Acetylcholine beyond bronchoconstriction: a regulator of inflammation and remodeling

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CHAPTER 2

REGULATION OF AIRWAY INFLAMMATION AND REMODELING BY MUSCARINIC RECEPTORS: PERSPECTIVES ON ANTICHOLINERGIC THERAPY IN ASTHMA AND COPD

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Abstract

Acetylcholine is the primary parasympathetic neurotransmitter in the airways and an autocrine/paracrine secreted hormone from non-neuronal origins including inflammatory cells and airway structural cells. In addition to the well-known functions of acetylcholine in regulating bronchoconstriction and mucus secretion, it is increasingly evident that acetylcholine regulates inflammatory cell chemotaxis and activation, and also participates in signaling events leading to chronic airway wall remodeling that is associated with chronic obstructive airways diseases including asthma and COPD. As muscarinic receptors appear responsible for most of the pro-inflammatory and remodeling effects of acetylcholine, these findings have significant implications for anticholinergic therapy in asthma and COPD, which is selective for muscarinic receptors. Here, the regulatory role of acetylcholine in inflammation and remodeling in asthma and COPD will be discussed including the perspectives that these findings offer for anticholinergic therapy in these diseases.

Introduction

Acetylcholine is the primary parasympathetic neurotransmitter in the airways and a paracrine/autocrine hormone released from non-neuronal origins. The role of acetylcholine in the regulation of bronchomotor tone and mucus secretion from airway submucosal glands is well established (1). More recent findings suggest that acetylcholine, acting on muscarinic receptors, regulates additional functions in the airways, including inflammation and remodeling in obstructive airways diseases such as asthma and COPD (2-4). Based on these findings, we have previously questioned the traditional view on the role of acetylcholine, and suggested new possibilities for therapeutic targeting of muscarinic receptors in asthma and COPD (2). In this review, we will discuss the role of muscarinic receptors in obstructive airways disease further and update the discussion in view of these recent research papers and trials. In view of the selectivity of currently used anticholinergics for muscarinic receptors, we will not elaborate on the role of nicotinic receptors in this review. Nicotinic receptors are, however, expressed in the airways and mediate anti-inflammatory effects of acetylcholine. For excellent reviews on the anti-inflammatory role of nicotinic receptors, we would like to refer to recently published reviews (5-7).

Acetylcholine and muscarinic receptors in the airways

Biosynthesis, metabolism and mode of action of acetylcholine

Acetylcholine is synthesized from choline and acetyl-CoA mainly by the enzyme choline acetyltransferase (ChAT) (7). Airway neurons and non-neuronal cells such as airway epithelial cells express ChAT and release acetylcholine (8). Further, macrophages, mast cells, lymphocytes, granolucytes, fibroblasts and smooth muscle cells all have been suggested to express ChAT (7), although the release of acetylcholine from these cells has not yet been demonstrated directly. Acetylcholine can bind to and activate a family of G protein coupled muscarinic receptors, but also a family of nicotinic receptors, which are ligand gated cation channels (9). Most inflammatory and airway structural cells express muscarinic and/or nicotinic receptors (2,7). The individual receptor subtypes and subunits expressed by these cells have been reviewed extensively by Wessler and Kirkpatrick (7).

The mechanisms that regulate the metabolism of non-neuronal acetylcholine by airway epithelial cells are still not fully established, although recent studies have yielded important new insights. The uptake of choline is the rate-limiting step in the synthesis of acetylcholine. Choline uptake in airway epithelial cells is regulated by the high affinity choline transporter (CHT1) and by choline-specific transporter-like proteins (CTL) (10,11). Organic cation transporter (OCT) subtypes 1 and 2 play a dominant role in the release of acetylcholine by airway epithelial cells (10,11). Furthermore, the expression of the vesicular acetylcholine transporter (VAChT) by some epithelial cell types, including secretory cells, neuroendocrine cells and brush cells has been reported, suggesting that storage and release of acetylcholine via vesicles may mediate acetylcholine release by non-neuronal cell types (10,11). The expression of muscarinic receptors, nicotinic receptors, synthesizing enzymes such as ChAT and the release of acetylcholine from non-neuronal cells is solid evidence for the existence of a non-neuronal cholinergic system in the airways next to the well-established neuronal cholinergic system.

Muscarinic receptor expression and function in the airways

Muscarinic receptors are the target for anticholinergic therapy in obstructive airways diseases as asthma and COPD and are the focus of this review. Muscarinic receptors are expressed by structural cells in the airways, predominantly airway smooth muscle, airway epithelium and airway fibroblasts. The parasympathetic neural network penetrates deep into the airway wall, and regulates bronchoconstriction, the release of mucus from submucosal glands, and to a lesser degree from goblet cells in the airway epithelium (2). The functional role of non-neuronal acetylcholine released from the airway epithelium is less well described, although recent studies suggest a role in airway smooth muscle contraction (12). It should be noted however that this finding is still controversial (13,14).

Additionally, acetylcholine, either neuronal or non-neuronal, may modulate airway inflammation and remodeling, as will be discussed further on.

The distribution of muscarinic receptor subtypes throughout the bronchial tree is mainly restricted to muscarinic M_1, M_2 and M_3 receptors (2). M_1 receptors are expressed by epithelial cells, where they play a modulatory role in electrolyte and water secretion, and in the ganglia, where they facilitate parasympathetic neurotransmission. M_2 receptors are expressed by neurons, where they function as autoreceptors, inhibiting the release of acetylcholine from both preganglionic nerves and from parasympathetic nerve terminals. M_2 autoreceptors are dysfunctional in allergic asthma due to eosinophil-derived release of major basic protein which acts as an allosteric antagonist of the M_2 receptor (15), augmenting acetylcholine release. Furthermore, M_2 receptors are widely expressed by airway mesenchymal cells such as fibroblasts and smooth muscle cells (2). Recent studies suggest that they may modulate cellular responses associated with airway remodeling (16). Also, a role in inhibition of G_s mediated airway smooth muscle relaxation has been proposed (1). M_3 receptors are probably the best characterized subtype and are the dominant receptor subtype in the regulation of mucus secretion from submucosal glands and airway smooth muscle contraction (2). As a result, M_3 receptors are the primary target for anticholinergics, and M_3 subtype-selectivity has been advocated for by several research groups (17-22).

Muscarinic receptors as therapeutic targets for asthma and COPD
Anticholinergic therapy in COPD, and to a lesser extent asthma, is mainly aimed at inhibition of bronchoconstriction by inhibition of muscarinic receptors. Although the term anticholinergic is most commonly used, all available anticholinergics used for the treatment of asthma and COPD are in fact specific antimuscarinics as they lack binding affinity at the nicotinic receptor. Clinically available anticholinergics are the short-acting ipratropium and the long-acting tiotropium. In addition to its longer duration of action, tiotropium has a considerably slower rate of dissociation from the M_1 and the M_3 receptor than from the M_2 receptor, making the drug 'kinetically selective' for M_1 and M_3 receptors (21). It is conceivable that this functional selectivity of tiotropium is beneficial, as smooth muscle contraction is primarily mediated by M_3 receptors, whereas M_2 receptor blockade facilitates acetylcholine release from parasympathetic nerves (2). However, direct evidence for a beneficial clinical effect of this functional M_3 selectivity of tiotropium is still lacking and the major difference between these drugs appears to be the duration of action.

The Understanding the Potential Long-term Impacts of on Function with Tiotropium (UPLIFT) trial has demonstrated that treatment with tiotropium provides a significant and sustained improvement in lung function and quality of life in COPD patients, and reduces exacerbations and hospitalizations (23). Currently available anticholinergics are the short-acting ipratropium and the long-acting tiotropium. These can be used either as monotherapy or in combination with β_2-agonists and provide significant improvement in FEV_1 in both asthma and COPD patients (24-27). The combination therapy with β_2-agonists is more effective than anticholinergic treatment alone; nonetheless, monotherapy is already markedly effective (28). The explanation for this relatively large effect of monotherapy may lie within the role that mediators of inflammation (e.g. thromboxane A_2, histamine) have in activating the airway cholinergic system. Airway inflammation has several ways to increase the output of neuronally released acetylcholine, as it results in exposure and activation of afferent C-fibres that facilitate ganglionic and central parasympathetic neurotransmission. Further, the release of acetylcholine can be facilitated directly via excitatory receptors for inflammatory mediators (e.g. prostaglandins, tachykinins) present on parasympathetic nerve terminals, and indirectly via inhibition of the M_2 autoreceptor through the release of eosinophil derived major basic protein that acts as an allosteric M_2 receptor antagonist (1,2). As a result, the bronchoconstrictor response (and perhaps additional responses) induced by pro-inflammatory mediators such as thromboxane A_2 is for a large part mediated by neuronally released acetylcholine (29). Further, bronchoconstriction induced by histamine after the early asthmatic response can be inhibited by ipratropium in a guinea pig model of asthma (30). This advocates for the use of anticholinergic therapy not only in COPD – where parasympathetic tone is the primary reversible component of airway obstruction (31) – but also in asthma. Indeed, recent clinical trials indicate significant improvements in lung function in asthma patients on top of usual care, and show that tiotropium therapy is non-inferior to β_2-agonist therapy when combined with corticosteroids in severe asthma patients (25,26,32). The additional observations that next to FEV_1 also exacerbation rate and lung function decline in subgroups of COPD patients are improved by treatment with tiotropium (33,34) has prompted speculations on the possible beneficial effects of anticholinergics on airway inflammation and remodeling (35).

Airway inflammation

Asthma and COPD are both characterized by chronic airway inflammation, albeit that the patterns of inflammation are markedly different. Different subtypes of T cells are involved in asthma and COPD: in asthma there is an increase in T_H2 (CD4^{+}) cells, whereas in COPD

CD8$^+$ T cells predominate. Furthermore, the inflammation that occurs in asthma can be described as eosinophilic, whereas that occurring in COPD is mainly neutrophilic. However, when disease severity increases these differences become less pronounced (36).

Inflammation and the non-neuronal cholinergic system
Increasing evidence suggests that acetylcholine contributes to airway inflammation. In 2004, Wessler et al. found that in patients with atopic dermatitis, a condition characterized by T_H2 type inflammation and often associated with bronchial asthma, expression of ChAT is increased in skin biopsies, with a consequent increase in acetylcholine (37). Further, Profita et al. (2011) demonstrated that cigarette smoke extract upregulated the non-neuronal cholinergic system in bronchial epithelial cells, by showing that expression of M_2 and M_3 receptors and ChAT mRNA and protein were increased, whereas M_1 receptor levels were not affected. Consequently, acetylcholine levels in cell extracts were significantly higher after stimulation with cigarette smoke extract. This increase could be reduced by tiotropium (38). In contrast, lungs of ovalbumin challenged rats and mice show a significant decrease in ChAT and other components of the cholinergic system, including the functionally relevant choline transporter CHT1 (39). Future studies are clearly warranted within this area to better understand the complex mechanism of regulation of the cholinergic system by inflammation and the significance of this process in asthma and COPD.
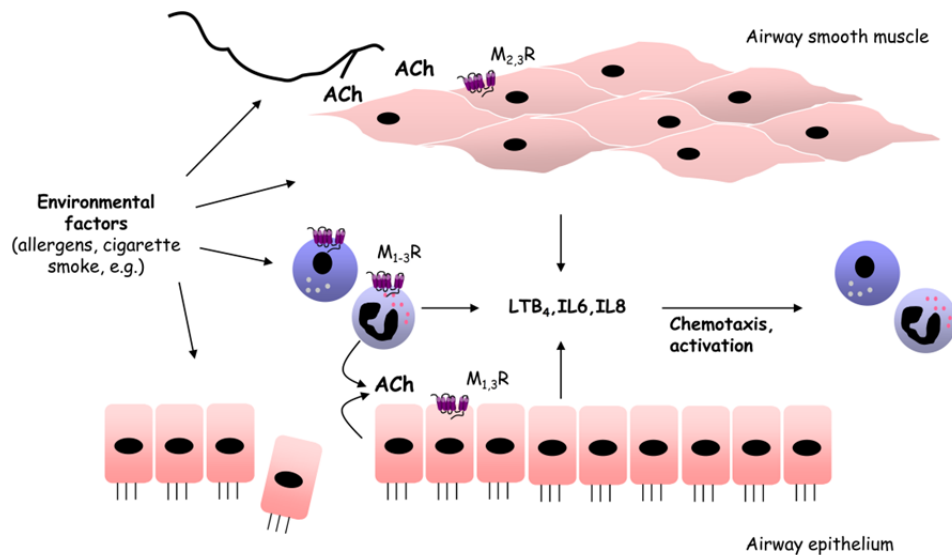
Inflammatory cells
Acetylcholine has been shown to affect inflammatory cells involved in asthma and COPD directly, by inducing proliferation or cytokine release from these cells. Carbachol can induce the proliferation of macrophages from mice *in vitro* (40). Also T-cell proliferation can be observed *ex vivo* after treatment of rats with the muscarinic agonist oxotremorine, whereas atropine suppresses the proliferation of T-cells (41). These anti-inflammatory properties of atropine were also demonstrated in rats *in vivo*, were it suppressed the turpentine-induced infiltration of leukocytes (41). Moreover, bovine alveolar macrophages exhibit neutrophil, eosinophil and monocyte chemotactic activity in response to acetylcholine, which is likely explained by cholinergic induction of leukotriene B_4 (LTB_4) release (42). Recently, this was confirmed for primary human macrophages (43). Moreover, it was shown that acetylcholine-induced release of chemotactic activity from monocytes, macrophages and epithelial cells could be inhibited by tiotropium (43). It has also been shown that acetylcholine can induce the release of LTB_4 from sputum cells of COPD patients (44). These results are consistent with a study demonstrating that tiotropium and also acetylcholinesterase, the degrading enzyme of acetylcholine, inhibited alveolar macrophage mediated migration of neutrophils from COPD patients (45). Using

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the M_3-selective antagonist 4-DAMP it was shown that this effect is mediated via the M_3 receptor (45). Further, although R,R-glycopyrrolate, a muscarinic receptor antagonist, did not inhibit LPS-induced TNF-α release by itself, it synergistically inhibited the rolipram and budesonide induced decrease in TNF-α release from human primary monocytes (46). All these findings support a broad role for acetylcholine acting on muscarinic receptors in the regulation of airway inflammatory cells (figure 1).

Epithelial cells
The expression of non-neuronal acetylcholine is relatively high in bronchial epithelial cells (8). Acetylcholine is known to induce eosinophil, monocyte and neutrophil chemotactic activity in bronchial epithelial cells (47,48). The increase in epithelial neutrophil chemotactic activity by acetylcholine could be inhibited by tiotropium, indicating the involvement of muscarinic receptors in this response (49). The acetylcholine-induced neutrophil chemotactic activity from epithelial cells is partially dependent on IL-8 release,

Figure 1. The regulatory role of acetylcholine in inflammatory cell chemotaxis and activation. Acetylcholine can be neuronally released or secreted as an autocrine or paracrine hormone from inflammatory cells and airway structural cells, most notably airway epithelial cells. In susceptible individuals, the release of acetylcholine may be enhanced in response to environmental factors such as cigarette smoke or allergens. As a consequence, pro-inflammatory cytokines including IL-6, IL-8 and LTB4 are produced, which attract and activate inflammatory cells, most notably neutrophils. Muscarinic M_3 receptors expressed on airway smooth muscle and muscarinic M_{1-3} receptors expressed by airway epithelial cells mediate the release of these factors via activation of ERK1/2 and NF-kB signaling pathways.

since it is inhibited by an anti-IL-8 monoclonal antibody (49). In line with this contention, the increase in IL-8 release in response to acetylcholine could be partially inhibited by tiotropium. In addition, acetylcholine induced LTB_4 release from bronchial epithelial cells in a tiotropium sensitive manner (38). Both IL-8 and LTB_4 release from bronchial epithelial cells is mediated via ERK1/2 and NF-κB signaling pathways and dependent on multiple muscarinic receptor subtypes ($M_1/M_2/M_3$) (38,49). Taken together, these studies implicate an important role for epithelial acetylcholine in airway inflammation, via the activation of muscarinic receptors (figure 1).

Another potential mechanism by which tiotropium could inhibit inflammation induced by epithelial cells is by attenuating respiratory syncytial virus (RSV) replication in these cells (50). RSV is one of the major causes of acute lower respiratory tract infection and has been detected in patients with exacerbations of asthma and COPD (51). In an *in vitro* study, Iesato et al. demonstrated that the attenuation of virus replication by tiotropium was partially due to inhibition of RhoA activity. Moreover, tiotropium inhibited epithelial IL-6 and IL-8 production induced by RSV infection (50). *In vivo* studies are needed to investigate the importance of inhibition of infection-induced airway inflammation by tiotropium.

Airway smooth muscle cells
The airway smooth muscle is increasingly recognized for its role in modulating inflammation by secreting cytokines and chemokines (52), and it has been shown that muscarinic receptors on airway smooth muscle cells are involved in these responses. Stimulation of bovine airway smooth muscle strips with the muscarinic agonist carbachol induces pro-inflammatory gene expression, including IL-6, IL-8 and cyclo-oxygenase-2 (53). Furthermore, carbachol augmented the cyclic stretch-induced expression of these genes (53). Stimulation of airway smooth muscle cells with carbachol also induces the protein release of IL-6 and IL-8 via M_3 receptors (54). Furthermore, methacholine strongly augmented cigarette smoke extract (CSE) induced IL-8 release (54). In line with findings in epithelial cells, IL-8 release induced by stimulation with methacholine and CSE in airway smooth muscle is ERK1/2 and NF-κB dependent (55).

In vivo studies
The regulatory role of muscarinic receptor signaling in inflammatory processes involved in asthma and COPD has been confirmed by *in vivo* studies, using animal models of these diseases.

Wollin and Pieper were the first to report anti-inflammatory properties of tiotropium in an animal model of cigarette smoke induced COPD (56). Total cell number and neutrophils in the bronchoalveolar lavage fluid (BALF) were concentration-dependently decreased after treatment with tiotropium. Furthermore, tiotropium inhibited the increase of several cytokines in the BALF, including IL-6, KC, TNF-α and LTB$_4$ (56). Similar inhibitory effects of tiotropium on airway neutrophilia were observed in a guinea pig model of LPS-induced COPD (57). Moreover, neutrophilia was inhibited by ipratropium in a cadmium-induced rat model of pulmonary inflammation (58), by tiotropium in a HCl-induced rat model of gastro-oesophageal reflux (59) and by bilateral vagotomy or treatment with atropine in a diesel particle-induced rat model of pulmonary inflammation (60). Of interest, the latter study found that atropine was more effective in inhibiting pulmonary inflammation than bilateral vagotomy, suggesting a role for non-neuronal acetylcholine in this response (60).

These findings may also be relevant for asthma. Our group has shown that tiotropium partially inhibits eosinophilia in a guinea pig model of asthma (61), which has been confirmed by Buels et al. (62). In line with these findings, infiltration of macrophages and eosinophils in the BALF was significantly inhibited by tiotropium treatment in a murine model of asthma. Furthermore, expression levels in BALF of IL-4, IL-5 and IL-13 were decreased by tiotropium treatment (63). In addition, aclidinium, a novel muscarinic receptor antagonist, inhibited infiltration of eosinophils in BALF in a mouse model of *Aspergillus fumigatus*-induced asthma (64). A recent study also suggested that M$_3$ receptors regulate these inflammatory responses, although the selectivity profile of the antagonist bencycloquidium that was used in this study precludes firm conclusions on the involvement of other receptor subtypes (65,65). Since both tiotropium and aclidinium are kinetically selective for the M$_3$ receptor, this suggests predominant involvement of this receptor subtype in the observed anti-inflammatory effects in asthma and COPD models described above. This is supported by our own data on M$_3$R$^{-/-}$ mice, in which neutrophilia and cytokine release in BALF were inhibited compared to wild-type mice after exposure to cigarette smoke (**chapter 3**).

Clearly, all these *in vivo* studies indicate a profound role for acetylcholine in inflammation in asthma and COPD, which is in accordance with results of *in vitro* studies that report pro-inflammatory effects of muscarinic receptors (figure 1). The implication of these findings is that treatment with anticholinergics may have beneficial effects that exceed their bronchodilatory properties, a contention confirmed in several models of pulmonary inflammation. However, the exact mechanism responsible for the regulatory role of acetylcholine in inflammation is far from understood.

Airway remodeling

Airway inflammation in chronic airway diseases such as asthma and COPD is often associated with cellular and structural alterations in the airways, referred to as airway remodeling (66). Airway remodeling is considered a major component of irreversible airflow limitation in these diseases (67), is progressive, and correlates with disease severity (68,69). Airway remodeling in asthma and COPD is characterized by mucus gland hypertrophy, goblet cell hyperplasia and pulmonary vascular remodeling (66). In addtion, in asthma the basement membrane is thickened, there is subepithelial fibrosis, and there is considerable thickening of the airway smooth muscle bundle (67). In contrast, in COPD the fibrosis is mostly peribronchial, and although increased airway smooth muscle mass may occur, this appears restricted to severe stages of COPD (68). Airway structural alterations may accelerate decline of lung function (70).

Epithelial cells and mucus production
The airway epithelial layer is in continuous interaction with the external environment. To protect itself from exogenous stimuli, mucus is secreted under the control of the cholinergic system by muscarinic receptors (71). Mucus secretion can be increased by electrical field stimulation of the vagal nerve in bronchial preparations, predominantly via M_3 receptors on the submucosal glands (71). In addition, electrolyte and water secretion are regulated by M_1 and M_3 receptors (72,73). Neuronal M_2 autoreceptors appears to regulate the extent of the secretory response, by limiting neuronally released acetylcholine (73). In response to acetylcholine, glandular goblet cells also produce mucus (71).

Mucus hypersecretion is an important pathological feature of chronic airway diseases contributing to airway obstruction (71). MUC5AC expression in airway epithelial cells and airway submucosal glands is directly correlated to airway obstruction in smokers (74) and in smokers, COPD patients and asthma patients, the expression of the MUC5AC gene is augmented (75). Also, the expression of MUC5B and the insoluble MUC2 are increased, particularly in COPD. The ratio of mucus cells to serous cells in the submucosal glands is also increased in COPD patients (76). In vitro studies demonstrated that aclidinium suppressed carbachol-induced MUC5AC overexpression in human bronchial tissue. Additionally, the increased expression of MUC5AC by the co-stimulation of cigarette smoke extract and carbachol could be attenuated by the use of aclidinium or atropine (77). Moreover, epidermal growth factor (EGF) stimulation enhanced the ACh-induced response on mucus cell activation in airway submucosal glands (78). *In vivo* studies confirm the role of acetylcholine in mucus hypersecretion and demonstrate that tiotropium reduces allergen-induced mucus gland hypertrophy and MUC5AC-positive

goblet cell number in guinea pigs (61). Further, it has been reported that tiotropium inhibits neutrophil elastase-induced goblet cell metaplasia in mice (79) and that treatment with tiotropium inhibited the increased MUC5AC expression and mucus gland hypertrophy in a guinea pig model of COPD (57). This demonstrates the important role of acetylcholine in the regulation of mucus secretion, both *in vitro* and in animal models of asthma and COPD *in vivo* (figure 2).

Acetylcholine may also regulate the proliferative and pro-fibrotic responses of airway epithelial cells. Bronchoconstriction induced by repeated challenges with methacholine induced epithelial cell proliferation and an increase in the expression of the profibrotic cytokine TGF-β by these cells in mild asthmatic subjects (80). In line with these findings, airway constriction induced by methacholine significantly increased the phosphorylation of the EGF receptor in airway epithelial cells (81). Moreover, in rat tracheal epithelial cells, acetylcholine induces proliferation mediated by M_1 receptors (82) and autocrine release of acetylcholine is sufficient to induce monkey airway epithelial cell proliferation (8). Thus, the cholinergic system is able to regulate epithelial cell proliferation, either through the induction of mechanical strain or in an autocrine/paracrine manner, which is required for the repair of the airway epithelial layer.

Mesenchymal cells
Airway mesenchymal cells (e.g. fibroblasts, airway smooth muscle cells) contribute to airway remodeling by means of proliferation, contractile protein expression and the release of components such as mediators, extracellular matrix proteins and matrix metalloproteinases (MMPs) (83,84). *In vitro* studies showed that the stimulation of muscarinic receptors on lung fibroblasts induces cell proliferation and the synthesis of collagen (16,85) through the activation of the mitogen-activated protein kinase pathway (85,86). This effect was mediated by the activation of M_2 receptors (16). Interestingly, acetylcholine-induced cell proliferation is enhanced in human lung fibroblasts from COPD patients compared with healthy non-smokers and healthy smokers without COPD (87). The higher activation of cell proliferation in fibroblasts from COPD patients was due to enhanced ERK1/2 and NF-κB phosphorylation. Notably, the synthesizing enzyme ChAT was also increased in lung fibroblasts from healthy smokers and COPD patients (87).

MMPs play a key role in airway remodeling, inflammation and emphysema (88). In COPD patients, increased expression levels of MMP-1, MMP-2 and MMP-9 have been reported (89,90). The activity of the MMPs can be inhibited by tissue inhibitor of matrix metalloproteinases (TIMPs) (88). Recently, it was demonstrated that tiotropium inhibited TGF-β-induced protein expression of both MMP-1 and MMP-2 in human lung fibroblasts,

but had no effect on the TGF-β-induced TIMP-1 and TIMP-2 expression (91,92). Therefore, these data suggest that treatment with tiotropium improves the balance between MMPs and TIMPs, inhibiting pro-fibrotic responses. As MMPs also play important roles in the infiltration of inflammatory cells, this effect could also contribute to the anti-inflammatory properties of anticholinergics.

Airway smooth muscle thickening is a characteristic pathological feature of asthma, and to a lesser extent of COPD. The induction of airway smooth muscle cell proliferation by growth factors, including PDGF and EGF, can be enhanced by the stimulation of muscarinic receptors (93-96). Specifically, Gβγ subunits derived from Gq protein coupled receptors cooperate with receptor tyrosine kinases (e.g. the PDGF/EGF receptor) to induce synergistic activation of PI3K/Akt/p70S6K signaling leading to cell proliferation (93,95,96).
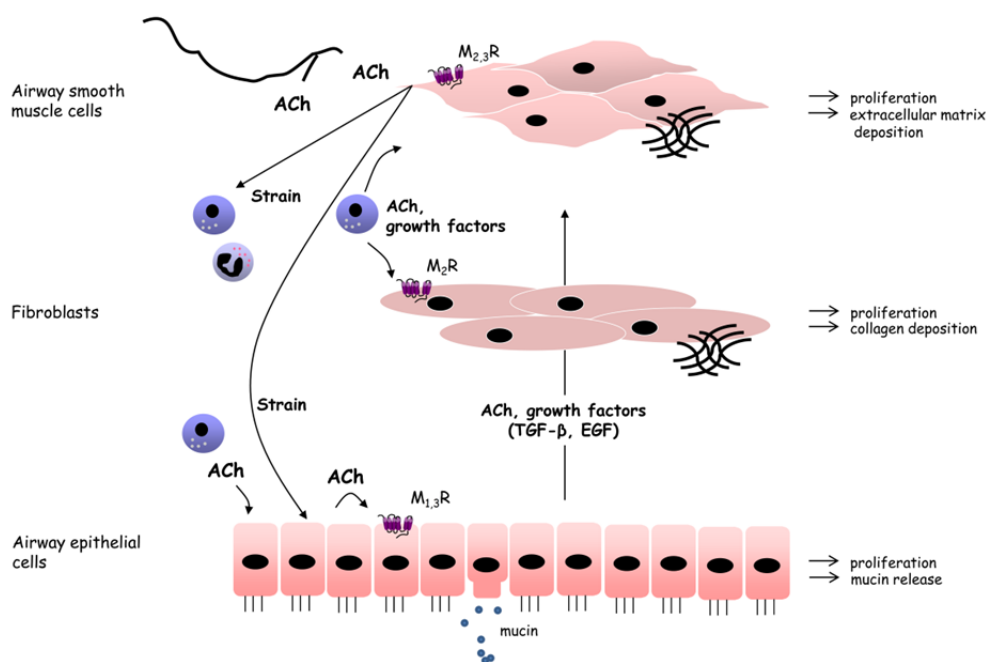
Figure 2. The regulatory role of acetylcholine in airway wall remodeling. Acetylcholine is neuronally released and secreted as an autocrine or paracrine hormone from airway structural cells and inflammatory cells. In the inflamed airway, inflammatory cells and airway epithelial cells also secrete growth factors that in concerted action with acetylcholine activate cell proliferation and matrix production by airway mesenchymal cells, including airway fibroblasts and airway smooth muscle cells. Furthermore, acetylcholine activates smooth muscle contraction leading to airway wall compression, which activates inflammatory cells and promotes remodeling responses by airway epithelial cells. Acetylcholine also directly promotes mucus production by and cell proliferation of airway epithelial cells.

Moreover, the activation of conventional PKC isoenzymes, likely via M_3 receptor mediated $G\alpha_q$ stimulation, leads to GSK-3 inactivation, which potentiates both translational and transcriptional processes (94). These pathways are also involved in the acquisition of contractile protein expression by TGF-β via transcriptional and translational processes (97-99) and can be activated by muscarinic receptor stimulation (100). Indeed, the expression of myosin light-chain kinase was augmented by carbachol in human airway smooth muscle cells exposed to cyclical mechanical strain (101). Additionally, we recently described that muscarinic receptor stimulation enhanced the TGF-β1-induced contractile protein expression in human airway smooth muscle cells (102). Collectively, these findings suggest an important role of muscarinic receptor stimulation in the proliferation and maturation of mesenchymal cells (figure 2).

In vivo studies

Inhibitory effects of anticholinergics on airway mesenchymal cell remodeling have indeed been reported in animal models of asthma and COPD. Treatment with tiotropium significantly inhibited airway smooth muscle remodeling in a guinea-pig model of chronic asthma using repeated challenges with ovalbumin (103). This was associated with the inhibition of increased contractile protein expression and of airway smooth muscle thickening. In a murine model of asthma, it was shown that tiotropium could also significantly inhibit smooth muscle thickening and the expression of TGF-β1 in BALF (63). Similar effects have been described for the M_3 receptor selective antagonist bencycloquidium bromide (65). Furthermore, bencycloquidium bromide reduced mucus production, globet cell metaplasia and collagen deposition and inhibited the upregulation of MMP-9, but not of TIMP-1 mRNA (65). Treatment with tiotropium also inhibited the increased peribronchial collagen deposition in a guinea pig model of COPD (57). Similarly, in a chronic gastro-oesophageal reflux model, tiotropium treatment prevented the increase in airway fibrosis (59). Taken together, these *in vivo* studies confirm *in vitro* studies showing that anticholinergics have anti-remodeling properties in asthma and COPD (figure 2).

Clinical implications

The above mentioned *in vitro* and *in vivo* studies indicate significant pro-inflammatory and remodeling effects for acetylcholine via muscarinic receptors, suggesting that anticholinergics may have anti-inflammatory and anti-remodeling properties in asthma and COPD patients. This hypothesis still needs to be proven in clinical studies, however. In the UPLIFT study, COPD patients treated with tiotropium during a 4 year period showed an improved quality of life and lung function, and a reduction in the frequency of

exacerbations. Although tiotropium did not reduce FEV_1 decline in the overall study population (23), in pre-specified post-hoc studies, GOLD stage II and young COPD patients with rapid lung function decline had a significant improvement in the accelerated post-bronchodilator FEV_1 decline (33,34). No notable reduction in exacerbation frequency was reported for ipratropium (104,105). This suggests a beneficial role for tiotropium as a long-acting anticholinergic or a possible role for M_3 receptor subtype selectivity, as tiotropium is kinetically selective for M_3 receptors compared with ipratropium. Moreover, it also indicates anti-inflammatory effects of tiotropium, since patients who have more exacerbations demonstrate increased levels of inflammatory markers at stable state (106). However, Powrie et al. (2007) were not able to demonstrate a reduction in sputum IL-6 or IL-8 levels in patients treated with tiotropium during one year, even though the number of exacerbations was significantly decreased (107). A possible explanation for this discrepancy proposed by the authors is that the reduction in amount of sputum after tiotropium treatment might result in an increase in cytokine concentrations. Measurement of cytokine concentrations in sputum might therefore not be the optimal method. Also, Perng et al. did not find a decrease in sputum IL-8 levels after tiotropium treatment (108). However, the treatment group in their study was small and patients only received tiotropium for 12 weeks. Further studies are therefore needed to elucidate the mechanisms by which tiotropium reduces exacerbations and FEV_1 decline in subgroups of COPD patients and whether this is based on the anti-inflammatory effects of tiotropium discussed in this paper or by other effects, including a reduction in dyspnea or mucus hypersecretion. Likewise, further studies on the beneficial effects of anticholinergics in asthma patients are warranted. In patients with severe, uncontrolled asthma it has recently been shown that treatment with tiotropium improves lung function (25). Furthermore, a recent clinical trial showed that repeated inhalations with the muscarinic receptor agonist methacholine induces airway remodeling in asthma patients, including the expression of TGF-β and collagen I in bronchial biopsies (80). Therefore, although a rationale for beneficial effects of anticholinergics beyond the well-described bronchodilator properties in asthma and COPD certainly exists, it is evident that this still needs to be confirmed in clinical studies.

Acknowledgements

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