchtwegvernauwing en de produktie van tachykininen kon worden geremd met een stof die zenuwgeleiding blokkeert, een geneesmiddel dat gebruikt wordt bij astma, en een experimenteel geneesmiddel dat het aangrijpingspunt (receptor) van deze eiwitten op de lichaamscellen blokkeert. Wij vonden dat capsaïcine slechts een geringe luchtwegvernauwing geeft die niet te remmen was met het

middel dat zenuwgeleiding blokkeert en ook niet met het geneesmiddel dat gebruikt wordt bij astma. Het experimentele geneesmiddel kon luchtwegvernauwing voorkómen en biedt wellicht mogelijkheden voor de behandeling van astma. De produktie van tachykininen door de zenuwen kon worden geremd door het middel dat zenuwgeleiding blokkeert maar wederom niet door het geneesmiddel dat gebruikt wordt bij astma. Uit deze experimenten blijkt dus dat prikkeling van blootgelegde zenuwen slechts een geringe vernauwing geeft van de luchtwegen. Echter de geproduceerde tachykininen bevorderen wellicht de slijm-produktie en het ontstekingsproces in de luchtwegen.

De **conclusies** uit de in dit proefschrift beschreven experimenten zijn als volgt:

1. Het luchtwegslijmvlies in de mens beschermt het onderliggende spierweefsel tegen prikkels van buiten.
2. Beschadiging van het luchtwegslijmvlies door stoffen die door ontstekingscellen gemaakt worden (zoals plaatsvindt bij astma) geeft een verhoging van zowel de prikkelbaarheid als de doorlaatbaarheid van de luchtweg voor prikkelende stoffen. Abnormale doorlaatbaarheid lijkt een belangrijke oorzaak van de verhoogde luchtwegprikkelbaarheid na schade aan het slijmvlies.
3. Eiwit afbrekende enzymen (peptidasen) op het luchtwegepitheel lijken geen sleutelrol te spelen bij het voorkomen van prikkelbaarheid van de luchtwegen: Het verwijderen van het epitheel heeft een groter effect op de prikkelbaarheid van de luchtwegen. Bovendien zijn dergelijke enzymen ook aanwezig op andere weefsels.
4. Bij prikkeling van zenuwuiteinden van het niet-adrenerge, niet-cholinerge (NANC) gedeelte van het onwillekeurige zenuwstelsel treedt slechts een geringe samentrekking van geïsoleerde menselijke luchtwegen op. De afgifte van tachykininen door de geprikkelde zenuwen is te remmen met het zenuwgif tetrodotoxine, en dit suggereert dat de afgifte van tachykininen via lokale zenuwreflexen plaatsvindt.

In figuur 3 worden de mechanismen die mogelijk een rol spelen bij de pathogenese van astma schematisch weergegeven weergegeven.

Dankwoord

Velen hebben een onmisbare bijdrage geleverd aan dit proefschrift. Allereerst wil ik de tientallen mensen bedanken die zich al meer dan 10 jaar inspannen om ons laboratorium te voorzien van operatiemateriaal. Het betreft longartsen, (thorax)chirurgen, pathologen, OK-assistenten, pathologie-assistenten en secretaresses van het Dijkzigt ziekenhuis, het Zuiderziekenhuis en het Ikazia ziekenhuis te Rotterdam, ziekenhuis Leyenburg te Den Haag, het Patholoog Anatomisch Laboratorium te Dordrecht en ziekenhuis De Baronie en het Ignatius ziekenhuis te Breda. Hun inzet maakt een unieke onderzoekslijn mogelijk.

Al even uniek is Rolien Raatgeep, analiste. Haar enorme inzet, enthousiasme en vaardigheid maakte dit onderzoek mogelijk. Haar kunde (kunst) maakte het mogelijk om ook uit kleine (perifere) luchtwegen reproduceerbare data te verkrijgen. Dat haar vaardigheid ook internationaal bekend is illustreert de volgende anekdote: Op het jaarlijks congres van the American Thoracic Society hield een vermaard onderzoeker een voordracht over het effect van een experimenteel geneesmiddel op humane grote (centrale) luchtwegen. Tijdens de discussie vroeg een toehoorder of hij het effect van het middel ook op humane perifere luchtwegen had onderzocht "..like they do in Rotterdam". Vertwijfeld stak de wetenschapper zijn grote handen in de lucht en riep: "Do you see these hands?".

Mijn promotor Prof. dr. K.F. Kerrebijn dank ik voor zijn visie, vertrouwen en begeleiding. Zelfs als decaan en lid van de Raad van Bestuur van het AZR vond hij tijd voor overleg.

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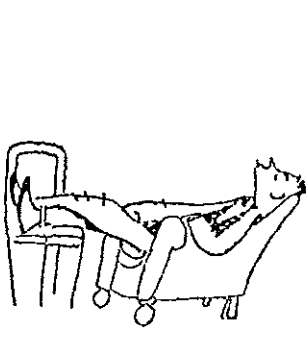
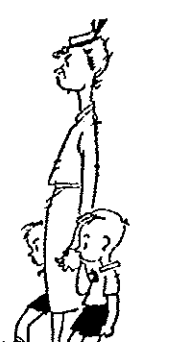


Curriculum Vitae

Anthon Hulsmann was born in Delft, The Netherlands, on 16 May 1961. He passed his secundary school exam (VWO) in 1980 at the Develsteincollege te Zwijndrecht. He started his medical training in 1980 at the Medical Faculty of the Erasmus University Rotterdam. During his studies he worked as a junior research assistant at the Pathophysiological Laboratory (head: Prof. Dr. A. Versprille), of the Department of Pulmonary Diseases (head: Prof. Dr. C. Hilvering), of the Erasmus University and as a laboratory worker (during weekends) at the Department of Haematology (head: Prof. Dr. J. Abels) of the University Hospital Dijkzigt, Rotterdam. In 1987 he obtained his medical degree (cum laude). From November 1987 to April 1989 he worked as a junior fellow in pediatrics in the Zuiderziekenhuis and the Sophia Children's Hospital, both in Rotterdam. In April 1989 he started his specialist training in pediatrics at the Sophia Children's Hospital (head: Prof. Dr. H.K.A. Visser). The training was interrupted from October 1991 to October 1993 when he followed an Asthma Foundation research fellowship at the subdivision of Paediatric Respiratory Medicine (head: Prof. Dr. K.F. Kerrebijn) of the Sophia Children's Hospital. The research bundled in this thesis was performed during this period at the Department of Pharmacology (head: Prof. Dr. P.R. Saxena) of the Erasmus University. In October 1993 he continued his training in pediatrics and was registered as a pediatrician in October 1995. He is married to Lena Adriana Lievaart, pediatric nurse, and they have two children: Simone and Peter.



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List of Abbreviations

^{14}C	radioactive carbon
^{111}In	radioactive indium
^{125}I	radioactive iodine
ACE	angiotensin converting enzyme
ANOVA	analysis of variance
AP	antipyrine
APUD cells	amine precursor uptake and deaminase cells
ATP	adenosine triphosphate
B-cells	lymphocytes of the bone marrow subtype
BAL	bronchoalveolar lavage
BCS	baseline contractile state
Bk	bradykinin
BM	basement membrane
BMDP	biomedical computer programs
BSA	bovine serum albumin
C-fibers	unmyelinated sensory nerve fibers
cAMP	cyclic adenosine-3',5'-monophosphate
CCRC	cumulative concentration response curve
CD	cluster of determination
cDNA	complementary desoxyribonucleic acid
C_{Dyn}	dynamic compliance
cGMP	cyclic guanosine-3',5'-monophosphate
CGRP	calcitonine gene-related peptide
Ci	Curie
cNOS	constitutive nitric oxide synthase
COPD	chronic obstructive pulmonary disease
cpm	counts per minute
dpm	desintegrations per minute
DTPA	diethylene triamine penta-acetic acid
EC_{50}	effective concentration that causes 50% of the maximal effect
ECP	eosinophil cationic protein
EDRF	endothelium-derived relaxing factor
EDTA	ethylene diamine tetra-acetic acid
EFS	electric field stimulation
eNANC	excitatory non-adrenergic, non-cholinergic
EpDRF	epithelium-derived relaxing factor
FITC	fluorescein isothiocyanate
FK-224	antagonist of NK_2- and NK_2 receptors
FPL55712	antagonist of the leukotriene C_4/D_4 receptor
FRC	functional residual capacity
g	gravity
G-protein	guanine nucleotide-binding regulatory protein
GM-CSF	granulocyte/macrophage colony-stimulating factor

GR32191	antagonist of the thromboxane prostanoid (TP) receptor
H_2O_2	hydrogen peroxide
HETE	hydroxyeicosatetraenoic acid
HLA	human leukocyte antigen
Hoe 140	antagonist of the bradykinin$_2$ (Bk_2) receptor
HPETE	hydroxyperoxyeicasotetraenoic acid
LT	leukotriene
HPS	heparan sulphate proteoglycan
HT	hydroxytryptamine = serotonine
Hz	Herz
IAR	immediate asthmatic reaction
ICAM	intercellular adhesion molecule
IgE	immunoglobulin E
IL	interleukin
iNANC	inhibitory non-adrenergic, non-cholinergic
iNOS	inducible nitric oxide synthase
K-H buffer	Krebs-Henseleit buffer
L_{max}	length at which airway smooth muscle develops maximal active tension
L_0	L_{max}
LAR	late asthmatic reaction
LECAM	leukocyte cell adhesion molecule
LFA	lymphocyte function-associated antigen
M-receptor	muscarinic receptor
N-receptor	nicotinic receptor
M	molar concentration
MACR	maximal active contractile range
MANOVA	repeated-measures analysis of variance
MBP	major basic protein
MCh	methacholine
mRNA	messenger ribonucleic acid
MW	molecular weight
NANC	non-adrenergic, non-cholinergic
NE cells	neuroendocrine cells
NEP	neutral metalloendopeptidase
NK receptor	neurokinin (tachykinin) receptor
NKA	neurokinin A
NKB	neurokinin B
NO	nitric oxide
NPK	neuropeptide K
NPY	neuropeptide Y
ns	not significant
O_2	oxygen
O_3	ozone
P	permeability (cm/s)
PAF	platelet-activating factor
PG	prostaglandin

PHI	peptide histidine isoleucine
PHM	peptide histidine methionine
PHV	peptide histidine valine
Pi	internal perimeter
RIA	radioimmunoassay
R_L	lung resistance
Rmax	maximal relaxing response
r_s	Spearman's rank correlation coefficient
SCG	sodium cromoglycate
SEM	standard error of the mean
SICA	spontaneous intrinsic contractile activity
Smax	maximal contraction
SP	substance P
SPSS	statistical package for social science
SR48968	antagonist of the neurokinin$_2$ (NK_2) receptor
T-cells	lymphocytes of the thymus subtype
TC	treshold concentration
TGF	transforming growth factor
Th2-cells	T-cells of the helper subtype No 2
TNF	tumor necrosis factor
TP receptor	thromboxane prostanoid receptor
TTX	tetrodotoxin
Tx	thromboxane
V	Volt
VCAM	vascular cellular adhesion molecule
VIP	vasoactive intestinal peptide
VLA	very late antigen