CHAPTER 9

GENERAL DISCUSSION AND SUMMARY

Preface

The objective of this thesis was to establish the role of acetylcholine in airway inflammation and remodeling in COPD and asthma. We aimed to investigate the role of individual muscarinic receptor subtypes, as well as the contribution of neuronal versus non-neuronal acetylcholine to inflammation and remodeling. The studies described in this thesis reveal that there is a critical regulatory role for the M₃ receptor in these processes, which is mediated via neuronal acetylcholine, but also involves non-neuronal acetylcholine.

Anticholinergics as bronchodilators in COPD and asthma

COPD and asthma are chronic obstructive airway diseases, and the incidence of both diseases is increasing (1). COPD is a leading cause of morbidity and mortality worldwide and is expected to become the third leading cause of death in 2020. This results in a substantial economic and social burden (2). With 180,000 deaths in the world each year, mortality from asthma is considerably lower, but around 300 million people in the world currently have asthma and its greatest burden lies in the morbidity and invalidity it causes, also in children (3). Inflammation and remodeling are hallmark features of both diseases, which contribute to the decline in lung function and the severity of the disease (4-6). Acetylcholine is the primary parasympathetic neurotransmitter in the airways, and induces bronchoconstriction and mucus secretion via M₃ receptors (chapter 2). The activity of the neuronal system is altered in asthma and COPD via several mechanisms, which can originate early in life or develop as an acute or chronic response after allergen challenge or stimuli such as cigarette smoke, leading to exaggerated acetylcholine release and airway narrowing (chapter 1). Strikingly, the increased cholinergic tone is the major reversible component of airflow limitation in COPD (7, 8). Therefore, anticholinergics are effective bronchodilators in this disease, and represent a first line of treatment (2). In asthma, use of anticholinergics is currently limited to the treatment of exacerbations (9), however, recent clinical trials indicate that anticholinergic therapy might also be beneficial for chronic treatment of moderate and severe asthma patients (10, 11).
Muscarinic receptor subtypes in the lung – is there a need for M₃ selective anticholinergics?

Increasing evidence suggests that the role of acetylcholine in the airways is not limited to bronchoconstriction and mucus secretion. In animal models of COPD and asthma, anticholinergics inhibit both airway inflammation and airway remodeling (chapter 2). However, it is not known which muscarinic receptor subtypes are involved in these responses. Multiple muscarinic receptor subtypes are expressed in the airways, that have differential roles in regulating bronchoconstriction, mucus secretion, inflammation and remodeling (chapter 2). Muscarinic receptor agonists and antagonists have limited selectivity towards individual muscarinic receptors, which limits the interpretation of the effects of these agents on functional parameters. Therefore, in this thesis, we used muscarinic receptor specific knock-out animals to investigate the role of individual muscarinic receptor subtypes in inflammation and remodeling. Here, we present evidence that the potentiating effects of acetylcholine on airway inflammation and remodeling are solely mediated via M₃ receptors in animal models of both COPD (chapter 3) and asthma (chapter 6).

COPD

In chapter 3, we investigated the contribution of individual muscarinic receptor subtypes to cigarette smoke-induced inflammation. Exposure to cigarette smoke results in neutrophilic inflammation, as is observed in patients with COPD. From different animals models of COPD, it was already known that neutrophilic inflammation, induced by cigarette smoke or LPS, can be prevented by pretreatment with anticholinergics, including tiotropium (12, 13), glycopyrrrolate (14) and aclidinium (15). Here, we demonstrated that the pro-inflammatory effects of acetylcholine are mediated via the M₃ receptor. Inhibition of the M₃ receptor, by total knock-out of the receptor or by a pharmacological approach using the M₃ antagonist 4-DAMP, prevented the inflammatory response induced by cigarette smoke. Thus, whole body exposure of wild-type animals for four days to an increasing number of cigarettes induced neutrophilic inflammation in the airways of wild-type animals, which was accompanied by an increase in the release of pro-inflammatory cytokines, including IL-8. This inflammatory response was almost completely prevented by inhibition of the M₃ receptor, suggesting that acetylcholine potentiates airway inflammation via this receptor subtype. This is further supported by the fact that the inflammatory response was enhanced after knock-out of the M₂ receptor. Loss of this autoinhibitory receptor results in enhanced acetylcholine release. With acetylcholine acting as a pro-inflammatory mediator, increased levels of acetylcholine in M₂ receptor knock-out (M₂R⁻/⁻) animals can explain the observed aggravated inflammatory response. Surprisingly, inflammation was also enhanced after knock-out of the M₃ receptor. This
might be explained via the role of the M₁ receptor on the airway epithelium, where it contributes to mucus production, by controlling electrolyte and water secretion. Impaired clearance of detrimental smoke particles from the airways after knock-out of the M₁ receptor could therefore underlie the enhanced inflammatory response observed in M₁R⁻/⁻ animals. This is further supported by a marked induction in the release of the cytokines IL-6 and MCP-1 in M₁R⁻/⁻ animals compared to wild-type animals. Taken together, the results described in chapter 3 indicate a pro-inflammatory role for acetylcholine via M₃ receptors.

The fact that acetylcholine has a pro-inflammatory effect on neutrophil inflammation via M₃ receptors is supported by various in vitro studies. Muscarinic receptor stimulation induced the release of IL-6 and IL-8 from airway smooth muscle cells, which has been shown to be mediated via M₃ receptors, and not via M₂ receptors (16). Moreover, tiotropium and the M₃ selective antagonists 4-DAMP and DAUS5884 inhibited methacholine and cigarette smoke-induced IL-8 release from airway smooth muscle cells, whereas there was no effect of the M₂ antagonist gallamine (16). Furthermore, acetylcholine can induce IL-8 release from bronchial epithelial cells, which can be inhibited by tiotropium and 4-DAMP, and not by the M₁ antagonist telenzepine or the M₂ antagonist gallamine (17). In addition, acetylcholine-induced neutrophil chemotactic activity from macrophages can be inhibited by 4-DAMP, and not by the M₁ antagonist pirenzepine or the M₂ antagonist gallamine (18). More recently, this was confirmed in macrophages from COPD patients. Thus, neutrophil chemotaxis induced by LPS-activated alveolar macrophages could be inhibited by tiotropium and 4-DAMP, and not by telenzepine, gallamine or tubocurarine (19). These in vitro findings, suggesting an important role for the M₃ receptor in inflammation, are now supported by conclusive in vivo findings, confirming the pro-inflammatory role of the M₃ receptor.

M₃ receptors are expressed on almost all cell types in the airways, including structural cells, like airway smooth muscle cells and epithelial cells, as well as inflammatory cells, like macrophages and neutrophils (table 1, chapter 1). As is evident from various in vitro studies, both structural cells and inflammatory cells can contribute to the pro-inflammatory effects of acetylcholine. Studies using airway structural cells demonstrated that muscarinic receptor stimulation can induce IL-8 release from airway smooth muscle cells and epithelial cells (16, 17). Moreover, acetylcholine can induce neutrophil and monocyte chemotactic activity from bronchial epithelial cells (20), as well as neutrophil migration by activating alveolar epithelial cells (21). In the latter study, acetylcholine also induced neutrophil migration by activating monocytes and alveolar macrophages, suggesting a direct effect of muscarinic receptor stimulation on inflammatory cells (21). Similar effects of muscarinic receptor stimulation on macrophages were observed in the
studies described above (18, 19). Moreover, proliferation of macrophages and T-cells has also been shown to be regulated by muscarinic receptors (22, 23). Thus, both structural and inflammatory cells can contribute to the pro-inflammatory effects of acetylcholine and it is not known whether this effect is primarily mediated via M₃ receptors on structural cells or via M₃ receptors on inflammatory cells.

Therefore, in chapter 4, we investigated the contribution of the M₃ receptor on structural cells versus inflammatory cells to cigarette smoke-induced inflammation. To distinguish between both cell types, bone marrow chimeric mice were generated. Bone marrow cells from M₃R⁻/⁻ animals were transplanted into irradiated wild-type animals, to investigate the contribution of the M₃ receptor on inflammatory cells, and bone marrow cells from wild-type animals were transplanted into irradiated M₃R⁻/⁻ animals, to investigate the contribution of the M₃ receptor on structural cells. Irradiated and transplanted wild-type animals and non-irradiated wild-type animals were used as controls. Mice were exposed to cigarette smoke, using a similar protocol as described in chapter 3. Exposure to cigarette smoke induced neutrophilic inflammation in non-irradiated and irradiated control animals. Interestingly, wild-type animals receiving M₃R⁻/⁻ bone marrow cells showed a similar increase in neutrophil number, suggesting that the M₃ receptor on inflammatory cells is not involved in the pro-inflammatory effect of acetylcholine. In contrast, no increase in the number of neutrophils was observed in M₃R⁻/⁻ animals receiving wild-type bone marrow cells, suggesting a critical role for airway structural cells in the pro-inflammatory effect of acetylcholine. Surprisingly, the increase in the release of IL-8 was similar among all smoke-exposed groups, both on mRNA and protein level. Thus, although the neutrophil chemoattractant is present in the airways of M₃R⁻/⁻ animals receiving wild-type bone marrow cells, no increase in neutrophil number is observed when compared to control animals. This suggests that neutrophil adhesion might be altered after knock-out of the M₃ receptor. Using a micro-array analysis, fibrinogen α and CD177 were identified as genes which might be involved in this process. Both genes are recently linked to neutrophil adhesion and transmigration (24-27). Interestingly, fibrinogen α and CD177 are both identified as inflammatory biomarkers for COPD, indicating the potential relevance of both genes for COPD pathophysiology (28-30). IL-8 can be released from structural cells, including epithelial cells, and from inflammatory cells, including macrophages. Our results suggest that the release of IL-8 from both structural cells and inflammatory cells is mediated via M₃ receptors, since IL-8 release is prevented after total knock-out of the M₃ receptor as demonstrated in chapter 3, but not after knock-out of the M₃ receptor on structural cells or inflammatory cells only. Moreover, this indicates a role for M₃ receptors on inflammatory cells, and suggests that acetylcholine can induce the release of IL-8 from inflammatory cells via M₃ receptors, as is described for macrophages.
in vitro (18, 19). However, knock-out of the M3 receptor on inflammatory cells in not sufficient to prevent neutrophilia, whereas knock-out of the M3 receptor on structural cells only is sufficient to prevent neutrophilia. This suggest that the contribution of the M3 receptor on inflammatory cells to the pro-inflammatory effect of acetylcholine is only small, and that this is mainly mediated via M3 receptors on structural cells. Taken together, the results from chapter 4 highlight the important role of airway structural cells in neutrophilic inflammation and the pathophysiology of COPD, and the involvement of acetylcholine in this response.

**Asthma**

In chapter 6, we demonstrated that the effects of acetylcholine in response to allergen exposure are also mediated via the M3 receptor. From animal models of asthma it was already known that acetylcholine plays a role in allergen-induced airway inflammation and remodeling. Pretreatment of guinea pigs with tiotropium partly prevented airway smooth muscle thickening, mucous gland hypertrophy and goblet cell metaplasia, as well as eosinophilic inflammation in response to allergen exposure (31, 32). Similar findings were observed in allergen-exposed mice, in which tiotropium partly prevented features of airway remodeling and airway inflammation. In addition to the findings in guinea pigs, tiotropium was also shown to inhibit excessive extracellular matrix deposition and to inhibit TNF-α cytokine release in mice (33). To investigate the effects of individual muscarinic receptor subtypes on allergen-induced inflammation and remodeling, muscarinic receptor subtype specific knock-out animals were exposed to ovalbumin, and lungs were collected for analysis of inflammation and remodeling. Allergen exposure induced features of remodeling, including goblet cell metaplasia, airway smooth muscle thickening, pulmonary vascular smooth muscle remodeling and enhanced deposition of extracellular matrix proteins in the airway wall of wild-type mice. These effects were absent or markedly lower in M3R/- mice, whereas M3R/- and M2R/- mice responded similar to wild-type mice with respect to remodeling. This suggests that the remodeling-promoting effects of acetylcholine in vivo in response to allergen exposure are solely mediated via M3 receptors, and not via M1 or M2 receptors.

Evidence from in vitro studies supports a role for acetylcholine in remodeling of the airways. It is known that muscarinic receptor stimulation can induce MUC5AC expression in epithelial cells, which can be inhibited by aclidinium (34). Moreover, tiotropium can inhibit IL-13-induced goblet cell metaplasia, as demonstrated in chapter 8. Muscarinic receptor stimulation is also shown to be involved in airway smooth muscle thickening and airway fibrosis, as it can enhance growth factor-induced proliferation (35, 36) contractile protein expression (37), and extracellular matrix deposition of airway smooth muscle cells.
and fibroblasts (38, 39). Furthermore, aclidinium has been shown to inhibit fibroblast to myofibroblast transition (40). Although some of these in vitro studies point to an important role for the M2 receptor in remodeling responses, we did not observe any effects of knock-out of the M1 receptor on allergen-induced remodeling. It is known that presynaptic M2 receptors on parasympathetic nerves are dysfunctional in asthmatic airways. This has been shown in animal models of allergen exposure, viral infection and ozone exposure, and also in patients with asthma (41-43). Although this effect is less well described for postsynaptic M2 receptors, dysfunction of M2 receptors after allergen challenge might explain why we did not observe any effects of knock-out of the M2 receptor. M2-mediated responses observed in vitro might therefore be less relevant to the in vivo situation. Moreover, M2 receptor dysfunction in response to allergen might also explain why there is no effect of M2 receptor knock-out on allergen-induced inflammation, whereas there is an effect of M2 receptor knock-out on cigarette smoke-induced inflammation. Furthermore, we did not observe any effects of knock-out of the M1 receptor on allergen-induced airway inflammation or remodeling. Whereas in the cigarette smoke model used in chapter 3, detrimental smoke particles had to be cleared from the airways in which the M1 receptor might be involved, this effect is likely less pronounced after inhalation of an allergen. Together, this might explain why there is no role for M1 or M2 receptors in allergen-induced airway remodeling, which is shown to be solely mediated by M3 receptors in vivo.

Next to its role in airway remodeling, the M3 receptor also seems to be involved in basal maintenance of airway structure. In chapter 3, we observed a decrease in basal gene expression of TGF-β1 and the extracellular matrix proteins collagen Iα1 and fibronectin in lung homogenates of M3R−/− mice compared to WT mice. Moreover, in chapter 6, we observed a decrease in basal levels of α-sm-actin in the airways and arteries of M3R−/− mice compared to WT mice. This may suggest that cholinergic regulation of airway tone, which is almost completely absent in M3R−/− mice, is important for the development and/or maintenance of airway structure, including the airway smooth muscle and the extracellular matrix.
Bronchoconstriction as a driver of airway remodeling

Lack of bronchoconstriction in M3R⁻/⁻ mice might also have other, far-reaching consequences in our model. We observed a marked inhibition of airway remodeling in M3R⁻/⁻ mice compared to wild-type mice, without inhibition of the allergic inflammatory response (chapter 6). Generally, airway structural changes after allergen challenge are attributed to eosinophilic inflammation (44), and tiotropium has previously been shown to inhibit eosinophilic inflammation in animal models of asthma (32, 33). The observation that there is no inhibition of the eosinophilic inflammation after knock-out of the M₃ receptor might be explained by the fact that this is orchestrated by both M₁ and M₃ receptors. Tiotropium also has a substantial dissociation half-life for the M₁ receptor (45), and we observed a trend towards lower eosinophil numbers in M₃R⁻/⁻ mice. However, the finding that remodeling can be inhibited after knock out of the M₃ receptor, without affecting inflammation, has significant implications. Recently, Grainge et al. demonstrated that repeated methacholine challenges in mild asthmatic patients is sufficient to induce airway remodeling, without affecting inflammation. Thus, methacholine challenge promoted TGF-β release, collagen deposition, goblet cell metaplasia and epithelial cell proliferation in airway biopsies, with no effect on eosinophil numbers (46). Interestingly, these effects on airway remodeling were similar to those induced by house dust mite, administered in an equi-effective dose with respect to bronchoconstriction but also causing eosinophilic inflammation. Moreover, effects on remodeling were prevented when methacholine was administered together with the β₂-agonist albuterol, to prevent methacholine-induced bronchoconstriction (46). These findings, together with our observations from chapter 6, raise the hypothesis that bronchoconstriction by itself might be sufficient to induce airway remodeling, and that the origin of airway remodeling is not inflammatory, but mechanical in nature.

Therefore, in chapter 7, we aimed to investigate the effects of bronchoconstriction on airway remodeling, and the involvement of the M₃ receptor in this response, by using mouse precision cut lung slices (PCLS). PCLS are a valuable tool to study bronchoconstriction-induced remodeling, since all lung cell types are present and cell-cell contacts and cell-matrix interactions are preserved, in contrast to cell cultures of single airway cell types. This was confirmed previously by our lab using guinea pig PCLS (47). In this study, Oenema et al. demonstrated that the effects of bronchoconstriction induced by methacholine on airway remodeling were similar to those of TGF-β, a pleiotropic cytokine known to be an important mediator of airway remodeling. Thus, stimulation of guinea pig PCLS for two days with TGF-β induced features of airway remodeling, including enhanced contractile protein expression, as assessed by Western Blot analysis and
immunohistochemistry. Interestingly, stimulation of guinea pig PCLS for two days with methacholine had similar effects on airway remodeling, which was shown to be mediated via enhanced release of TGF-β (47). In chapter 7, we aimed to investigate the involvement of the M₃ receptor in this response, by exposing PCLS from wild-type and M₃R⁻/⁻ mice to TGF-β and methacholine. First, we confirmed that bronchoconstriction in response to methacholine is almost completely abolished in M₃R⁻/⁻ mice, which is in line with the literature (48, 49). Next, PCLS from wild-type and M₃R⁻/⁻ mice were exposed to TGF-β and methacholine for two days. Exposure of PCLS from wild-type mice to TGF-β induced an increase in the expression of the contractile protein α-sm-actin and the extracellular matrix proteins fibronectin and collagen type I. A similar increase in TGF-β-induced airway remodeling was observed in M₃R⁻/⁻ mice, suggesting that the muscarinic receptor regulation of airway remodeling occurs upstream of TGF-β signaling. In contrast to findings in guinea pigs PCLS, exposure to methacholine did not induce airway remodeling in murine PCLS. Species differences might underlie the differences observed in guinea pig PCLS versus murine PCLS. Whereas airway constriction in guinea pig PCLS is maintained over days in the presence of methacholine, unpublished observations from our lab indicate that the methacholine-induced constriction is rapidly lost in murine PCLS. Moreover, the contraction rate in response to methacholine is also greater in guinea pig PCLS compared to murine PCLS. Further studies, using different experimental approaches, are therefore needed to fully elucidate the effect of M₃ receptor activation on airway remodeling. Next, we analyzed whether TGF-β-induced remodeling altered the airway contractility. We did not observe any functional effects of exposure of PCLS to TGF-β or methacholine, since contraction against the thromboxane A2 agonist U46619 was not altered in wild-type or M₃R⁻/⁻ mice after stimulation for two days. Thus, despite clear effects of TGF-β on the expression of fibronectin, collagen Iα1 and α-sm-actin, this did not translate into changes in airway contractility, suggesting that it is difficult to demonstrate a direct link between airway remodeling and airway contractility using lung slices.

The hypothesis that bronchoconstriction by itself might be sufficient to induce remodeling, independent of the inflammatory response, is still ongoing, and an increasing number of studies support this hypothesis. In vitro, it has been shown that muscarinic receptor stimulation, in combination with mechanical strain, can induce α-sm-actin and sm-myosin mRNA expression in bovine tracheal smooth muscle strips, and myosin light-chain kinase expression in human airway smooth muscle cells (50, 51). Moreover, contraction of airway smooth muscle cells induces TGF-β activation (52). In intact airways, the epithelium is compressed by bronchoconstriction, and this induces the activation of epidermal growth factor (EGF) (53). Compression of epithelial cells in vitro results in enhanced TGF-β expression (54) and an increase in epithelial thickness (55). Moreover,
mechanical stress applied to the epithelium has been shown to increase the expression of fibronectin, collagen and matrix metalloproteinase type 9 (MMP-9) in airway fibroblasts in a co-culture system (56). Taken together, the studies outlined above suggest that bronchoconstriction may lead to airway remodeling in asthma via the release of growth factors from epithelial and smooth muscle cells in response to mechanical stress. This is important from a therapeutic perspective, as this would imply that prevention of bronchoconstriction, probably already in an early stage of disease, might prevent airway remodeling in asthma.

**Neuronal and non-neuronal acetylcholine**

In the last decades, it has become clear that acetylcholine is not only released as a neurotransmitter from nerve terminals, but also as a hormone from non-neuronal cells, including cells in the airways, acting in an autocrine and/or paracrine manner. However, it is not known to which extent the pro-inflammatory and remodeling-promoting effects of acetylcholine in the airways as discussed above are mediated by neuronal or by non-neuronal acetylcholine. It has been suggested that non-neuronal acetylcholine contributes to these effects, however, evidence for such a role is still limited (chapter 2, 57, 58). In this thesis, we present evidence that both neuronal (chapter 5) and non-neuronal (chapter 8) acetylcholine contribute to airway inflammation and remodeling in COPD and asthma.

**COPD**

In chapter 5, we investigated the effect of targeted lung denervation (TLD) on inflammation in patients with COPD. TLD is a novel potential therapy for patients with COPD, in which parasympathetic airway nerves are ablated by locally applying radiofrequency energy in the main bronchi using bronchoscopy. Inhibition of acetylcholine by ablating airway nerves is expected to inhibit bronchoconstriction and the first experimental data support this notion. In a small group of patients, TLD was shown to increase FEV₁, 6 minute walk-test distance and the St. George’s Respiratory Questionnaire score (59). We hypothesized that TLD would also inhibit airway inflammation. We demonstrated that 30 days after TLD of the right lung, airway inflammation is attenuated. TLD resulted in a reduction of inflammatory cells and cytokine release in the bronchial wash, and a reduction in gene expression of inflammatory mediators, including the expression of IL-6, IL-8, TGF-β and MUC5AC, in bronchial brush specimen. This is the first study reporting a direct inhibitory effect of acetylcholine on inflammation in patients with COPD and suggests that this is mediated by neurally released acetylcholine. The fact that TGF-β levels were significantly reduced after TLD suggests that TLD might also affect remodeling in COPD. This implies that airway remodeling in COPD is also mediated by
neuronal acetylcholine. However, evidence for such a role is still limited, and clearly further studies are needed to elucidate the role of neuronal versus non-neuronal acetylcholine in airway remodeling in COPD.

A role for neuronal acetylcholine in inflammation in COPD is further supported by the results from chapter 3, in which the cigarette smoke-induced inflammatory response was enhanced in M_2R<sup>−/−</sup> mice compared to wild-type mice. Knock-out of prejunctional autoinhibitory M_2 receptors results in enhanced acetylcholine release. With acetylcholine acting as a pro-inflammatory mediator, knock-out of the autoinhibitory receptors enhances the inflammatory response. A similar autoinhibitory mechanism for the release of non-neuronal acetylcholine has not been demonstrated until now, thereby suggesting that neurally released acetylcholine is the main driver of this inflammatory response. Furthermore, we did not observe any regulation of expression of components of the non-neuronal cholinergic system after exposure to cigarette smoke. Moreover, the importance of structural cells over inflammatory cells in cigarette smoke-induced inflammation, as observed in chapter 4, might further support a role for neuronal acetylcholine in airway inflammation in COPD.

**Asthma**

The hypothesis that bronchoconstriction might be the driver of allergen-induced airway remodeling suggests that also in allergic asthma, neuronal acetylcholine plays an important role. Moreover, we did not observe any regulation of expression of components of the non-neuronal cholinergic system after allergen challenge. Thereby, we cannot confirm a role for non-neuronal acetylcholine in allergen-induced remodeling (chapter 6). However, we did observe a clear role for non-neuronal acetylcholine in goblet cell metaplasia in chapter 8. In this chapter, we investigated the direct effects of endogenous non-neuronal acetylcholine on epithelial cell differentiation. It was already known that tiotropium can inhibit allergen-induced goblet cell metaplasia and MUC5AC expression in vivo (32, 33), an effect also observed after knock-out of the M<sub>3</sub> receptor in chapter 6. Epithelial cells have been shown to produce and release acetylcholine (60). Therefore, the observed effects on epithelial cell differentiation might be mediated by neuronal acetylcholine, or by a direct effect of non-neuronal acetylcholine on the airway epithelium. To investigate the latter hypothesis, human airway epithelial (HAE) cells were cultured at an air-liquid-interface (ALI) and exposed to tiotropium and/or IL-13 during differentiation. IL-13 is a main driver of goblet cell metaplasia, and plays a central role in the pathogenesis of asthma (61). Epithelial cells used for this study expressed all components of the non-neuronal cholinergic system, suggesting production of acetylcholine by these cells. Previously, it has been shown that epithelial cells can indeed
release acetylcholine (60). Interestingly, carnitine acetyltransferase (CarAT) was identified as the synthesizing enzyme of acetylcholine. Tiotropium had no effects on epithelial cell differentiation following air exposure, however, significantly inhibited IL-13-induced goblet cell metaplasia. Tiotropium inhibited the increase in MUC5AC positive cells and goblet cells, and the increase in MUCSAC gene expression. Moreover, we demonstrated that the effects of tiotropium might be mediated via effects on the transcription factors FoxA2 and FoxA3, whereas increased expression of SPDEF by IL-13 was not affected by tiotropium. This indicates that non-neuronal acetylcholine contributes to goblet cell metaplasia by a direct effect on epithelial cells. In the airways, mucus is secreted by goblet cells and submucosal glands. Submucosal glands are innervated by airway nerves (chapter 1) and the release of mucus from glands is under cholinergic neural control (62). It is still a matter debate whether goblet cells can also release mucus in response to neuronal acetylcholine, but data from our study suggest at least a role for non-neuronal acetylcholine in this response.

Based on the in vivo studies described in this thesis, I propose that the potentiating effects of acetylcholine on airway inflammation and remodeling are mainly mediated by neuronally released acetylcholine. However, a number of in vitro studies, including the study described in chapter 8, indicate that non-neuronal acetylcholine might contribute to the effects of acetylcholine. Tiotropium and 4-DAMP have been shown to inhibit alveolar macrophage mediated migration of neutrophils from COPD patients (19). Moreover, tiotropium inhibited TGF-β-induced MMP-1 and MMP-2 expression in human lung fibroblasts (63), and aclidinium has been shown to inhibit TGF-β and cigarette smoke-induced fibroblast to myofibroblast differentiation (40, 64). These studies indicate that non-neuronal acetylcholine might contribute to airway inflammation and remodeling of airways cells in an autocrine or paracrine manner. However, the relative contribution of these effects to the effects of neuronal acetylcholine in vivo is still uncertain. Vagotomy of animals under controlled experimental conditions might definitely establish the contribution of neuronal versus non-neuronal acetylcholine to inflammation and remodeling in disease models. Bilateral vagotomy is, however, not possible in the mouse (unpublished observations). In the literature, bilateral vagotomy of bigger laboratory animals has been reported, and this might be a way to answer this question. Moreover, targeted lung denervation in patients with asthma can answer whether neuronal acetylcholine is also the main contributor to airway inflammation in asthma.
Clinical implications

COPD
Evidence for a role of acetylcholine as a driver of airway inflammation and remodeling in patients with COPD or asthma is still limited. In this thesis, we demonstrate for the first time that acetylcholine might act as a pro-inflammatory mediator in patients with COPD, since airway inflammation is attenuated after TLD (chapter 5). There are some limitations to this study, of which the major limitations are the limited number of subjects included in the study and the lack of a control arm. However, we believe that the findings from this small study are very promising. A large-scale, multicenter, sham-controlled study into the effectiveness of TLD is planned and this will provide more conclusive evidence on the role of neuronal acetylcholine as a pro-inflammatory mediator. There is no additional evidence until now that demonstrates direct effects of acetylcholine on inflammation in patients with COPD. From different trials, including the UPLIFT (Understanding Potential Long-term Impacts on Function with Tiotropium) trial, it is known that tiotropium reduces the number of exacerbations (65). Treatment with glycopyrrolate or aclidinium, albeit for a shorter period, was also shown to affect exacerbations, as the time to the first exacerbation was increased (66, 67). This might suggest an anti-inflammatory effect of anticholinergic therapy, since the inflammatory response is enhanced during exacerbations, with increased expression of pro-inflammatory cytokines like IL-8 (68, 69). Moreover, patients who have more exacerbations demonstrate increased levels of inflammatory markers at stable state (70, 71). Until now, methodological problems complicated the evaluation of airway inflammation in drug studies. In a study by Powrie et al., treatment with anticholinergics reduced the amount of sputum, which might result in increased cytokine concentrations in the sputum. This has been suggested to explain why no reduction in IL-6 or IL-8 sputum levels was observed in patients with COPD after tiotropium treatment (72, 73). In chapter 5, we used a bronchoscopic intervention, which enabled us to collect a bronchial wash and brush and examine the effects of acetylcholine on airway inflammation in a direct manner. Here, we demonstrate that acetylcholine might indeed affect airway inflammation in patients with COPD, as is expected from the increasing body of evidence from in vitro and in vivo studies reviewed in chapter 2, and from the studies described in this thesis. Moreover, TLD reduced the levels of TGF-β, suggesting effects of acetylcholine on airway remodeling in patients with COPD. In the overall study population of the UPLIFT trial, tiotropium did not affect the rate of decline in lung function (65). However, tiotropium did inhibit the accelerated decline in lung function in specific subgroups of the trial, including young patients and patients with moderate disease (74, 75). Interestingly, young patients are also highly represented in the TLD study. Clearly, future studies are needed to understand the effects of acetylcholine on airway
remodeling in patients with COPD. Accelerated decline in lung function might not be the optimal outcome parameter for future studies to analyze remodeling, since it is known from the ECLIPSE study that this is highly variable between patients with COPD, and lung function might even increase in a subset of patients, irrespective of treatment (76). Instead, more direct measurements of remodeling parameters, using airway biopsies from patients, taken before and after long-term anticholinergic therapy, either after TLD or anticholinergic treatment, might help to elucidate the role of acetylcholine in airway remodeling in patients with COPD.

Asthma
Anticholinergics are currently not included as controller therapy for the treatment of asthma. However, recent trials suggest that patients with asthma might benefit from anticholinergic controller therapy, since tiotropium has been shown to induce bronchodilation in moderate and severe asthma patients (10, 11). In severe asthma patients, addition of tiotropium to standard therapy with inhaled glucocorticosteroids and long-acting β2-agonists did not only induce bronchodilation, but was also shown to increase the time to the first exacerbation, and reduce the risk of a severe exacerbation (10). This suggests that acetylcholine also exerts pro-inflammatory effects in patients with asthma. However, direct evidence for such a role is still lacking. Moreover, long-term studies into the effects of anticholinergic therapy on airway remodeling in asthma are needed to confirm the remodeling-promoting effects of acetylcholine as described in this thesis. Repeated challenges with methacholine in mild asthma patients did demonstrate that muscarinic receptor stimulation can induce airway remodeling (46). Moreover, methacholine challenge induced epithelial cell proliferation and goblet cell metaplasia, suggesting a role for acetylcholine in mucus hypersecretion. This is supported by results from chapter 8, in which tiotropium inhibited goblet cell metaplasia of human airway epithelial cells. Tiotropium has been shown to reduce sputum levels in patients with chronic mucus hypersecretion (77). Although the concern has been expressed that anticholinergics desiccate mucus, thereby increasing the viscosity and making the mucus more difficult to clear, there is now some data to support that anticholinergics are beneficial for patients with mucus hypersecretion (78). This is relevant for both COPD and asthma, in which mucus hypersecretion can occur, which contributes to airflow obstruction of the smaller airways and increases the risk of exacerbations.
M₃ selective anticholinergics

The studies described in this thesis point to a critical regulatory role for the M₃ receptor in airway inflammation and remodeling. Although current anticholinergics are kinetically selective for the M₃ receptor, they also have a substantial dissociation half-life from the M₁ receptor (table 2, chapter 1). For example, dissociation half-life of tiotropium from the M₁ receptor is 10.5 hours (45). Our studies suggest that an even more selective muscarinic antagonist, solely inhibiting M₃ receptors, is desirable and may lead to improved effects on airway inflammation and remodeling. Inhibition of acetylcholine release by TLD might also further enhance outcomes on inflammation and remodeling, however, this does not inhibit non-neuronal acetylcholine release.

Conclusions

In conclusion, the described studies have revealed that acetylcholine contributes to airway inflammation and remodeling in COPD and asthma, which is mediated via M₃ receptors. This involves neuronally released acetylcholine, but also non-neuronal acetylcholine, which was shown to contribute to goblet cell metaplasia (figure 1). Together, this suggests that patients with COPD or asthma might benefit from anticholinergic therapy to a much larger extent than previously appreciated. Moreover, as bronchodilators, anticholinergics might also affect airway remodeling by preventing mechanical stress. Therefore, I believe that the combined effects of anticholinergic therapy on bronchoconstriction, mucus secretion, inflammation and remodeling may together lead to a positive outcome for patients with COPD or asthma.
Acetylcholine released from nerve terminals and airway cells contributes to inflammation and remodeling of the airways via $M_1$ receptors. Environmental factors, including [1] cigarette smoke (CS), [2] allergens and [3] bronchoconstricting agents, can induce or enhance acetylcholine release, and thereby contribute to inflammation and remodeling (chapter 2). CS exposure results in enhanced cytokine release, including IL-8, IL-6 and MCP-1, and TGF-β release, mediated via $M_1$ receptors (chapter 3). This is primarily mediated via $M_1$ receptors on structural cells, although $M_1$ receptors on inflammatory cells are also involved (chapter 4). The CS-induced inflammatory response is inhibited by $M_2$ and $M_3$ receptors. $M_2$ receptors on the airway epithelium control electrolyte and water secretion, and might affect the clearance of smoke particles from the airways. $M_3$ receptors on nerve endings are auto-inhibitory receptors, and might inhibit the inflammation by inhibition of acetylcholine release (chapter 3). Exposure to allergens also enhances inflammation and remodeling of the airways. Enhanced goblet cell metaplasia, airway smooth muscle thickening and extracellular matrix deposition is mediated via $M_3$ receptors. No role for $M_2$ and $M_3$ receptors is observed, and the latter might be dysfunctional after allergen exposure (chapter 6). Allergen-induced inflammation is not affected by knock-out of the $M_3$ receptor. This suggests that bronchoconstriction might drive airway remodeling, independent of the inflammatory response. Bronchoconstriction induces mechanical strain, which results in enhanced expression of airway smooth muscle α-actin and extracellular matrix proteins (chapter 7). Both neuronally released acetylcholine (chapter 5) and non-neuronally released acetylcholine (chapter 8) contribute to inflammation and remodeling processes in the airways.
Taken together, the studies described in this thesis have revealed that:

- The neurotransmitter acetylcholine is not only involved in bronchoconstriction and mucus secretion in the airways, but also contributes to airway inflammation and remodeling (chapter 2). Like the effects on bronchoconstriction and mucus secretion, effects on inflammation and remodeling are primarily mediated via M₃ receptors (chapter 3 and chapter 6).

- The M₃ receptor plays a pro-inflammatory role in cigarette smoke-induced inflammation, whereas M₁ and M₂ receptors exert an anti-inflammatory effect (chapter 3). This suggests that M₃ selective antagonists can reduce neutrophilia.

- The pro-inflammatory effect of acetylcholine on cigarette smoke-induced inflammation is primarily mediated via M₃ receptors located on structural cells (chapter 4).

- The M₃ receptor contributes to allergen-induced airway remodeling, whereas no role for M₃ receptors in allergen-induced inflammation is observed (chapter 6). This suggests that bronchoconstriction might drive airway remodeling, independent of the inflammatory response (chapter 6 and chapter 7).

- M₁ and M₂ receptors are not involved in allergen-induced inflammation and remodeling (chapter 6).

- Targeted lung denervation attenuates inflammation in patients with COPD, providing the first direct evidence that acetylcholine might affect inflammation in humans. This implies an important role for neuronal acetylcholine in the inflammatory response (chapter 5).

- Tiotropium prevents IL-13-induced goblet cell metaplasia of human airway epithelial cells, which indicates that non-neuronal acetylcholine contributes to epithelial cell differentiation (chapter 8).

- Patients with COPD and asthma may benefit from anticholinergic therapy to a much broader extent than previously appreciated, since anticholinergic therapy might also inhibit inflammation and remodeling of the airways. This can be achieved via TLD or via anticholinergic treatment. Treatment with an even more selective muscarinic antagonist, solely inhibiting M₃ receptors, might further enhance this beneficial effect (this thesis).
References


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