The airways are innervated by efferent (motor) as well as afferent (sensory) autonomic nerves, and this autonomic nervous system controls many aspects of airway function. In addition to cholinergic and adrenergic innervation, nonadrenergic noncholinergic (NANC) innervation of the airways is importantly involved in the regulation of many processes in the airways which may influence airway function, such as modulation of airway smooth muscle tone, mucus secretion from submucosal glands, transport of fluid across the airway epithelium, permeability and blood flow in the bronchial circulation, and the release of mediators from mast cells and other inflammatory cells.

The NANC nervous system has inhibitory (iNANC) as well as excitatory (eNANC) properties. iNANC responses are mediated by nitric oxide (NO), which may be derived from neural as well as non-neural sources in the airways, and vasoactive intestinal polypeptide (VIP), causing bronchodilation, vasodilation and inhibition of inflammatory cell activity. On the other hand, eNANC responses are mediated by the release of tachykinins (e.g. substance P, neurokinin A), causing bronchoconstriction, vasodilation, plasma leakage, mucus secretion and activation of inflammatory cells (collectively referred to as ‘neurogenic inflammation’).

Thus far, little is known about the role of (neural or non-neural) NO, VIP and endogenous tachykinins in allergen-induced early and late asthmatic reactions, airway hyperreactivity and airway inflammation. The aim of the experiments described in this thesis was to investigate the role of eNANC and iNANC neurotransmission, and non-neural NO in allergic asthma, using a guinea pig model of allergic asthma, which has recently been developed in our department (1,2). This model is characterized by allergen-induced early and late asthmatic reactions, airway hyperreactivity after these reactions, and airway inflammation. Both in a qualitative and a quantitative sense, these responses are remarkably similar to those observed in human asthma (3).

In Chapter 2 the role of endogenous NO in the regulation of histamine- and allergen-induced airway obstruction was investigated. For this purpose, the effect of the NOS inhibitor L-NAME on airway reactivity to inhaled histamine was determined before, and after the allergen-induced early and late asthmatic reaction. In addition, the effect of L-NAME on the early and late asthmatic reactions was investigated.

Histamine-induced bronchoconstriction was potentiated by the non-selective NOS inhibitor L-NAME before allergen provocation, indicating that endogenous NO exerts a functional antagonism against agonist-induced airway smooth muscle contraction. The cellular source of histamine-induced eNOS-derived NO in vivo is unknown. Since histamine-induced bronchoconstriction in guinea pigs is partly mediated by a vasoactive intestinal peptide (VIP), the local release of NO may play a role in the modulation of airway smooth muscle tone.

The allergen-induced bronchoconstriction is prevented by L-NAME before allergen provocation, indicating that endogenous NO may be involved in the maintenance of airway smooth muscle tone. The role of NO in the regulation of airway smooth muscle tone may be relevant to the pathogenesis of asthma.

After the late asthmatic reaction, which is in line with the early asthmatic reaction, the bronchoconstrictor response to histamine was not significantly enhanced by L-NAME, indicating that the role of endogenous NO in the regulation of airway smooth muscle tone may be limited in this model of allergic asthma.

In allergic asthma, the role of endogenous NO in the regulation of airway smooth muscle tone may be relevant to the pathogenesis of asthma.
mediated by a vagal reflex mechanism (4), neurally formed nNOS-derived NO may be involved (5). In addition, in vitro studies, using luminal perfusion of guinea pig tracheal tube preparations, have indicated that histamine-induced non-neural cNOS activity in the airway epithelium may be involved (6,7).

The allergen-induced early asthmatic reaction was also increased by inhalation of L-NAME before allergen provocation, suggesting that allergen-induced endogenous NO not only counteracts agonist-induced bronchoconstriction, but also acts as a feedback mechanism against allergen-induced bronchoconstriction. This is in line with observations of Persson et al. (8), who demonstrated in OA-sensitized guinea pigs that allergen-induced bronchoconstriction was potentiated by intraperitoneally administered L-NAME, which was reversed by inhalation of NO (8). After the allergen-induced early asthmatic reaction, increased airway reactivity to inhaled histamine was not affected by L-NAME, indicating that a deficiency of NO may be involved in allergen-induced airway hyperreactivity. These in vivo observations extend the previous observation by De Boer et al. (7), who demonstrated ex vivo, using perfused airway preparations, that hyperreactivity to contractile agonists after the allergen-induced early asthmatic reaction was associated with a deficiency of NO. Very remarkably, in a study of Persson et al. (9), in OA-sensitized guinea pigs it was demonstrated that allergen provocation caused a rapid but transient increase in exhaled (cNOS-derived) NO, which dropped below basal levels in progress of the early asthmatic reaction. The latter observation indicates that an impaired cNOS function may already develop during the early asthmatic reaction. As indicated above, the deficiency of NO presumably reflects a dysfunctional production or effectiveness of cNOS-derived NO.

After the late asthmatic reaction, the AHR to histamine was partially reversed, which is in line with previous observations in the same animal model (3,10,11), as well as in mild asthmatic patients (12,13). Very surprisingly, the partial reversal of AHR to histamine after the late asthmatic reaction could be completely overcome by L-NAME, indicating that restoration of production or effectiveness of NO may account for this reversal. The latter observation may represent an important endogenous regulation mechanism for the recovery of allergen-induced AHR.

In allergic asthmatic patients, the allergen-induced late asthmatic reaction has been shown to be associated with enhanced exhaled NO levels (14). In addition, allergen provocation has been reported to cause enhanced pulmonary NO production in sensitized rats (15) and guinea pigs during the late asthmatic response (16), which was due to the induction of iNOS (15-17). Therefore, the partial reversal of allergen-induced airway hyperreactivity after the late asthmatic reaction could well be due to a beneficial bronchodilatory action of iNOS-derived NO. This hypothesis
was investigated in Chapter 3, using the selective iNOS inhibitor aminoguanidine. As observed with the non-selective NOS inhibitor L-NNAME (Chapter 2), the partial reversal of the allergen-induced airway hyperreactivity to histamine after the late asthmatic reaction was equally inhibited by inhalation of the selective iNOS inhibitor aminoguanidine, indicating that the NO involved in the reversal of hyperreactivity is indeed derived from iNOS.

However, high concentrations of iNOS-derived NO may not only have a bronchodilatory action, but also have cytotoxic and cytostatic effects (18). Furthermore, high concentrations of iNOS-derived NO may induce cell damage via its reaction with inflammatory cell-derived superoxide anions (O$_2^-$) to produce peroxynitrite (19,20). Possible detrimental effects of iNOS activation on airway inflammation and the airway reactivity to inhaled histamine were investigated by administration of the iNOS inhibitor aminoguanidine shortly before the onset of the late asthmatic reaction. A deleterious effect of iNOS-derived NO on the airway hyperreactivity to histamine after the late asthmatic reaction could indeed be demonstrated, since aminoguanidine inhaled at this time point reduced the airway hyperreactivity after the late asthmatic reaction. Bronchoalveolar lavage studies indicated that airway inflammation and epithelial damage induced by iNOS-derived NO may contribute to the airway hyperreactivity after the late asthmatic reaction, since aminoguanidine inhaled before the late asthmatic reaction also reduced the number of neutrophils, lymphocytes and ciliated epithelial cells in the airways.

This study for the first time shows that endogenous iNOS-derived NO is involved in the allergen-induced infiltration of inflammatory cells in the airways, as well as in the induction of epithelial damage. Since neutrophils have the capacity to generate enhanced levels of O$_2^-$ in patients with asthma, which correlates with the airway reactivity to methacholine in these patients (21), enhanced production of peroxynitrite in the vicinity of epithelial cells that express iNOS could be one mechanism of the observed NO-induced epithelial cell damage and subsequent hyperreactivity. Thus, it has recently been demonstrated that exogenous peroxynitrite may cause epithelial damage and hyperreactivity of guinea pig airways (22). Moreover, peroxynitrite was demonstrated to increase the release of major basic protein from eosinophils in tracheal preparations (22), which may similarly cause epithelial damage in the airways (23). Furthermore, this study for the first time indicates that iNOS-derived NO may have simultaneous beneficial and detrimental effects on the allergen-induced AHR to histamine after the LAR, by functional antagonism of histamine-induced bronchoconstriction and by promoting airway inflammation and epithelial damage, respectively. Considering the detrimental proinflammatory effects of iNOS-derived NO on airway function, a selective iNOS inhibitor could be beneficial in asthma patients treated with corticosteroids due to enhanced iNOS expression. Furthermore, selective iNOS inhibitors might be beneficial in bronchodilating effects of aminoguanidine and non-favorable effects monitored carefully.

Evidence for impaired NO production initially found in our study in asthma treated with corticosteroids. Thus, while the airway responsiveness was increased in severe asthma (29), a similar response in severe asthmatics was observed in severe asthmatics with a deficiency of NO compared to healthy controls. A deficiency of histamine, was recently observed in parainfluenza type 3 infected patients. A deficiency of histamine was inversely correlated with a protective role of endogenous NO in hyperreactivity (31).

It has previously been speculated that NO activity may be involved in the protective role of constutive NO synthases. The development of this airway reactivity to methacholine indicates that NO deficiency after L-NNAME inhalation of L-NNAME on basal conditions, indicates a protective role of NO on bronchoconstriction, as the early asthmatic reaction effect of L-NNAME on airway hyperreactivity is impaired. After the allergen challenges in Chapter 2 and 3. After the allergen challenges in Chapter 2 and 3.
inhibitor could be beneficial in the treatment of allergic asthma. Indeed, in stable asthmatic patients the exhaled NO concentration, which is presumably increased due to enhanced iNOS activity (24-26), could be reduced after inhalation of the selective iNOS inhibitor aminoguanidine (27). However, in view of the beneficial bronchodilating effects of iNOS-derived NO observed in our study, the effectiveness of such a treatment will depend on the balance between the favorable and non-favorable effects of these inhibitors in the airways, and should thus be monitored carefully.

Evidence for impaired cNOS-derived NO modulating airway reactivity as initially found in our studies has recently also been found in patients with severe asthma treated with corticosteroids, which suppresses the expression of iNOS (28). Thus, while the airway reactivity to bradykinin (and to methacholine) could be significantly increased by inhalation of the NOS inhibitor L-NMMA in patients with mild asthma (29), a similarly enhanced airway hyperreactivity to bradykinin present in severe asthmatics was insensitive to the NOS inhibitor, indicating that a deficiency of NO contributed to the enhanced airway responsiveness in these patients. A deficiency of cNOS-derived NO, causing airway hyperreactivity to histamine, was recently also demonstrated in guinea pigs with a respiratory parainfluenza type 3 infection (30). Moreover, in allergic asthmatics that were experimentally infected with rhinovirus-16, the increase in airway reactivity to histamine was inversely correlated with the NO level in exhaled air, indicating a protective role of endogenous (iNOS-derived) NO in virus-induced airway hyperreactivity (31).

It has previously been demonstrated that enhanced vagal cholinergic reflex activity may be involved in the allergen-induced airway hyperreactivity to inhaled histamine after the early asthmatic reaction (32), which may indicate that an impaired function of constitutive neural NOS (nNOS) in iNANC nerves is implicated in the development of this airway hyperreactivity. In order to test this hypothesis, the regulatory role of endogenous nNOS-derived NO in vagally-induced bronchoconstriction was investigated in vivo in Chapter 4. It was demonstrated that inhalation of L-NAME potentiated the vagally-induced bronchoconstriction under basal conditions, indicating that vagally-induced NO antagonizes this bronchoconstriction, as has also been observed previously by Lei et al. (33). After the early asthmatic reaction, when the airway reactivity to histamine is increased, the effect of L-NAME on vagal bronchoconstriction had vanished, suggesting that an impairment of nNOS function may at least partially account for the observed cNOS dysfunction after the allergen-induced early asthmatic reaction as observed in Chapter 2 and 3. After the late asthmatic reaction, when airway hyperreactivity to
histamine was partially restored, inhalation of L-NAME again potentiated the vagally-induced bronchoconstriction, indicating the reversal of nNOS function.

The observation that nNOS may be reduced after the allergen-induced early asthmatic reaction was confirmed in Chapter 5, in which the role of iNANC neurotransmitters NO and VIP in the electrical field stimulation (EFS)-induced relaxation of histamine precontracted proximal tracheal preparations obtained from non-challenged control animals and from animals at 6 h (i.e. after the early asthmatic reaction) and 24 h (i.e. after the late asthmatic reaction) after allergen provocation was investigated ex vivo. After the allergen-induced early asthmatic reaction, iNANC, NO, and VIP responses were significantly reduced compared to non-challenged controls, while the sympathetic response was unchanged. After the allergen-induced late asthmatic reaction, the NO response was still reduced (though not significantly), whereas the VIP response was fully restored again. The results indicate that a reduced iNANC activity may indeed contribute to the airway hyperreactivity that is observed after the early asthmatic reaction (Chapter 2), and that the reduced iNANC activity may be due to a deficiency of neurally released NO as well as VIP. In addition, restoration of iNANC activity could contribute to the partial reversal of the allergen-induced airway hyperreactivity after the late asthmatic reaction, although the NO response may not have been fully recovered at this time point. The latter observation appears to be at variance with the observation in Chapter 4, that the vagally-induced NO response was completely restored after the late asthmatic reaction. The cause of this discrepancy is presently unclear, and further experiments are needed to clarify the role of vagally-induced NO in neural bronchoconstriction.

In Chapter 5 it was demonstrated that the contribution of the iNANC neurotransmitter NO to the EFS-induced relaxation of tracheal preparations was inhibited by the β-adrenoceptor antagonist timolol, while, conversely, the contribution of the sympathetic neurotransmitter noradrenaline (NA) was inhibited by the NOS inhibitor L-NAME, indicating an interaction between iNANC and sympathetic neural pathways in guinea pig airways. In Chapter 6, we investigated the possible effect of the sympathetic system on the iNANC response (NO and VIP) into more detail. As in Chapter 5, it was found that EFS-induced NO-mediated relaxation was significantly decreased in the presence of timolol; in contrast, the EFS-induced VIP-mediated appeared to be potentiated in the presence of timolol.

However, increased endogenous sympathetic activity (as obtained in the presence of the α2-adrenoceptor antagonist yohimbine through antagonism of autonomic α2-adrenoceptors) did not affect the EFS-induced NO-response via β-adrenoceptor stimulation. In addition, exogenous β-adrenoceptor stimulation with the non-selective β-adrenoceptor antagonist timolol EFS-induced NO release observed in response to a partial modulation of the contractions. In contrast, the sympathetic activity via α2-adrenoceptors was not affected.

It has been shown that the pro-inflammatory effect of iNANC is mediated through NK2 receptors, which are also involved in the development of airway hyperreactivity. In Chapter 5, it was demonstrated that the NO response was partially restored after the late asthmatic reaction. However, increased endogenous sympathetic activity (as obtained in the presence of the α2-adrenoceptor antagonist yohimbine through antagonism of autonomic α2-adrenoceptors) did not affect the EFS-induced NO-response via β-adrenoceptor stimulation. In addition, exogenous β-adrenoceptor stimulation with the non-selective β-guanethidine did not affect the NO response.
selective β-adrenoceptor agonist isoprenaline in the presence of the sympatholytic guanethidine did not affect the EFS-induced NO-response, while, surprisingly, the EFS-induced VIP-ergic relaxation was inhibited. These results indicate that the observed synergistic interaction between the EFS-induced NO- and NA-responses cannot be explained by sympathetic facilitation of the NO-response; instead, modulation of sympathetic activity by neurally-derived NO might be implicated. In contrast, the VIP-response appeared to be negatively regulated by sympathetic activity via β-adrenoceptors. Finally, endogenous as well as exogenous stimulation of α2-adrenoceptors did not affect iNANC-mediated relaxation. This is in line with previous observations (34) and indicates that in contrast to other organs (35-37), prejunctional α2-adrenoceptors are not involved in the modulation of the iNANC response in the guinea pig trachea.

It has been proposed that mechanisms underlying neurogenic inflammation are involved in the pathophysiology of allergic asthma, and that both NK1 and NK2 receptors may be involved (38). However, direct evidence for the involvement of these receptors in allergen-induced obstructions, airway hyperreactivity, and airway inflammation by use of specific NK receptor antagonists is sparse. Only recently, the effectiveness of the specific non-peptide NK2 receptor antagonist SR48968 on NKA-induced bronchoconstriction in asthmatic patients has been established (39). Therefore, we investigated the role of both NK1 and NK2 receptors in allergen-induced early and late reactions, AHR after these reactions, and airway inflammation in our guinea pig model, using recently developed selective non-peptide NK1 and NK2 receptor antagonists, SR140333 and SR48968, respectively.

In Chapter 7, the role of the NK1 receptor in allergic responses was investigated. Inhalation of SR140333 had no effect on basal airway reactivity to inhaled histamine. However, when inhaled before allergen provocation and after the allergen-induced early asthmatic reaction, SR140333 significantly attenuated the development of allergen-induced airway hyperreactivity to histamine after both the early and late asthmatic reaction. The SR140333-induced inhibition of allergen-induced airway hyperreactivity after the late asthmatic reaction was accompanied by a reduced number of eosinophils, neutrophils and lymphocytes in the bronchoalveolar lavage fluid, while there was a tendency of reduced epithelial cell number. These observations for the first time indicate that NK1 receptor-mediated infiltration of pro-inflammatory cells and subsequent epithelial damage may be involved in the development of allergen-induced airway hyperreactivity.

In Chapter 8, in a very similar study design as used in Chapter 7, the role of the NK2 receptor in allergen-induced asthmatic reactions, airway hyperreactivity, and airway inflammation, was investigated using the selective non-peptide NK2 receptor
antagonist SR48968. Very importantly, inhalation of SR48968 before allergen provokeation and after the allergen-induced early asthmatic reaction attenuated the allergen-induced late asthmatic reaction as well as the airway hyperreactivity after this reaction, whereas the early asthmatic reaction and the airway hyperreactivity after this reaction were not affected. The SR48968-induced attenuation of the late asthmatic reaction and subsequent airway hyperreactivity was paralleled by a reduction of neutrophils and lymphocytes in the bronchoalveolar lavage fluid collected after the late asthmatic reaction, whereas the influx of eosinophils had not significantly changed. In addition, as with SR140333, SR48968 tended to reduce the epithelial cell number in the bronchoalveolar lavage fluid. These observations may indicate that both NK1- (Chapter 7) and NK2 receptor-induced inflammatory cell infiltration and subsequent epithelial damage may be (differentially) involved in development of allergen-induced AHR. The observation that SR140333 treatment inhibited the influx of eosinophils, while the numbers of epithelial cells in the bronchoalveolar lavage fluid tended to be reduced, indicates that eosinophils are importantly involved in NK1 receptor-mediated epithelial damage. NK1 receptor-mediated priming of eosinophils for chemotaxis (40) may be a crucial step in the migration of these cells to the airway lumen, where they become activated. Subsequently, eosinophil-derived mediators, such as major basic protein, may induce airway hyperreactivity by damaging the integrity of the epithelial layer (23). Although eosinophil activation was not measured, an NK2 receptor-mediated mechanism for epithelial damage may be eosinophil-independent, since influx of eosinophils was not affected by SR48968. In contrast, SR48968 inhibited the influx of neutrophils, suggesting that this inflammatory cell type may be implicated in the observed SR48968-induced attenuation of airway hyperreactivity after the late asthmatic response. As already mentioned above, neutrophils have the capacity to generate enhanced levels of superoxide anions (O2-) in patients with asthma, which correlates with the airway reactivity to methacholine in these patients (21). Enhanced production of peroxynitrite in the vicinity of epithelial cells that express iNOS could be one mechanism of the observed epithelial cell damage and subsequent hyperreactivity. The observation, that iNOS is not induced before the late asthmatic response (Chapter 3), may explain why SR48968 reduced the airway hyperreactivity after the late, but not after the early asthmatic reaction.

Allergen provocation has been reported to increase the tachykinin-immunoreactivity of excitatory nonadrenergic noncholinergic (eNANC) neurons and the tissue level of tachykinins in the airways of ovalbumin-sensitized guinea pigs (41) and augmented the sensitivity of sensory nerve fibers (42,43). In addition, the accessibility of these afferents for stimuli may be increased after allergen challenge,

as a consequence, infiltrated i
accessibility allergen-inc
increase hyp
bronchodilator conscious a
provocation
bronchodilator
Similarly, re
effectiveness ovalbumin p
increase the
nerves in th
consequence,

The final conclusions are summarized as follows:

• A deficiency in hyperreactivity deficiency (presumably neurotr

• Reversal late asthma
this resp

• iNOS-deficient allergen-
reaction on the o

• In guinea p
adrenocorti
adrenocorti
Summary and conclusions

as a consequence of epithelial damage caused by cytotoxic mediators derived from infiltrated inflammatory cells (23). Therefore, increased density, excitability, and accessibility of cNANC nerves caused by allergen provocation may contribute to the allergen-induced late asthmatic reaction and airway hyperreactivity after this reaction via increased N\(_2\) receptor-mediated bronchoconstriction. To investigate this hypothesis, in Chapter 9 vagally-induced N\(_2\) receptor-mediated bronchoconstriction was assessed in chronically instrumented, ovalbumin-sensitized, conscious and unrestrained guinea-pigs after single and repeated allergen provocation, using the selective N\(_2\) receptor antagonist SR48968. Single allergen provocation did not change the effect of SR48968 on vagally-induced bronchoconstriction, as established after the early and late asthmatic reaction. Similarly, repeated allergen provocations every other day did not affect the effectiveness of SR48968, as determined 24 h after 4, 8, and 12 subsequent ovalbumin provocations. Thus, although allergen provocation has been reported to increase the level of neuropeptides and the density of tachykinin-immunoreactive nerves in the airways of guinea pigs, these changes appear not to have functional consequences with respect to vagally-induced N\(_2\) receptor stimulation.

The final conclusions derived from investigations described in this thesis are summarized below:

- A deficiency of cNOS-derived NO contributes to the allergen-induced airway hyperreactivity towards histamine after the early asthmatic reaction. This deficiency may include both neural (iNANC nerve-derived) and non-neural (presumably epithelium-derived) NO. In addition, the activity of the iNANC neurotransmitter VIP is also reduced after the early asthmatic reaction.
- Reversal of the airway hyperreactivity to histamine after the allergen-induced late asthmatic reaction is importantly due to induction of iNOS activity during this response. In addition, restoration of the iNANC activity, particularly the response mediated by VIP, may also be implicated.
- iNOS-derived NO may have both beneficial and detrimental effects on the allergen-induced airway hyperreactivity to histamine after the late asthmatic reaction, by functional antagonism of histamine-induced bronchoconstriction on the one hand, and by promoting airway inflammation and epithelial damage on the other hand, respectively.
- In guinea pig trachea, the release or effectiveness of the iNANC neurotransmitter NO is not under regulation of pre- or postjunctional \(\beta\)-adrenoceptor stimulation; nor is it under regulation of prejunctional \(\alpha_2\)-adrenoceptors. Prejunctional \(\alpha_2\)-adrenoceptors are similarly not involved in the
modulation of iNANC nerve-derived VIP activity; however, the VIP response is negatively regulated by pre- and/or postfunctional β-adrenoceptors.

- Both NK₁ and NK₂ receptors are differentially involved in the development of allergen-induced airway hyperreactivity and airway inflammation. NK₁ receptors are implicated in the allergen-induced early as well as late airway hyperreactivity, presumably by an effect on the influx of eosinophils, neutrophils and lymphocytes in the airways. In contrast, NK₂ receptors are only involved in the allergen-induced late airway hyperreactivity, and promote the influx of neutrophils and lymphocytes, but not eosinophils, in the airways. In addition, NK₂ receptors, but not NK₁ receptors, are involved in the severity of the allergen-induced late asthmatic reaction.

- Single and chronic allergen provocation has no effect on the severity of vagally-induced NK₂ receptor-mediated bronchoconstriction, indicating that previously reported changes in immunoreactive eNANC nerves are not associated with a functional increase in eNANC activity.

- Considering the beneficial effects of NK₁ and NK₂ receptor antagonists on allergen-induced asthmatic responses, airway hyperreactivity after these responses, and airway inflammation, these antagonists may be very useful in the treatment of allergic asthma. Especially, combined treatment with both NK₁ and NK₂ receptor antagonists could be favorable, since NK₁ and NK₂ receptors are differentially involved in allergic asthma. The effectiveness of therapy with selective iNOS inhibitors to inhibit detrimental effects of high NO concentrations in allergen-induced AHR remains to be established.

References

Summary and conclusions


Chapter 10


