

# The Guinea Pig as an Animal Model for Asthma

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**Abstract:** Experimental guinea pig asthma is a reliable and clinically relevant facsimile of human disease. The guinea pig is the preferred choice for use as a model of allergic bronchial asthma in the evaluation of anti-asthmatic drugs, since the airway anatomy and the response to inflammatory mediators is similar to humans. Further, the great strength of this model is the direct anaphylactic bronchoconstriction upon antigen challenge. Under certain conditions a late asthmatic response can be measured and airway hyperresponsiveness is observed *in vitro* and *in vivo*. Moreover, the inflammatory response is comparable with the human situation. More recent studies describe a chronic model for asthma in which airway remodeling is induced as can be observed in the asthmatic patients. The focus here is to demonstrate that guinea pig asthma models are useful for testing novel therapeutics.

**Key Words:** Allergy, asthma, guinea pig, early asthmatic reaction, late asthmatic reaction, airway hyperresponsiveness, airway inflammation, airway remodelling.

## INTRODUCTION

Guinea-pigs represent one of the oldest animal models of allergic airway responses, with studies conducted on these animals over 100 years ago [1]. The guinea-pig is the most widely used model and test system for contact hypersensitivity to chemical irritants and proteins. Guinea pig asthma models are so popular that they have even been patented (US patent 5602302). Guinea-pigs are limited in terms of mechanistic studies, particularly those involving genetics, due to the low number of inbred strains and lack of guinea-pig-specific reagents available. As with mice and rat models of asthma, guinea-pigs are readily sensitized to ovalbumin [2, 3] and it is easy to elicit a response to challenge that is similar to an asthmatic phenotype that involves eosinophilia and increased airway responsiveness [4].

In mimicking the physiological abnormalities of human asthmatics, guinea-pigs may be seen as an ideal model as they develop a well-characterized early asthmatic reaction (EAR) and late asthmatic reaction (LAR) to allergen challenge following sensitization [5-8]. From an immunological point of view, IgG1 seems to play a critical role in allergic responses in guinea-pigs [9]. Guinea-pigs are useful as models of immediate hypersensitivity to irritants and have pharmacological responses similar to humans. They have been employed in the study of asthma because they develop classic allergic responses to foreign protein antigens such as ovalbumin and cockroaches and exhibit histological airway changes similar to those in human asthmatic airways. Moreover, useful guinea pig models have been developed for occupational asthma and virus-induced asthma. Rather new

in the field is the development of a chronic asthma model in guinea pigs [10]. In this review we will briefly introduce asthma and the different guinea pig models that are used. Accordingly, the pathophysiological features observed in asthmatic patients will be compared with those observed in the guinea pig models with special emphasis on the ovalbumin model.

## ASTHMA

Asthma affects about 10% of the population, and the prevalence is rising in many parts of the world [11], especially in children [12, 13]. Atopy or hypersensitivity to common environmental antigens is a major cause of asthma in both children and adults. However, air pollution, infections, working environment (occupational asthma) and vaccination policies have also been incriminated. Moreover, more evidence arises for the contribution of genetic factors. Besides the increasing prevalence, deaths from asthma are also increasing, yet the exact reason for this is unclear. Since 1958, asthma is defined as the condition of subjects with widespread narrowing of the bronchial airways which changes in severity over short periods of time either spontaneously or under treatment. However, nowadays the definition 'chronic inflammatory disease of the airways associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and cough; these episodes are associated with widespread but variable airflow obstruction that is reversible either spontaneously or with treatment' [12] is used.

Asthmatic responses can be divided into early and late asthmatic responses. Characteristic features of chronic asthma are airway inflammation, airway hyperresponsiveness [14] and epithelial damage. Several experimental models of asthma have been developed in laboratory animals. The ovalbumin model of airway inflammation is characterized by

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high levels of ovalbumin specific IgE, an eosinophil and T-cell predominant bronchial inflammatory response and the development of airway hyperresponsiveness Fig. (1).

### EARLY AND LATE ASTHMATIC RESPONSES

Following bronchial allergen challenge in nearly all atopic asthmatics, there is a rapid fall in pulmonary function (forced expiratory volume in one second (FEV<sub>1</sub>)) at 5-10 minutes, which gradually recovers over the following 2 hours, termed the early asthmatic reaction. In about 50% of subjects, there is a second fall in FEV<sub>1</sub> between 4 and 6 hours, the late asthmatic reaction. This may last up to 12 hours and in some individuals it may be followed by recurring airway obstruction for several days or even weeks [15].

Exposure to common environmental allergens in atopic (allergic) subjects induces the production of immunoglobulin-E (IgE) [16], which binds to mast cells *via* the high affinity IgE receptor, FcεRI. Subsequent allergen exposure promotes cross-linking of FcεRI-bound IgE molecules, mast cell activation and the release of numerous preformed (histamine), newly synthesized (arachidonic acid metabolites) or formed (bradykinin) and nerves-derived mediators. These mediators cause bronchial obstruction *via* smooth muscle contraction and increase vascular permeability, which results in oedema in the airway wall [17]. This phase is designed as

the early asthmatic reaction. Other newly synthesized mediators by mast cells, such as cytokines, including interleukin-1 (IL-1) and IL-5, interferon-γ (IFN- γ) and tumor necrosis factor-α (TNF-α), are involved in the orchestrated recruitment and activation of inflammatory cells into the airway wall [18-23]. Subsequent mediator release by inflammatory cells and tissue damage following these events account for the ensuing airway obstruction and the associated increase in airway hyperresponsiveness associated with the late asthmatic reaction.

### IMMUNE-INFLAMMATORY RESPONSE IN ASTHMA

It has now been widely accepted that asthma is fundamentally a disease of airway inflammation [24]. Histological analysis of bronchial biopsies from patients with atopic asthma show airway inflammation characterized by the presence of activated eosinophils, lymphocytes and macrophages, some neutrophils, increased numbers of degranulated mast cells and epithelial injury.

Eosinophils are clearly most characteristic of asthma, and correlate with its severity [25]. A number of cytokines regulates the function of eosinophils in asthma. Among others, like RANTES and eotaxins, IL-5 is the most predominant cytokine, since this cytokine stimulates the maturation of the

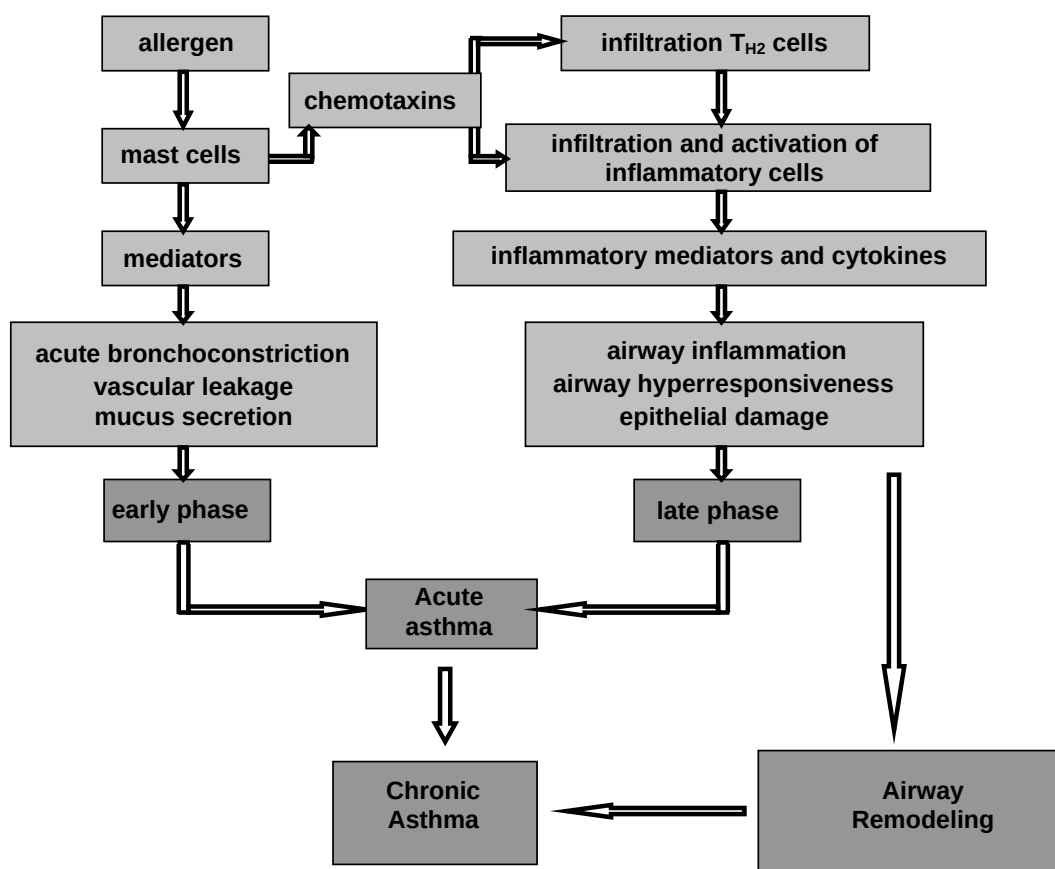


**Fig. (1). Schematic diagram of the pathogenesis of allergic asthma in sensitized subjects.** Upon second encounter of the allergen, cross-linking of FcεRI receptors on mast cells leads to the release of mediators and chemotaxins from these cells. The mediators cause acute bronchoconstriction, vascular leakage and mucus secretion (the early phase). Chemotaxins released by mast cells cause the infiltration of inflammatory cells, which in turn release inflammatory mediators and cytokines. This leads to asthmatic cardinal signs, such as chronic airway inflammation, airway hyperresponsiveness, epithelial damage and airway remodeling.

progenitor CD34<sup>+</sup> cells in mature eosinophils and the release of eosinophils from bone marrow to the circulation, and also prolongs their survival [26].

Macrophages are the most prevalent cells in the airways, both in airways from normal patients and asthmatics [27, 28]. However, the release of (inflammatory) mediators in asthma is greater than in patients without asthma [29]. Furthermore, macrophages are recruited to the airways of allergic subjects following allergen challenge [30]. Macrophages may play a pivotal role in asthma and allergic disease by directing the recruitment and activation of inflammatory cells in conjunction with activated T-lymphocytes, by generating factors that directly promote bronchospasm and tissue injury (e.g. reactive oxygen species), and by the production and release of cytokines that modify airway inflammation responses.

A considerable amount of evidence has accumulated over the last 10 years that the T-lymphocyte, particularly those with Th2 cytokines profiles, plays a key role in the pathogenesis of inflammation [31]. Disease severity can be correlated with airway inflammation and the presence of activated T cells and their products in lung tissue, BAL fluid and sputum [32]. The regulation and activation of eosinophils and mast cells by products of T cells, particularly cytokines released by CD4<sup>+</sup> Th2 cells, has been demonstrated [33-36]. Furthermore, the efficacy of immunosuppressants such as cyclosporin and steroids in resolving inflammation can be directly related to the down-regulation of T cells [37-39].

Interestingly, several studies found that allergic inflammation in asthma arises from an imbalance between Th1 and Th2 cells [40]. Th1 cells produce IL-2 and IFN- $\gamma$ , which are essential for cellular defence mechanisms. Th2 cells produce cytokines (IL-4, 5, 6, 9, and 13) that mediate allergic inflammation. Furthermore, there is reciprocal inhibition, in that Th1-type cytokines inhibit the production of Th2-type cytokines and vice versa [41, 42]. The so-called 'hygiene hypothesis' states that the rising incidence of asthma in developed countries is due to the declining exposure in childhood to both bacterial and viral infections (which stimulate Th1 responses), with a consequent polarization towards Th2 immune environment [43-45].

## AIRWAY HYPERRESPONSIVENESS

One of the key features of asthma is the development of airway hyperresponsiveness [46]. Airway hyperresponsiveness equates to an exaggerated bronchoconstrictor response, not only to allergens to which the subjects are sensitized, but also to a range of non-specific stimuli, including agents as diverse as cold air, fog or histamine [47]. Airway hyperresponsiveness can be quantified in humans by measurements of forced expiratory flow rates (FEV<sub>1</sub>) before and after the administration of increasing quantities of these agents. A number of studies have demonstrated that the degree of sensitivity of the airways to bronchoconstrictor agents correlates with the clinical severity of asthma [48]. However, how the hyperresponsive state is acquired is not exactly understood. At present, it is believed that airway hyperresponsiveness can result from the co-ordination of several mechanisms. A relationship exists between the inflammatory state of the airways, especially the prominence of eosinophils, and the

severity of hyperresponsiveness [25, 49, 50]. Accumulation of activated eosinophils, which release potent mediators, causes damage to the epithelial layer, which leads to bronchoconstriction [51]. Moreover, there is increasing evidence that airway hyperresponsiveness is a consequence of epithelial damage (see below) resulting from the accumulation of large numbers of activated eosinophils in the airway wall. In addition to eosinophils, T cells constitute a large proportion of the inflammatory cells within the lungs of asthmatics. Indeed, T-cell-mediated immune responses are believed to be important contributors to airway hyperresponsiveness in asthmatic patients through the release of chemokines and cytokines that enhance lung inflammation [48], favor the production of IgE [52], activate eosinophils [26, 32] and mast cells [53], and directly enhance airway hyperresponsiveness [32].

On the other hand, some studies pointed out the discrepancies between inflammation and AHR. In particular, Folkerts *et al.* showed that inhaled bradykinin is able to increase AHR to histamine in guinea pig model *in vivo* and that this increase is equipotent to the enhanced AHR to histamine produced by parainfluenza-3 virus infection [54]. The pretreatment with the bradykinin B2 receptor antagonist HOE 140 inhibited virus-induced AHR to histamine without affecting bronchoalveolar cell influx suggesting that bradykinin is a major factor in leading to AHR and that the presence of inflammatory cells is not a prerequisite to the development of AHR.

In recent study it has been shown that substance P plays a role in the induction of AHR in guinea pig model of allergic asthma which is most likely mediated by the neurotrophin nerve growth factor (NGF) *via* its high affinity receptor tyrosine kinase A (trkA) [55]. The tachykinin NK1 receptor antagonist significantly reduced the ovalbumin-induced AHR to histamine, both after the EAR at 5 h after challenge and after the LAR at 23 hours after challenge [56]. In addition, in the same study it has been shown that NK1 receptor antagonist significantly inhibited the allergen-induced infiltration of eosinophils, neutrophils and lymphocytes in the airways. These results suggest that tachykinin NK1 receptor is involved in the development of allergen-induced AHR to histamine associated with inflammatory cell infiltration in the guinea pig airways. In another study the same authors found that the tachykinin NK2 receptor activation is involved in the development of allergen-induced LAR (but not early) AHR to histamine and that NK2-mediated infiltration of neutrophils and lymphocytes in the airways may contribute to these effects [57]. In allergic guinea pig model, intranasal pretreatment with the specific PI3-Kinase inhibitor wortmannin abolished the AHR to histamine in association with a dose-dependent reduction in eosinophil peroxidase [58] activity, but without affecting BAL eosinophilia. These data suggest that wortmannin is a potent inhibitor of eosinophil degranulation and can prevent AHR in allergen-challenged guinea pigs [59].

Recent findings also showed that the tissue levels of IL-13 induced by allergen challenge of sensitized guinea pigs stimulate AHR and cellular (macrophages, lymphocytes, eosinophils and neutrophils) inflammation and that IL-13 is required for the expression of allergen-induced AHR in the guinea pig ovalbumin model [60].

Besides inflammation, airway remodelling, including smooth muscle hyperplasia and hypertrophy, collagen deposition and sub-epithelial fibrosis, might contribute to the development of airway hyperresponsiveness.

### EPITHELIAL CELLS

The airway is lined with epithelial cells. The airway epithelium is involved in asthma, since several studies have demonstrated abnormal epithelial histology and epithelial damage in asthmatics [61, 62]. Several reasons can account for the involvement of the epithelium. First, the epithelium acts as a physiological barrier to diffusion. After epithelial damage, inhaled contractile agonists, such as allergens, may reach the underlying smooth muscle more easily. Secondly, the epithelial layer protects nerves from stimulation by inhaled irritants. If the epithelium is damaged, the sensory nerves involved in neuropeptide release are exposed and bronchoconstriction is induced. Thirdly, the airway epithelium releases a variety of mediators, such as cytokines and chemokines and epithelium-derived relaxing factors (Epi DRFs) such as nitric oxide (NO) and prostaglandin (PG) E<sub>2</sub>. The latter two may protect the airways from excessive bronchoconstriction [63]. Therefore, damage or disturbed function of the epithelial layer, associated with decreased synthesis of epithelium-derived relaxing factors may contribute to the development of airway hyperresponsiveness in asthma [64].

### ACUTE AND CHRONIC OVALBUMIN MODELS

The most widely used ovalbumin model is that described by Andersson [65]. In this study the temporal development of the acute anaphylactic bronchoconstriction in guinea-pigs sensitized to ovalbumin by different regimens, including IgE-antibody promoting ones was investigated. Guinea-pigs sensitized with low amounts (1-10 micrograms) of ovalbumin together with alum produce the most pronounced bronchospasm when challenged with an intravenous injection of a low dose of antigen. Guinea-pigs sensitized with small amounts of antigen together with alum produced IgE and IgG1 antibodies. However, in sera from animals immunized with large amounts of antigen, only IgG1 antibodies could be detected. Recently, this model has been optimized by Smith & Broadley [66]. They demonstrated that a single sensitisation with 10 µg OA did not reveal an EAR, LAR or AHR following exposure to OA. However, total and differential cell counts (eosinophils and macrophages) were significantly greater 24 h post-challenge, when compared to saline-challenged sensitised animals. The addition of a booster injection of 10 µg revealed an EAR, but no LAR or AHR after ovalbumin inhalation. Surprisingly, there was a significant cell influx. Sensitisation with 100 µg OA (single and booster injections) revealed all four parameters of the asthmatic response (EAR, LAR, AHR and cell influx). The incorporation of the booster sensitisation injection resulted in a prolongation of the LAR, and the AHR was more pronounced and cell influx increased significantly, when compared to all other sensitisation protocols. Thus, sensitisation with 100 µg OA (with a booster injection) can reveal an EAR, LAR, AHR and cell influx following inhalation exposure to OA (10 µg).

Interestingly, the ovalbumin guinea pig model has been used to investigate age and sex differences [67]. In unsensitized animals, density of resident inflammatory cells as well as baseline pulmonary function differed with age and sex. Maximum OVA-induced eosinophilia in females occurred at a lower sensitizing dose of OVA than in males, and the slopes of the dose-response relationship differed significantly between sexes. Young females had more pronounced increases in eosinophils compared with some adult treatment groups. The concentrations of OVA-specific antibodies were not directly related to differences in cellular infiltration. Airway hyperresponsiveness to methacholine challenge was observed in all treatment groups. It can be concluded that young animals require major reductions in allergen exposure compared with adults to effectively limit airway inflammation in primary prevention. Heterogeneity of asthma symptoms seen with age and sex suggests that primary prevention by limiting allergen exposure or treatment with anti-inflammatory or bronchodilator drugs may be more effective strategies for specific age and gender populations [67].

Due to the gaining interest in airway remodeling, chronic models of asthma have been developed which has been described in detail by Meurs *et al.* [68]. To sensitize animals to the antigen with IgE-class antibodies, 100 mg Al(OH)<sub>3</sub> per ml of OA was injected (0.5 ml intraperitoneally and 0.5 ml intradermally divided over seven injection sites). After one month the animals received 12 weekly ovalbumin aerosol challenges. Increasing concentrations of 0.05%, 0.1%, 0.3%, 0.5% and 1.0% (w/v) OA solution in saline for 3 min, separated by 7-min intervals were performed. This induces airway remodeling such as an increase in airway smooth muscle mass in the noncartilaginous airways, hypercontractility of tracheal smooth muscle and an increased pulmonary contractile protein (sm-myosin) abundance [69-71].

### COCKROACH MODEL

To develop this semichronic asthma model, cockroach antigen is nebulized to a semiclosed acrylic chamber housing to immunize the animals. The aerosol exposure was carried out for a period of 45 min repeated twice a day, 5 days a week, for 4 weeks. After 4 weeks of immunization, development of anaphylactic sensitivity to cockroach antigen was confirmed by both allergic skin test and PCA test as described. Five days after the last aerosol exposure, the asthmatic responses *in vivo* were measured using a double chamber plethysmograph following antigen challenge (1 mg/ml × 10 ml) in conscious state. Immediately after completion of a 24-hour study of the lung function, bronchoalveolar lavage was performed to determine inflammatory state of the airway as described [72]. A comparative study was performed between cockroach and ovalbumin sensitization [73]. The cockroach antigen induced a dual-asthmatic response, whereas ovalbumin induced only an early asthmatic response. The average total of leukocytes in the lungs after cockroach was almost 10x higher (predominantly neutrophils) than after ovalbumin challenge (predominantly eosinophils). Tracheal smooth muscle responses to antigen-specific contractions were more brisk in the OA group than those of the cockroach group. Thus, cockroach induced a more severe and persistent late-phase inflammation than OA.

This appears to be related to an influx of neutrophils rather than anaphylactic bronchospasm.

### OCCUPATIONAL ASTHMA

Most, if not all, occupational respiratory low molecular weight (LMW) allergens known to date have been identified by recognition of asthmatic symptoms in exposed workers. The serious health problems of asthma together with the continuous introduction of new chemicals to workers and consumers (e.g. cleaning agents, epoxy glues, and hairdressing products; emphasize the importance of a predictive test or testing strategy for LMW chemicals [76]. Guinea-pig models have been developed for assessment of allergenicity of occupational agents [74-76], and has received rigorous review [1]. The model assesses a chemical's ability to cause airway hypersensitivity *via* a "relevant" route of exposure. The model utilizes inhalation as the route of exposure to the allergen, for both the sensitization and the elicitation phases of the response. Pulmonary function is measured using plethysmography. It allows observation of the breathing pattern of animals, which is used to detect airway constriction, the major parameter employed to indicate sensitization. A further benefit from use of whole body plethysmography is the ability to monitor breathing patterns continuously over a period of 24 h. Because airflow obstruction in patients may occur hours after cessation of exposure, the continuous monitoring of breathing pattern is an essential feature of the guinea-pig model to identify late-onset responses. Moreover, microvascular leakage is observed in this model [74].

### VIRUS-INDUCED ASTHMA

Virus-associated bronchial hyperresponsiveness is an important model for studying basic mechanisms in the pathogenesis of asthma. Epidemiologic studies have shown a close temporal association between respiratory viral infections and exacerbations of asthma. Abundant evidence indicates that viral respiratory infections induce airway hyperresponsiveness to bronchoconstrictor agents in asthmatic and healthy persons [77]. Likewise, guinea pigs inoculated intra nasally or intratracheally with parainfluenza or respiratory syncytial virus (RSV) showed an increased airway responsiveness to nonspecific stimuli showed [78]. Moreover, light microscopic and ultrastructural examination of the central airways of animals inoculated with virus revealed stratification of the epithelial lining, with pronounced loss of cilia and granule-depleted goblet cells. In the peripheral airways, typical lesions of patchy alveolitis and bronchiolitis were found. Virus inoculation resulted in a reduction in the number of airway mucosal mast cells, particularly in the bronchioles, and in a change of density of the granules of mast cells. Further, a significant rise (100%) in histamine concentration is observed in lung lavage fluid after virus inoculation, which indicates that mast cells are activated.

One of the pathways by which viruses trigger bronchoconstriction and airway hyperresponsiveness is by causing dysfunction of inhibitory  $M_2$  muscarinic receptors on the airway parasympathetic nerves. These receptors normally limit acetylcholine (ACh) release from the parasympathetic nerves. Loss of  $M_2$  muscarinic receptor function increases

ACh release, thereby potentiating vagally mediated bronchoconstriction. Viral infection causes an influx of macrophages into the lungs, and it has been shown that rhinoviruses induced activation of NF- $\kappa$ B and a time- and dose-dependent increase in TNF- $\alpha$  release by macrophages in conjunction with viral replication suggesting a role for macrophages, as an important source of proinflammatory cytokines, in the pathogenesis of virus-induced asthma [79] Fig. (2). Recently it has been studied the role of macrophages in virus-induced airway hyperresponsiveness. Guinea pigs infected with parainfluenza virus were hyperresponsive to electrical stimulation of the vagus nerves and in the above-cited animals the muscarinic agonist pilocarpine was not able to inhibit vagally induced bronchoconstriction suggesting  $M_2$  receptor dysfunction. Furthermore, in guinea pigs treated to be macrophage-depleted viral infection did not cause airway hyperresponsiveness and  $M_2$  receptor dysfunction suggesting that macrophages mediate virus-induced airway hyperresponsiveness [80].

In virus-induced asthma the intensity of the inflammatory response has been directly correlated to the expression of chemokines by virally infected pulmonary structural cells (e.g. epithelial cells), resident immune cells and infiltrating leukocytes. A number of chemokines, including CXCL8 (IL-8), CCL3 (macrophage inflammatory protein 1a) and CCL5 (RANTES) have incriminated as major mediators released during respiratory viral infection, and the level of these chemokines correlates with the severity of the disease. Many studies collectively indicate an intimate connection between chemokine expression and respiratory viral infections [81]. A recent study showed that RSV can establish persistent infection of guinea pig lung regardless of host Th1/Th2 background; however a host Th1 background limits the extent of virus-associated AHR and airway inflammation, whilst Th2-skewed guinea pigs had virus-associated AHR and significantly greater levels of airway T cells and eosinophils. Heterogeneity in virus-host interactions may be relevant to understanding why some children hospitalized for RSV bronchiolitis go on to develop recurrent wheezing/asthma symptoms [82].

### INFLAMMATORY CELL INFILTRATION AND AIRWAY REMODELING IN ASTHMA

Although airway inflammation and airway hyperreactivity are observed after allergen inhalation both in allergic humans and guinea pigs, little is known about the mechanisms by which inflammatory cells can contribute to allergen-induced airway hyperreactivity. However, cytokines, chemokines and T lymphocytes are likely to be involved.

Airway inflammation is already observed 3-6 h post challenge. Westerhof *et al.* [83] investigated the inflammatory cell infiltration in different airway compartments at 6 and 24 h. Adventitial eosinophil and neutrophil numbers in both bronchi and bronchioli significantly increased at 6 h post-allergen provocation, while there was a strong tendency to enhanced eosinophils in the bronchial submucosa at this time point. At 24h CD3+ T lymphocytes increased in the adventitial compartment of the large airways and in the parenchyma at 24h post-allergen, while numbers of CD8+ cells did not differ. The results indicate that, after allergen provocation,

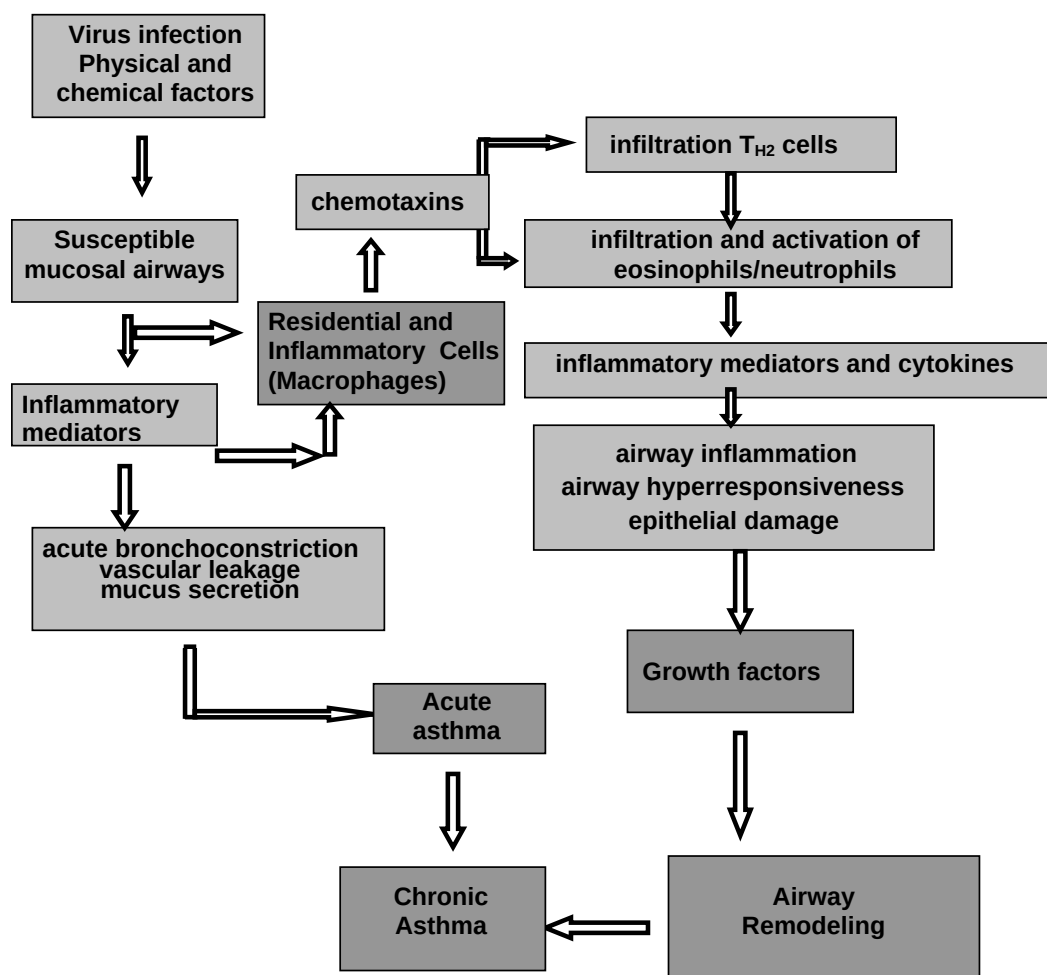


**Fig. (2). Schematic diagram of the pathogenesis of non-allergic asthma.** Virus infection and physical/chemical agents in susceptible airways may provoke the release of peptide and non-peptide inflammatory mediators (kinins, prostaglandins, leukotrienes, neuropeptides etc.) provoking acute bronchoconstriction, plasma exudation and mucus secretion. These mediators activate residential (epithelial cells, fibroblasts, smooth muscle cells) and inflammatory cells (e.g. macrophages) in releasing chemotactic factors (e.g. RANTES, eotaxins, LTB<sub>4</sub> and IL-8) with the infiltration of Th<sub>2</sub> cells and eosinophils/neutrophils provoking chronic airway inflammation, airway hyperresponsiveness, epithelial damage and additionally the above-cited mediators with the products (proteases, oxidants etc.) released by various inflammatory cells may induce the release of growth factors with the formation of airway remodeling, a feature of chronic asthma even at very young age.

inflammatory cell numbers in the airways are mainly elevated in the adventitial compartment.

Many researchers measured the number of inflammatory cells in the bronchoalveolar lavage fluid. As in healthy human airways, eosinophils are present in the airways of guinea pigs under basal conditions. At 24h after ovalbumin challenge a tremendous increase in the number of macrophages and eosinophils are observed, where the eosinophils may even outnumber the macrophages [66, 84]. Eotaxin, leukotrienes and interleukin-5 may be responsible for the attraction of eosinophils in this model. Eotaxin is an eosinophil-specific chemoattractant originally identified in bronchoalveolar lavage fluid after allergen challenge of sensitized guinea-pigs. Li *et al.* [85] determined and quantified the cellular sources of guinea-pig lung eotaxin and localized gene expression in structural cells of large and small airways and in alveolar macrophages. Compared with naive animals, there was a fivefold increase of eotaxin protein and a 25 fold upregulation of eotaxin gene expression in the airway epithelium 3 h after ovalbumin challenge. The average percentages

of alveolar macrophages staining for eotaxin protein increased significantly in ovalbumin-challenged animals. The upregulation in bronchial epithelium and alveolar macrophages showed a strong positive association. The results indicate that there are multiple cellular sources of guinea-pig lung-derived eotaxin, including bronchial and bronchiolar epithelial cells, airway smooth muscle, bronchial vascular endothelium, and chondrocytes and alveolar macrophages, and that there are relatively rapid and marked increases of eotaxin protein and gene expression in airway epithelium and alveolar macrophages following allergen challenge.

Further, immunoreactive eotaxin levels rose in airway tissue and BAL fluid to a peak at 6 h falling to low levels by 12 h [86]. Eosinophil numbers in the tissue correlated with eotaxin levels until 6 h. In contrast, few eosinophils appeared in BAL over the first 12 h, major trafficking through the airway epithelium occurring at 12-24 h when eotaxin levels were low. Allergen challenge of the lung resulted in a rapid release of bone marrow eosinophils into the blood. An antibody to IL-5 suppressed bone marrow eosinophil release and

lung eosinophilia, without affecting lung eotaxin levels. Thus, IL-5 and eotaxin appear to cooperate in mediating a rapid transfer of eosinophils from the bone marrow to the lung in response to allergen challenge [86]. In addition to the eotaxin, leukotrienes may collaborate with IL-5. Nabe *et al.* [87] demonstrated that the leukotrienes produced after antigen provocation sequentially induce IL-5 production from some immune component cells *via* leukotriene receptor activation. Thus, it is likely that leukotrienes indirectly cause antigen-induced eosinophilia through IL-5 production. Therefore, a leukotriene-receptor antagonist such as Montelukast, may not only directly prevent the mode of action of leukotrienes but may also indirectly prevent the release of IL-5. Besides the effect of Montelukast on IL-5 levels, it may also influence the production of IL-10 [88]. IL-10 is an important immunoregulatory and anti-inflammatory cytokine that may interfere with nuclear factor-kappaB (NF-kappaB) which plays an important role in the pathogenesis of asthma. Montelukast, prevented the decrease of IL-10 in bronchoalveolar lavage fluid and inhibited the activation of NF-kappaB in lung tissues of ovalbumin challenged guinea pigs. There is a significantly negative correlation between the level of IL-10 and the activation of NF-kappaB in lung tissues. This may be new important mechanisms of Montelukast's anti-airway-inflammation effects in asthmatic guinea pigs [88]. Another cytokine that may suppress airway inflammation is interferon (IFN)- $\beta$ . Satoh *et al.* [89] investigated the anti-asthmatic effects of IFN- $\beta$  in ovalbumin challenged guinea pig. IFN- $\beta$  suppressed the eosinophil infiltration and the increase in respiratory resistance. Moreover, IFN- $\beta$  inhibited the LAR. These data suggest that IFN- $\beta$  would have some clinical benefit for patients with asthma.

The recruitment of leukocytes to sites of inflammation is dependent on the expression and function of a class of receptors called cell adhesion molecules. They are expressed on both the surface of leukocytes as well as the endothelial cells that line blood vessel walls. Leukocytes loosely associate and "roll" on endothelial cell layers that have been activated by cytokines such as IL-1 or tumor necrosis factor- $\alpha$ . The plasma concentrations of soluble intracellular cell adhesion molecule (sICAM-1) and P-Selectin are increased in ovalbumin challenged animals [90]. After administration of rhIL-1 receptor antagonist solution as ultrasonic spraying, the asthmatic symptoms in guinea pigs were obviously attenuated. The plasma sICAM-1 and P-selectin levels in asthmatic guinea pigs were each dose-dependently reduced with the increase of rhIL-1ra dose.

Oxidative stress, resulting from an increase in local (airway) and systemic reactive oxygen species (ROS) production and relative antioxidant systems deficiency, has been also implicated in asthma pathophysiology [91]. Increased amount of ROS have been reported in exhaled breath condensates as well as in airway eosinophils and macrophages from asthmatic patients [92], along with decreased levels of antioxidants (vitamin C and E) in lung fluid and plasma [93]. ROS can lead to airway hyperresponsiveness by increasing airway smooth muscle contractility and by inducing airway wall edema, epithelial desquamation and mucus plugging. The microsomal enzyme heme oxygenase (HO), catalyzing the oxidation of heme to biliverdin and carbon monoxide (CO), is involved in the protection against airway inflamma-

tion and oxidative aggression [94]. Three main isoforms have been identified: the inducible form HO-1 (also known as heat shock protein), and the two constitutive isoforms HO-2 and HO-3. In the ovalbumin-immunized and multiple aerosol challenged guinea pigs airway inflammation, oxidative stress and HO activity were increased compared to the control group, and the HO upregulation by repeated intraperitoneal administration of the HO-1 inducer hemin significantly decreased airway inflammation and oxidative stress in ovalbumin-treated animals suggesting that upregulation of HO pathway has a protective effect against oxidative stress in a guinea pig model of allergic asthma [95].

In the chronic ovalbumin model airway inflammation is present as well; however, researchers concentrate especially on airway remodeling and mucus secretion [71]. Ovalbumin-sensitized guinea pigs challenged for 12 weeks with aerosolised ovalbumin, demonstrate airway smooth muscle thickening, hypercontractility of tracheal smooth muscle, increased pulmonary contractile protein (smooth-muscle myosin) abundance, mucous gland hypertrophy, an increase in mucin 5 subtypes A and C (Muc5AC)-positive goblet cell numbers and eosinophilia. It is likely that the chronic inflammation contributes to the airway remodeling. However, in the acute model it was demonstrated that enhanced mucin gene expression appears to occur before eosinophil infiltration [96]. Levels of both Muc2 and Muc5ac mRNA in lungs of OVA-sensitized guinea pigs increased significantly by 30 min after an acute exposure to OVA. In addition, levels of eotaxin mRNA also increased in these tissues, but the increases were not significant until 2 h after challenge. Correspondingly, the number of eosinophils in bronchoalveolar lavage fluid did not increase until 4 h postchallenge, as also mentioned above in another study.

In sensitized guinea pigs at different stages of maturation (juvenile, adult and old animals) chronic exposures to antigen (twice a week for 6 weeks) induced airway wall thickening regardless of their stages of maturation [97]. In particular, the thickness of the inner wall area increased in juvenile and adult animals, whilst smooth muscle area increased only in old guinea pigs suggesting that the development of antigen-induced airway remodeling is partly modified by the degree of maturation [97].

In guinea pig asthma model, ovalbumin-challenged sensitized animals showed high levels of matrix metalloproteinase 9 (MMP-9) in both structural and inflammatory cells in lung biopsies compared to control animals. These results implied that MMP-9 may contribute to tissue remodeling in asthma and both structural and inflammatory cells are the main cellular sources of MMP-9 [98].

In an experimental model of chronic allergic pulmonary inflammation in guinea pigs after antigen challenge the pre-treatment with the non-specific Nitric Oxide synthase inhibitor L-NAME increased total pulmonary resistance and collagen deposition in the airways whilst the administration of the specific iNOS inhibitor reduced total pulmonary resistance, eosinophilic recruitment and collagen and elastic fibers content in the airways. This study indicates that the selective blockage of iNOS attenuates bronchoconstriction and inflammatory-remodeling process [99].

Thrombin, a serine protease activated at the sites of inflammation and abundant in BAL fluid, is both a contractile agonist and a mitogen of airway smooth muscle. Recently, it has been shown that tyrosine kinase inhibitors, mitogen-activated protein kinase (MAPK) inhibitors and phosphatidylinositol 3-kinase (PI3K) inhibitor significantly attenuated thrombin-induced airway smooth muscle proliferation in guinea pigs. This study suggests that thrombin-induced protease activated receptor (PAR) stimulation may activate Src and Syk tyrosine kinases or transactivate EGFR, via an autocrine/paracrine release of EGF-like ligands at the cell surface, leading to the activation of the downstream MAPK signaling cascade and subsequently to smooth muscle proliferation [100].

### MICROVASCULAR LEAKAGE

Airway wall remodelling in asthma involves a number of changes including increased vascularity, vasodilation and microvascular leakage. Evidence suggests that the number and size of bronchial vessels is increased in patients with asthma compared with normal controls. In particular, there may be increased numbers of vessels in patients with fatal asthma. Treatment with inhaled corticosteroids is now known to reduce this vascularity. Bronchial vessels may undergo proliferation in response to inflammatory stimuli. Many factors can induce angiogenesis including a range of mediators and growth factors. Others can cause a vascular response by causing vasodilation and microvascular leakage. Airway wall oedema is likely to be important in asthma but has not yet been quantified. It is thought that mast cells play a key role in modulating these vascular remodelling changes by releasing cytokines and growth factors [101].

In guinea pigs, ovalbumin challenge results in leakage in trachea, intrapulmonary airways, and total lung (parenchyma and intrapulmonary airways) which can be prevented by leukotriene antagonists [102] or potassium channel openers [103]. While the guinea pig is the preferred choice for use as a model of allergic bronchial asthma in the evaluation of anti-asthmatic drugs, it has been shown that antigen-induced bronchoconstriction in guinea pigs is attenuated by epinephrine released from the adrenal gland. In order to investigate the possible influence of the adrenal gland on the effects of antiexudative and bronchodilative drugs on antigen-induced airway responses, Ikezono *et al.* [104] examined the inhibitory effects of procaterol, a selective beta(2)-adrenoceptor agonist, on antigen-induced airway microvascular leakage and bronchoconstriction in adrenalectomized guinea pigs and compared them with the drug's effects in sham-operated animals. Procaterol dose-dependently inhibited OA-induced airway microvascular leakage, and its effects were more potent in adrenalectomized animals than in sham-operated animals. Although the plasma concentration of epinephrine during OA inhalation was approximately 3 times higher than that during saline inhalation in sham-operated animals, no difference was seen in adrenalectomized animals. Therefore, while procaterol essentially possesses pronounced inhibitory effects on antigen-induced airway microvascular leakage and bronchoconstriction in guinea pigs, the effects are considerably masked by epinephrine released from the adrenal gland.

The actions of tachykinins are terminated by the proteolytic cleavage that is mainly due to neutral endopeptidase

[105], a membrane-bound enzyme present on various types of airway cells. Therefore, the effects produced by exogenous or endogenous tachykinins in the airways are exaggerated by inhibiting NEP activity. Plasma extravasation following exposure of either the trachea or nasal mucosa of sensitized guinea pigs to antigen is increased by pretreatment with phosphoramidon, a high affinity NEP inhibitor, indicating that one or more peptides metabolized by NEP are involved in this response [106, 107]. Of note, phosphoramidon enhances the increase in plasma extravasation in the trachea 10 min (or 15 min in the nasal mucosa) after antigen exposure, but not 5 min before, suggesting that NEP-sensitive peptides are not involved in the immediate response, and that a relative delay between antigen provocation and peptide-mediated plasma extravasation [108]. In the guinea pig, plasma extravasation in the trachea induced by exogenous or endogenous tachykinins is mediated by NK1 receptor activation. Using the selective NK1 receptor antagonist CP96345, the antigen-induced plasma extravasation was reduced 10 min after allergen challenge and this inhibition was greater in the presence of phosphoramidon [106]. Similar findings have been obtained in the guinea pig nasal mucosa where CP96345 markedly reduces plasma extravasation induced by the topical application of ovalbumin in the nostril of sensitized animals [107]. The involvement of tachykinins is not evident immediately after antigen challenge suggesting that the participation of tachykinins in the anaphylactic reaction of the airways is indirect. Many inflammatory mediators are released early in the anaphylactic reaction including histamine, leukotrienes and kinins and all of these mediators may release sensory neuropeptides [108]. In the guinea pig trachea and nasal mucosa most of the tachykinin-evoked plasma extravasation caused by antigen challenge is due to kinins, which are first released by antigen and then act on sensory nerves to release neuropeptides [107, 109].

### AGONIST-INDUCED BRONCHOCONSTRICTION

Many inflammatory mediators, nerves-derived agonists and chemical (e.g. low pH) or physical stimuli are able to provoke acute bronchoconstriction in guinea pig airways Fig. (3). The classical direct pharmacological stimuli used for inducing acute bronchoconstriction either in guinea pig or in humans are histamine and cholinergic agonists (acetylcholine and methacholine). Both agonists are able to directly provoke airway smooth muscle contraction mainly via the activation of their own receptor subtypes: histamine H<sub>1</sub> and muscarinic M<sub>3</sub> receptors respectively. In guinea pig the density of muscarinic M<sub>3</sub> receptors in the lung tissue of ovalbumin-sensitized animals was higher than in control group suggesting a major role for this receptor subtype in allergen-induced dysfunction of the bronchial tone [110]. Besides histamine and acetylcholine another mediator, the nonapeptide bradykinin, has been extensively studied in guinea pigs because of the complexity of its action in evoking bronchoconstriction *in vivo*.

Bradykinin, which is formed from a plasma precursor, participates in the allergic inflammatory response because increased kinin levels have been detected in early and late phase reactions in guinea pigs [111]. Administration of bradykinin induces bronchoconstriction in guinea pigs by multiple mechanisms which depend on the route of admini-

stration of the peptide [112]. Most of the response to aerosolized bradykinin is due to activation of neural pathways, e.g. cholinergic reflexes and axon reflexes and the subsequent release of tachykinins [112]. A minor component is apparently due to the direct action of bradykinin on smooth muscle cells. The intravenous administration of bradykinin causes bronchoconstriction by activating cholinergic reflexes and by releasing prostanoids. Furthermore, bronchodilator catecholamines are released from the adrenal medulla following intravenous bradykinin [113]. Irrespective of the method of administration, bradykinin is also able to release the bronchoprotective epithelium-derived nitric oxide (NO) by activation of the enzyme NO synthase (NOS) [114, 115] Fig. (3). NO is formed by NOS which is structurally differentiated in 3 isoforms: 1) endothelial constitutive NOS (eNOS); 2) inducible NOS [112]; 3) neuronal NOS (nNOS). Bradykinin, after coupling with the B2 receptor, is able to increase intracellular calcium levels in epithelial cells and subsequently to activate the calcium-dependent constitutive isoforms of NOS with the rapid release of small amounts of bronchoprotective NO [116].

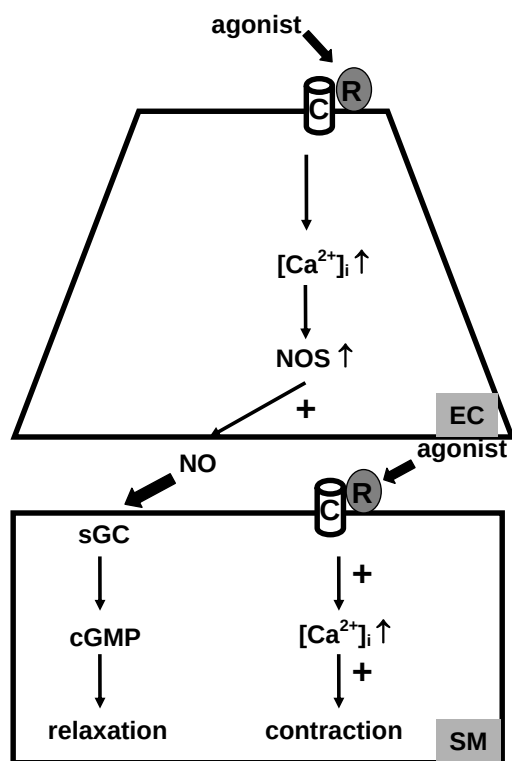


**Fig. (3). Effect of agonist-stimulation on airway smooth muscle contractions.** In normal situations, there is a balance between calcium influx in the smooth muscle (SM) leading to a contraction on one hand and calcium influx in the epithelial cell (EC) which results in production of NO, leading to relaxation of the smooth muscle on the other hand (biochemical antagonism). In cases of NO deficiency or epithelial damage this route of antagonism is disrupted, which leads to an enhanced smooth muscle contraction or airway hyperresponsiveness. R receptor, C calcium channel,  $[Ca^{2+}]_i$  intracellular calcium concentration, NOS nitric oxide synthase, sGC soluble guanylate cyclase, cGMP cyclic guanosine monophosphate.

Cold air and citric acid can also stimulate the sensory nerves C and A $\delta$ -fibers provoking different effects in the

airways including cough. Tachykinins released from the capsaicin-sensitive sensory nerve endings so-called C-fibers in the guinea pig airways induce a series of inflammatory responses referred to as "neurogenic inflammation" [117]. Tachykinins are the peptide transmitters substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) belonging to a large family of small peptides that share a common C-terminus sequence [118]. Tachykinins stimulate 3 types of receptors, namely the NK<sub>1</sub>, NK<sub>2</sub> and NK<sub>3</sub> receptors. NK<sub>1</sub> receptors are activated by all 3 tachykinins with similar potency, whereas NK<sub>2</sub> and NK<sub>3</sub> receptors are preferentially activated by NKA and NKB respectively [119]. SP and NKA released from terminals of capsaicin-sensitive primary sensory neurons in peripheral tissues cause all the major signs of inflammation by means of activation of NK<sub>1</sub> and NK<sub>2</sub> receptors, including plasma protein extravasation in post-capillary venules (NK<sub>1</sub>), arteriolar vasodilation (NK<sub>1</sub>), leukocyte adhesion to the endothelium of postcapillary venules (NK<sub>1</sub>) and activation of inflammatory cells (NK<sub>1</sub>). In the guinea pig airways tachykinins may also cause contraction (mainly NK<sub>2</sub>, but also NK<sub>1</sub>) or relaxation (NK<sub>1</sub>) of smooth muscle cells, secretion from seromucous cells (NK<sub>1</sub>), hyperresponsiveness after a variety of stimuli (NK<sub>2</sub> and NK<sub>1</sub>) and cough. Using guinea pig model of acute bronchoconstriction it has been demonstrated the major role of tachykinins and particularly of the NK<sub>2</sub> receptor on citric acid- and cold air-induced bronchoconstriction [120, 121]. Additionally, the bronchoconstriction evoked by citric acid or cold air was halved by the pre-treatment with the bradykinin B2 receptor antagonist HOE140 suggesting a local formation of kinins in the airways after exposure to acid or cold air and a significant involvement of locally formed kinins in acute bronchoconstriction induced by airway acidification and cooling. Thus, bradykinin (endogenously formed by the above-cited chemical and physical stimuli) may amplify acute bronchoconstriction indirectly by increasing the release of tachykinins through activation of C-fibers. In recent studies it has been established that citric acid-induced non-cholinergic bronchoconstriction in guinea pigs was attenuated by pretreatment with either a mast cell degranulating agent compound 48/80 or a mast cell stabilizer cromolyn sodium [122] and citric acid inhalation caused an increase in BAL histamine level suggesting the activation of mast cells by citric acid. Both leukotriene synthesis inhibitor and histamine H1 receptor prevented citric acid-induced bronchoconstriction indicating that the involvement of mast cells in citric acid induced airway constriction may be mediated via leukotrienes and histamine which subsequently may activate tachykinin-containing afferent C-fibers [123].

In sensitized guinea pigs the combination of NK<sub>2</sub> and NK<sub>1</sub> antagonists markedly reduced the bronchoconstriction induced by low dose of antigen and partially inhibited the delayed component of the bronchoconstriction induced by a single high dose of antigen [124] suggesting that tachykinins released following antigen challenge are involved in anaphylactic-induced bronchoconstriction. There is no evidence that the antigen-antibody complex *per se* stimulates sensory nerves, but the experimental observations suggest that mediators, such as bradykinin, released during an early phase of anaphylaxis are involved in the stimulation of capsaicin-sensitive sensory nerves [125].

In sensitized guinea pigs, an antigen challenge can initiate the release of inflammatory mediators such as histamine, kinins, tachykinins, leukotrienes and prostaglandins in the presence of cells such as macrophages and mast cells producing contraction of smooth muscle and bronchospasm (early asthmatic response). T cells can also be activated by the allergen to release cytokines and tumor necrosis factor resulting in the recruitment of eosinophils to the airways. Eosinophils and their mediators are involved in the development of the LAR following allergen challenge. In a recent study has been shown that an early and late (at 6 hours) bronchoconstrictor response in conjunction with AHR to histamine (12 hour post-challenge) and with the increase of macrophage cell counts is produced following low dose (3 mM) of adenosine in sensitized guinea pigs but not in unsensitized animals [2]. Furthermore, after exposure with high dose of adenosine (300 mM) sensitized guinea pigs showed an early bronchodilatation followed by late bronchoconstriction a 6 hours. AHR to histamine occurred after 12 hours in association with an increase in eosinophils and macrophages. It is known that the immediate bronchoconstrictor response (EAR) to adenosine is mast cell dependent and both  $A_{2B}$  and  $A_3$  receptors may mediate mast cell activation [126] which then results in the release of histamine from mast cells. The bronchodilator effect following exposure to high dose of adenosine has been reported in another study [127] and it has been assigned to the activation of  $A_{2A}$  subtype on the airway smooth muscle [128].

In a more recent study it has been shown that the inhibitor of p42/44 mitogen-activated protein kinases (MAPK), but not p38 MAPK, can reduce antigen-induced release of peptide leukotrienes leading to a rapid resolution of anaphylactic bronchial contraction, and may have therapeutic potential for allergic asthma [129].

## BRONCHORELAXANT PATHWAYS

### Neural Pathways

Cholinergic and adrenergic systems modulate the bronchomotor tone together with the non-adrenergic non-cholinergic neural (NANC) system which mediates contraction (excitatory NANC [eNANC]) or relaxation (inhibitory NANC [iNANC]) of airway smooth muscle [130]. Nitric Oxide (NO) is a neurotransmitter of iNANC system, and nitrgergic neurotransmission is present in several organs including the airways. Immunostaining studies demonstrated that nNOS is localised into nerves of guinea pig and human airways which supply vessels, smooth muscle and lamina propria [131]. NOS immunoreactive neurons are found in parasympathetic ganglia and also in sympathetic and sensory (more in jugular than in nodose) ganglia supplying the airways [132]. They are more prominent in proximal than in distal airways in agreement with the distribution of iNANC functional responses. NO is released from peripheral nerves by nNOS and is activated by calcium entry when the nerve is depolarised.

NO mediates approximately half of the iNANC (relaxant) response in guinea pig trachea *in vitro*, and the neuropeptide vasoactive intestinal peptide (VIP) should be involved in the second half of iNANC relaxant response [133]. VIP-mediated guinea-pig airway smooth muscle relaxation is preceded

by release of S-nitrosothiols into the airways [134]. The human iNANC response in central and peripheral airways is completely mediated by NO [135]. The neurons, which release NO, are probably part of the cholinergic pathway. However, stimulation of the preganglionic cervical vagus nerve in an *in vitro* guinea-pig tracheal tube preparation did not cause NO-mediated bronchodilatation, while activation of postganglionic intrinsic nerves provoked bronchodilatation suggesting that NO-dependent NANC relaxations of the airways are mediated by post-ganglionic parasympathetic nerves [136]. It has also been shown that a NO-dependent component of non-cholinergic parasympathetic nerves modulates airway smooth muscle tone at baseline, pointing out the spontaneous activity of non-cholinergic nerves during tidal breathing [137]. Fischer and co-workers provided the first evidence that NOS-immunoreactive neurons intrinsic to the guinea pig oesophagus project axons to the adjacent trachealis, showing that these neurons could be the postganglionic parasympathetic neurons mediating iNANC relaxation of the trachealis [138]. Furthermore, inhibition of nitric oxide synthase potentiates cholinergic neural bronchoconstriction without affecting neural acetylcholine release [139], suggesting that nNOS-derived NO is a functional antagonist to excitatory cholinergic pathway at post-junctional level.

Physiologic and morphologic studies of iNANC nerves indicate that they represent a distinct parasympathetic pathway from the well-characterised cholinergic-parasympathetic pathways innervating the airways [140]. Consequently, it seems likely that interactions between these nerve pathways occur postjunctionally and are manifested through their opposing actions on airway smooth muscle. In particular, Canning *et al.* observed that stimulation of capsaicin-sensitive visceral afferent fibers activates, upon peripheral release of tachykinins, iNANC neurons innervating guinea pig trachealis *via* activation of both  $NK_3$  and  $NK_1$  receptors [141]. It has also been observed that endogenous NO released in association with nerve stimulation regulates the magnitude of eNANC response in guinea pig airways [142]. Finally, neural NO-induced relaxation is impaired in guinea pig airways after allergen exposure, without affecting nNOS expression, suggesting a reduced neural NOS activity when allergic inflammation is exacerbated.

In guinea pig tracheal preparations it has been shown that endogenous arginase activity attenuates iNANC nerve-mediated NO production and airway smooth muscle relaxation under basal conditions, *via* competition with nNOS for the substrate of NO L-arginine [143]. In a more recent study it has been shown that allergen challenge reduced iNANC relaxations that were not affected by the NOS inhibitor L-NNA indicating that a deficiency of nNOS-derived NO underlies the impaired iNANC relaxations. Furthermore, inhibition of arginase activity restored the impaired iNANC relaxations indicating that increased arginase activity strongly restricts iNANC nerve mediated airway smooth muscle relaxation [144].

### Non Neural Pathways

Endogenous NO is also able to modulate excitatory airway responses induced by different mediators in animal

models. Nijkamp *et al.* showed in guinea pigs that aerosolised NOS inhibitors enhanced bronchoconstriction induced by increasing intravenous doses of histamine *in vivo* [145] suggesting a modulator role for endogenous NO in airway reactivity Fig. (2). Furthermore, Ricciardolo *et al.* found a L-arginine/NO-dependent modulation of bradykinin-induced bronchoconstriction in guinea pigs that originates independently from the simultaneous activation of the excitatory neural component: post-ganglionic cholinergic nerves and capsaicin-sensitive afferent nerves [114]. It has also been noted that after acid inhalation tachykinin- and bradykinin-mediated bronchoconstriction in guinea pigs is reduced by endogenous release of NO [120]. The NK<sub>1</sub> receptor is likely to be responsible for bronchoprotective NO release in the airways after tachykinin stimulation [146]. Interestingly, bronchoconstriction provoked by stimulation of protease activated receptor-2 (PAR-2), after intratracheal instillation or intravenous injection of trypsin or the tethered ligands for PAR-2, was inhibited by tachykinin antagonists and potentiated by NOS inhibitor [147] suggesting the involvement of tachykinin-containing sensory nerves to activate the bronchoconstrictor mechanism and the release of NO to obtain the counteracting relaxant effect.

Different groups of investigators have shown that acute bronchoconstriction induced by allergen inhalation is potentiated by NOS inhibitors in sensitised guinea pigs *in vivo*, suggesting a modulation by endogenous protective NO on early asthmatic reaction in animal model [148, 149]. Other *in vivo* studies in guinea pigs have shown that the enhanced airway reactivity induced by allergen (6 hours after exposure) is not further potentiated by pre-treatment with NOS inhibitors [150, 151] and that virus-induced airway reactivity is completely blocked by low doses of inhaled L-Arginine (NO substrate) suggesting that allergen- or virus-induced airway hyperreactivity is due to the impairment of endogenous release of protective NO. More specifically, it can be postulated that a deficiency in eNOS-derived NO contributes to the increased airway reactivity after EAR to allergen (4-6 hours), whilst a recovery in iNOS-derived NO production aids the reversal of airway reactivity after the LAR (24-48 hours) in guinea pigs. This is supposed by the lack of effect of the specific iNOS inhibitor aminoguanidine on airway reactivity to histamine after EAR and by a significant potentiation of the partially reduced airway reactivity to histamine after the LAR induced by inhalation of the specific iNOS inhibitor aminoguanidine [151]. More recently, it has been noted that expression of nNOS is reduced at 6 h, but not at 24 h after allergen challenge in association with a decrease in constitutive NOS activity and in the amounts of exhaled NO. Together with maximal airway hyperresponsiveness to histamine this suggests that the transient down-regulated nNOS may have a role in airway hyperresponsiveness [152]. In agreement with the previous studies, Toward and Broadley found that exposure to inhaled LPS initially inhibited NO synthesis and the reduced NO levels coincided with the period of increased airway reactivity to histamine (1 hour after exposure) in guinea pig [153]. In contrast, 48 h after LPS exposure the bronchoconstrictor response to histamine was attenuated (airway hyporesponsiveness) in association with increased levels of NO metabolites in the bronchoalveolar lavage fluid. This suggests a renewal of NO synthesis proba-

bly derived by cytokine-induced NF- $\kappa$ B activation of iNOS gene, with a bronchial relaxant effect.

Several mediators including bradykinin, substance P and endothelin-1, applied to the inside of intact tracheal tubes, provoke concentration-dependent relaxations [154]. These agonist-mediated relaxations are reversed into contractions (or contractions are markedly potentiated) by NOS inhibitors, indicating that the relaxant effect in the airways is mediated by the release of endogenous NO [115, 155] suggesting that airway epithelium releases NO, which counteracts smooth muscle contraction induced by different spasmogens. These striking results demonstrate the functional importance of epithelium in airway reactivity, not merely considered as a physical protective barrier between spasmogens and smooth muscle but as a modulator of bronchomotor tone *via* the release of relaxant substances (so-called epithelium-derived relaxing factors). Treatment of guinea pig trachea *in vitro* with an inactivator of guanylyl cyclase caused a 5-fold increase in the sensitivity to histamine contractile response indicating the involvement of NO/cGMP pathway in the development of airway hyperresponsiveness [156]. A further study showed that the electrochemical detection of bradykinin-induced NO release in guinea pig airways was fast (duration: ~ 2 sec), mainly dependent on the epithelium and absent in Ca<sup>2+</sup>-free medium. Suggesting that a Ca<sup>2+</sup>-dependent eNOS pathway seems to be involved in the endogenous release of bronchoprotective NO [116].

The subsequent step of epithelial-derived NO release is the paracrine effect on airway smooth muscle that is dependent on cGMP increase in the effector cell. In fact, it has been shown that bradykinin raises significantly cGMP levels in guinea pig airways and that this effect is blocked by the pre-treatment with NOS inhibitors and in epithelium-denuded preparations. This suggests that cGMP is the final mediator of the bronchoprotection dependent on epithelium-derived NO in this species [115].

Meurs *et al.* showed that polycation-induced airway hyperreactivity to methacholine is dependent on the deficiency of endogenous NO, suggesting that polycationic peptides released by activated eosinophils in the inflamed airways may contribute to the deficiency of bronchoprotective NO [157]. In a further study these authors found that endogenous arginase activity potentiates methacholine-induced airway constriction by inhibition of NO production in naive guinea pig, presumably by competition with eNOS for the common substrate L-arginine [158]. In an elegant study Ten Broeke *et al.* showed that calcium-like peptides (CALP1 and CALP2) targeting calcium binding EF hand motif of calcium sensors (calmodulin and calcium channels) may have a role in regulating airway responsiveness by controlling [Ca<sub>2</sub><sup>+</sup>]<sub>i</sub> and, consequently, modulating the activity of eNOS [159]. In fact they observed that CALP2 inhibition of CALP1-induced airway hyperresponsiveness was Ca<sup>2+</sup>-epithelium-dependent and NO-mediated. Interestingly, they found that bradykinin-induced [Ca<sub>2</sub><sup>+</sup>]<sub>i</sub> increase in epithelial cells was markedly higher after incubation with CALP2. In allergen-challenged guinea pigs, the enhanced contractile response to agonists in tracheal preparations after early reaction was not augmented by NOS inhibition as shown in naive animals suggesting an impairment of protective NO [160]. In a further study the

same authors showed that L-arginine administration reduced methacholine-induced contraction in isolated perfused tracheae from guinea pigs indicating that limitation of the substrate may underlie the reduced eNOS activity and the excessive contractile response [161]. Finally, it has also been demonstrated that increased arginase activity contributes to allergen-induced deficiency of eNOS-derived NO and airway hyperresponsiveness after early allergen reaction in guinea pigs, presumably by direct competition with eNOS for L-arginine [162]

## GENERAL CONCLUSIONS

The guinea pig has a number of advantages to be used for respiratory research. It is a small docile and relative inexpensive animal. Sensitization to the allergen is possible *via* inhalation and systemically. In contrast to mice and rats, the guinea pig airways are sensitive to histamine and demonstrate an early and late allergic reaction. Moreover, during the late asthmatic reaction both eosinophils and neutrophils are observed. Finally, airway hyperresponsiveness is observed *in vitro* and *in vivo*. Disadvantages are that only a few inbred strains exist and only a few species-specific reagents are available. Instead of IgE, IgG<sub>1</sub> seems to be the major class of anaphylactic antibody and guinea pigs are rather insensitive to cromolyn.

Guinea-pigs are also often used as a 'screening' model for drugs that act through particular pathways that are seen to be relevant to human asthma and have been useful in the development of drugs such as corticosteroids and  $\beta_2$  receptor-agonists [9]. The response of isolated guinea-pig airways to pharmacological agonists has been compared directly with humans to determine whether they are a good model for human airways [163]. It was found that there were similar responses between human and guinea-pig airways when exposed to methacholine, histamine and allergen following sensitization.

Both acute and chronic model of guinea pig asthma showed a closely link with the human asthma permitting the understanding of deeper intimate mechanisms underlying airway inflammation, hyperresponsiveness and remodeling through the use of newly developed synthetic antagonists or inhibitors for selective pathways which may anticipate clinically-relevant human usage. Finally, the guinea pig is also considered a useful model for exploring the basic aspects of the development of post-bronchiolitis asthma in children following respiratory virus infections opening new horizons for the most promising therapeutic strategies able to suppress the formation of asthma as *sequela* of bronchiolitis.

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