Acetylcholine beyond bronchoconstriction

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Review

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Acetylcholine beyond bronchoconstriction: roles in inflammation and remodeling

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Acetylcholine is the primary parasympathetic neurotransmitter in the airways, where it not only induces bronchoconstriction and mucus secretion, but also regulates airway inflammation and remodeling. In this review, we propose that these effects are all primarily mediated via the muscarinic M_3 receptor. Acetylcholine promotes inflammation and remodeling via direct effects on airway cells, and via mechanical stress applied to the airways sequential to bronchoconstriction. The effects on inflammation and remodeling are regulated by both neuronal and non-neuronal acetylcholine. Taken together, we believe that the combined effects of anticholinergic therapy on M_3-mediated bronchoconstriction, mucus secretion, inflammation, and remodeling may account for the positive outcome of treatment with these drugs for patients with chronic pulmonary obstructive disease (COPD) or asthma.

Acetylcholine: not only a contractile neurotransmitter

It is generally accepted that acetylcholine (see Glossary), released from parasympathetic nerve endings, induces airway smooth muscle contraction. For this reason, anticholinergics are used as bronchodilators in obstructive airway diseases [1]. However, recent research has revealed that the (patho)physiological role of acetylcholine exceeds smooth muscle contraction, because resident and inflammatory cells that control inflammation and remodeling produce acetylcholine and express muscarinic receptors [2]. Moreover, anticholinergics reduce neutrophilia and small airway remodeling in animal models of COPD [3,4], and eosinophilia and airway remodeling in animal models of allergic asthma [5–7].

Despite these recent developments, anticholinergics are still primarily used for their bronchodilatory effects. Selectivity for the muscarinic M_3 receptor subtype is considered beneficial, because this is the primary muscarinic receptor subtype mediating the contractile effects of acetylcholine. Until recently, it was not known whether M_3 subtype selectivity of anticholinergics was a desired property for anti-inflammatory and remodeling effects, and whether the selective focus on the bronchodilatory capacities of anticholinergics is justified. Here, we synthesize the recent literature on the pro-inflammatory and pro-remodeling actions of acetylcholine in humans, human cell systems, and animal models of airways inflammation and remodeling, focusing on three key questions: (i) what is the role of individual muscarinic receptor subtypes in these responses; (ii) what is the role of the bronchoconstrictor effects of acetylcholine via the M_3 receptor in inflammation and remodeling; and (iii) what is the role of neuronal and non-neuronal acetylcholine?

Anticholinergics as bronchodilators in COPD and asthma

COPD and asthma are chronic obstructive airway diseases, and the incidence of both has increased over the past decades [8]. COPD is a leading cause of morbidity and mortality worldwide and is expected to become the third leading cause of death in 2020, which results in a substantial economic and social burden [9]. With 180 000 deaths worldwide each year, mortality from asthma is

Glossary

Acetylcholine: a primary parasympathetic neurotransmitter in the airways, where it induces bronchoconstriction and mucus secretion via muscarinic receptors, and a hormone released from non-neuronal cells.
Airway remodeling: structural changes that occur in both large and small airways in airway diseases, including asthma and COPD.
Anticholinergics: muscarinic receptor antagonists used for the treatment of COPD and, to a lesser extent, asthma, to inhibit the effects of acetylcholine.
Asthma: a chronic inflammatory disorder of the airways associated with airway hyper-responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing.
COPD: a chronic inflammatory disorder of the airways associated with airflow limitation that is usually progressive.
Eosinophilia: increase of the number of eosinophils, which are leukocytes involved in the allergic asthmatic response.
Forced expiratory volume in 1 s (FEV_1): the volume of air that can forcibly be blown out in 1 s after full inspiration.
Lipopolysaccharides (LPS): major component of the outer membrane of Gram-negative bacteria, which generates an inflammatory response.
Muscarinic receptors: G protein-coupled receptors that are expressed by almost all cell types in the airways and are target receptors for acetylcholine.
Neutrophilia: increase in the number of neutrophils, which are granulocytes involved in the inflammatory response in COPD.
St George's Respiratory Questionnaire: an index designed to measure and quantify health status in patients with chronic airflow limitation.

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Box 1. Subtype selectivity of anticholinergics

Tiotropium was the first long-acting anticholinergic introduced to the market 10 years ago. Tiotropium is a potent muscarinic receptor antagonist; however, onset of action is slower compared with the short-acting anticholinergic ipratropium [80]. Although the steady-state affinity of tiotropium for M_1, M_2, and M_3 receptors is similar, dissociation from the M_3 receptor is slower compared with the other receptor subtypes, in particular M_2 receptors (Table I) [81,82]. Used as a bronchodilator, this is a desired property, because inhibition of M_3 receptors inhibits airway smooth muscle contraction, whereas antagonizing autoinhibitory M_2 receptors on vagal nerve terminals would enhance acetylcholine release and thereby enhance airway smooth muscle contraction. Moreover, this long duration of action at the M_3 receptor allows for once-daily dosing. Slow dissociation of tiotropium from the M_3 receptor is attributed to interactions at the binding site, which prevents rapid dissociation via a snap-lock mechanism [83]. Recently, inhaled glycopyrronium, umeclidinium, and aclidinium were also introduced to the market. As becomes clear from Table I, all these long-acting anticholinergics are kinetically selective for the M_3 receptor. However, the dissociation half-life from the M_3 receptor of these compounds is shorter compared with tiotropium [81].

Table I. Binding affinity and half-life time at the human M_1, M_2 and M_3 receptor for different anticholinergics[a]

Anticholinergic	Binding affinitiy (-log M)			$t_{1/2}$ (h)		
	M_1R	M_2R	M_3R	M_1R	M_2R	M_3R
Ipratropium	9.40	9.53	9.58	0.1	0.03	0.22
Tiotropium	10.80	10.69	11.02	10.5	2.6	27
Aclidinium	10.78	10.68	10.74	6.4	1.8	10.7
Glycopyrronium	10.09	9.67	10.04	2.0	0.37	6.1

[a]Binding affinity was determined in heterologous competition experiments against [^{3}H]NMS. Data represent pK_i values of at least three independent experiments performed in triplicate and the standard error was 0.1 or less. Dissociation half-life was determined by the dissociation constants, by analyzing competition kinetics curves in the presence of [^{3}H]NMS and different concentrations of antagonist. At least three independent experiments were performed in triplicate. Data from [81]. Available data for umeclidinium indicate comparable affinity and half-life to tiotropium [84].

considerably lower, but around 300 million people currently have asthma and its greatest burden lies in the morbidity it causes, including in children [10]. Inflammation and remodeling are hallmark features of both diseases, which contribute to the decline in lung function and the severity of the disease [11–13]. Acetylcholine is the primary parasympathetic neurotransmitter in the airways, and induces bronchoconstriction and mucus secretion via M_3 receptors [14]. The activity of the neuronal system is altered in COPD and asthma 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. Enhanced activity of the neuronal system leads to exaggerated acetylcholine release and airway narrowing [15]. Strikingly, the increased cholinergic tone is the major reversible component of airflow limitation in COPD [16,17]. Therefore, anticholinergics are effective bronchodilators in this disease, and represent a first line of treatment [9]. In asthma, use of anticholinergics is most commonly limited to the treatment of exacerbations [18]; however, recent clinical trials indicate that they might also be beneficial for chronic treatment of patients with moderate or severe asthma [19,20]. Currently available long-acting anticholinergics are kinetically selective for M_3 receptors (Box 1).

Muscarinic receptor subtypes in the lung: is there a need for M_3 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 [14]. Multiple muscarinic receptor subtypes are expressed in the airways, which may have differential roles in regulating bronchoconstriction, mucus secretion, inflammation, and remodeling [14]. Muscarinic receptor agonists and antagonists have limited selectivity towards individual muscarinic receptors (Box 1), which hinders the interpretation of the effects of these agents on functional parameters. Therefore, muscarinic receptor-specific knockout animals have proven useful to investigate the role of individual muscarinic receptor subtypes in inflammation and remodeling in animal models of COPD and asthma.

COPD

It was shown in different animal models of COPD that neutrophilic inflammation induced by cigarette smoke or lipopolysaccharides (LPS) can be prevented by pretreatment with anticholinergics, including tiotropium [3,4], glycopyrrolate [21], and aclidinium [22]. Studies in muscarinic receptor subtype-deficient mice support a role for the M_3 receptor in inflammation in response to cigarette smoke [23]. Knock-out of the M_3 receptor or inhibition using the M_3 receptor preferring antagonist 4-DAMP prevented the inflammatory response induced by cigarette smoke in mice, characterized by reduced numbers of neutrophils and reduced expression of the neutrophil chemotactic factor KC [interleukin (IL)-8 in humans] [23]. Interestingly, this inflammatory response was enhanced after knock out of the M_2 receptor. Loss of this autoinhibitory receptor results in enhanced acetylcholine release. With acetylcholine acting as a pro-inflammatory mediator, increased levels of acetylcholine in M_2 receptor subtype-deficient ($M_2R^{-/-}$) animals can explain the observed aggravated inflammatory response. Inflammation was also enhanced after knock out of the M_1 receptor. This might be explained via the role of the M_1 receptor on the airway epithelium, where it contributes to mucus production, by controlling electrolyte and water secretion. Therefore, impaired clearance of detrimental smoke particles from the airways after knock out of the M_1 receptor could underlie the enhanced inflammatory response observed in $M_1R^{-/-}$ animals. This is further supported by a marked induction in the release of the cytokines IL-6 and monocyte chemoattractant protein-1 (MCP-1) in $M_1R^{-/-}$ animals compared with wildtype animals [23] (Figure 1).

The fact that acetylcholine has a pro-inflammatory effect on neutrophilic inflammation via M_3 receptors is supported by various *in vitro* studies. Muscarinic receptor stimulation enhances the release of IL-6 and IL-8 in combination with cigarette smoke extract from airway smooth muscle cells [24]. Tiotropium and the M_3 selective antagonists 4-DAMP and DAU5884 inhibit this response, whereas no effect of the M_2 antagonist gallamine is observed [24]. Furthermore, acetylcholine can induce IL-8 release from bronchial epithelial cells, which can be inhibited by

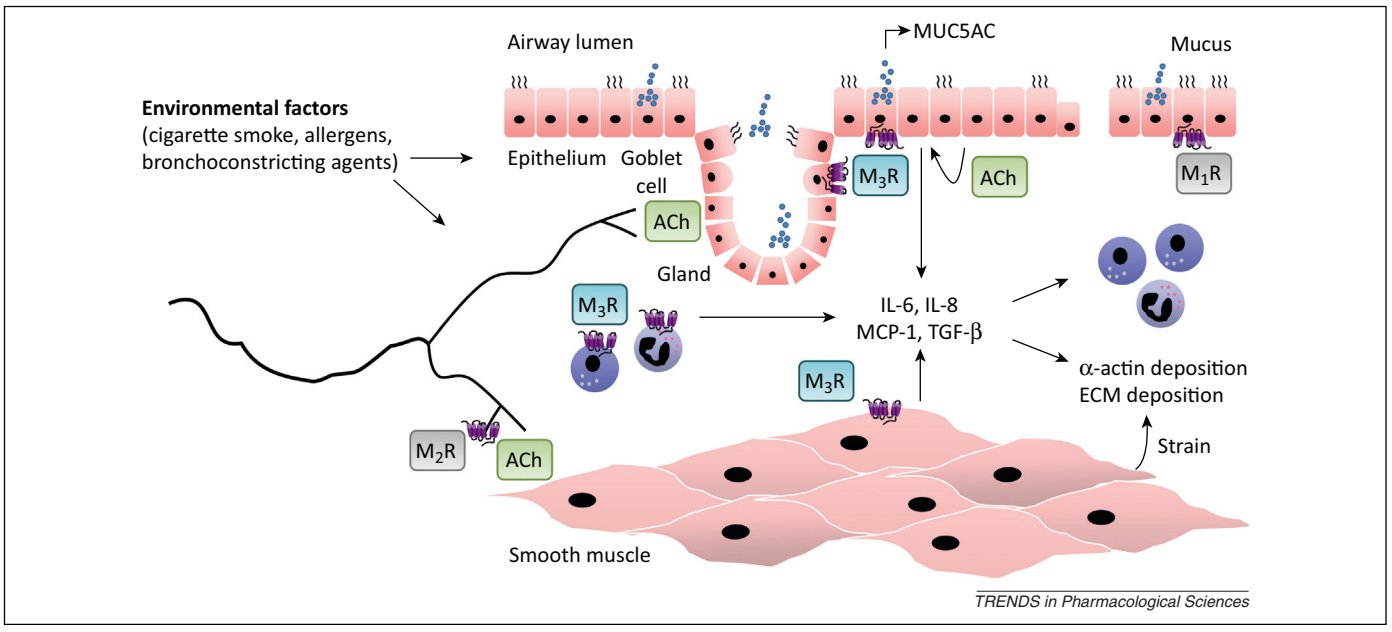

Figure 1. Acetylcholine (ACh) released from nerve terminals and airway cells contributes to inflammation and remodeling of the airways via M_3 receptors. Environmental factors, including cigarette smoke (CS), allergens, and bronchoconstricting agents, can induce or enhance acetylcholine release, and thereby contribute to inflammation and remodeling. CS exposure results in enhanced cytokine release, including interleukin (IL)-8, IL-6, and monocyte chemoattractant protein-1 (MCP-1), and transforming growth factor (TGF)-β release, mediated via M_3 receptors on structural cells. Exposure to allergens also enhances inflammation and remodeling of the airways. Enhanced goblet cell metaplasia, airway smooth muscle thickening, and extracellular matrix (ECM) deposition are mediated via M_3 receptors. Allergen-induced inflammation is not affected by knock out of the M_3 receptor. This suggests that bronchoconstriction drives airway remodeling, independent of the inflammatory response. Both neuronally released acetylcholine and non-neuronally released acetylcholine contribute to inflammation and remodeling processes in the airways. Abbreviation: MUC5AC, mucin 5AC, oligomeric mucus/gel-forming.

tiotropium and 4-DAMP, but not by the M_1 antagonist telenzepine or the M_2 antagonist gallamine [25]. In addition, acetylcholine-induced neutrophil chemotactic activity from macrophages can be inhibited by 4-DAMP, but not by the M_1 antagonist pirenzepine or the M_2 antagonist gallamine [26]. More recently, this was confirmed in macrophages from patients with COPD. Thus, neutrophil chemotaxis induced by LPS-activated alveolar macrophages could be inhibited by tiotropium and 4-DAMP, but not by telenzepine, gallamine, or tubocurarine [27].

M_3 receptors are expressed on almost all cell types in the airways, including structural cells, such as airway smooth muscle cells and epithelial cells, as well as inflammatory cells, such as macrophages and neutrophils (Table 1). As is evident from the various *in vitro* studies described above, both structural cells and inflammatory cells can contribute to the pro-inflammatory effects of acetylcholine, and it is not known whether this effect is primarily mediated via M_3 receptors on structural cells or via M_3 receptors on inflammatory cells. Recently, using bone marrow chimeric animals, it was shown that this is primarily mediated via M_3 receptors on structural cells [28]. Exposure to cigarette smoke induced neutrophilic inflammation in non-irradiated and irradiated control animals. Interestingly, wildtype animals receiving $M_3R^{-/-}$ bone marrow cells showed a similar increase in neutrophil number, suggesting that the M_3 receptor on inflammatory cells is not involved in the pro-inflammatory effect of acetylcholine. By contrast, no increase in the number of neutrophils was observed in $M_3R^{-/-}$ animals receiving wildtype bone marrow cells, suggesting a critical role for airway structural cells in the pro-inflammatory effect of acetylcholine [28].

Asthma

Recently, the effects of acetylcholine on airway remodeling in response to allergen exposure were also demonstrated to be mediated via the M_3 receptor [29]. From animal models of asthma, it was already known that acetylcholine has 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 [5,6]. 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

Table 1. Expression of muscarinic receptors on airway cells and their major effects[a]

Cell	Muscarinic receptor expression	Functional effect
Neuron	M_1, M_2	Neurotransmission
Airway smooth muscle cell	M_2, M_3	Bronchoconstriction via M_3
Epithelial cell	M_1, M_2, M_3	Mucus secretion via M_3
Submucosal gland	M_1, M_3	Mucus secretion via M_3
Fibroblast	M_1, M_2, M_3	Proliferation, extracellular matrix production
Mast cell	M_1, M_3	Inhibition of histamine release
Macrophage	M_1, M_2, M_3	Cytokine production
Lymphocyte	M_1, M_2, M_3	Cytokine production
Neutrophil	M_1, M_2, M_3	Cytokine production
Eosinophil	M_1, M_2, M_3	Unknown

[a]From [15,85–87].

inhibit excessive extracellular matrix deposition and to inhibit T_H2 cytokine release in mice [7]. Using muscarinic receptor-subtype-deficient mice, we demonstrated that allergen-induced goblet cell metaplasia, airway smooth muscle thickening, and enhanced deposition of collagen I and fibronectin is absent or markedly lower in $M_3R^{-/-}$ mice, whereas $M_1R^{-/-}$ and $M_2R^{-/-}$ mice responded similarly to wildtype 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 M_3 receptors, but not via M_1 or M_2 receptors (Figure 1).

Evidence from *in vitro* studies supports a role for acetylcholine in remodeling of the airways. It is known that muscarinic receptor stimulation can induce mucin 5AC, oligomeric mucus/gel-forming (MUC5AC) expression in epithelial cells, which can be inhibited by aclidinium [30]. Moreover, tiotropium can inhibit IL-13-induced goblet cell metaplasia [31]. Muscarinic receptor stimulation is also shown to be involved in airway smooth muscle thickening and airway fibrosis, because it can enhance growth factor-induced proliferation [32,33], contractile protein expression [34], and extracellular matrix deposition of airway smooth muscle cells and fibroblasts [35,36]. Furthermore, aclidinium has been shown to inhibit fibroblast to myofibroblast transition [37]. Although growth factor-induced proliferation was dependent on the M_3 receptor, matrix protein deposition was dependent on the muscarinic M_2 receptor *in vitro*. However, *in vivo*, knock out of the M_2 receptor did not affect allergen-induced remodeling [29], which might be explained by M_2 receptor dysfunction after allergen challenge [15,38].

Interestingly, in $M_3R^{-/-}$ mice, a marked inhibition of airway remodeling was observed, without inhibition of the allergic inflammatory response [29]. Generally, airway structural changes after allergen challenge are attributed to eosinophilic inflammation [39], and tiotropium has previously been shown to inhibit eosinophilic inflammation in animal models of asthma [5,7]. The observation that there is no inhibition of the eosinophilic inflammation after knock out of the M_3 receptor might be explained by the fact that this is orchestrated by both M_1 and M_3 receptors. Tiotropium also has a substantial dissociation half-life for the M_1 receptor (Box 1), and a trend towards lower eosinophil numbers was observed in $M_1R^{-/-}$ mice.

Bronchoconstriction as a driver of airway remodeling

The finding that remodeling can be inhibited after knock out of the M_3 receptor, without affecting inflammation, has significant implications. Recently, it was demonstrated that repeated methacholine challenges in patients with mild asthma is sufficient to induce airway remodeling, without affecting inflammation. Thus, methacholine challenge promoted transforming growth factor (TGF)-β release, collagen deposition, goblet cell metaplasia, and epithelial cell proliferation in airway biopsies, with no effect on eosinophil numbers [40]. 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 β_2-agonist albuterol, to prevent methacholine-induced bronchoconstriction [40]. Together, these findings 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 (Figure 1).

These observations put the role of the M_3 receptor described above in an interesting perspective, because in fact the protective effects of inhibition or knock out of this receptor subtype may in part be explained by the inhibition of bronchoconstriction [41]. In support, in lung slices from guinea pigs, it was demonstrated that bronchoconstriction induced by methacholine results in airway remodeling. Of note, the effects of methacholine were similar to those of TGF-β, and shown to be mediated via enhanced release of TGF-β [42]. *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 [43,44]. Moreover, contraction of airway smooth muscle cells induces TGF-β activation [45]. In intact airways, the epithelium is compressed by bronchoconstriction, and this induces the activation of the epidermal growth factor receptor (EGFR) [46]. Compression of epithelial cells *in vitro* results in enhanced TGF-β expression [47] and an increase in epithelial thickness [48]. 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 [49]. Taken together, the studies outlined above suggest that bronchoconstriction leads 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, because it would imply that prevention of bronchoconstriction might prevent airway remodeling in asthma. However, it seems unlikely that the entire anti-inflammatory and remodeling activity of anticholinergics is due to these biomechanical effects, as in the above mentioned studies bronchoconstriction was selectively coupled to remodeling, and not to inflammation, whereas anticholinergics and M_3 receptor knockout have clear anti-inflammatory effects on neutrophilic inflammation [4,23]. Moreover, over the past 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 epithelial cells and inflammatory cells, acting in an autocrine and/or paracrine manner [2,14].

Neuronal and non-neuronal acetylcholine

It is not known to what 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 [50,51].

Recently, the first evidence was provided showing that inflammation in COPD is, at least in part, mediated via neuronal acetylcholine. The effect of targeted lung denervation (TLD) on inflammation was investigated 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 forced expiratory volume in 1 min (FEV_1), 6-min walk-test distance and the St George's Respiratory Questionnaire score [52]. It was demonstrated that, 30 days after TLD of the right lung, airway inflammation was 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 was the first study reporting a direct inhibitory effect of acetylcholine on inflammation in patients with COPD, and suggests that this is mediated by neuronally released mediators, possibly acetylcholine. Next to ablation of airway nerves, therapies activating airway nerves via implants or external stimulation are under development [53]. However, the mechanism by which vagal stimulation can suppress bronchoconstriction is unclear and needs to be determined.

A role for neuronal acetylcholine in inflammation in COPD is further supported by studies on muscarinic receptor subtype-deficient mice, in which the cigarette smoke-induced inflammatory response was enhanced in $M_2R^{-/-}$ mice compared with wildtype mice [23]. Knock out of prejunctional autoinhibitory M_2 receptors results in enhanced acetylcholine release and thereby enhanced inflammation. A similar autoinhibitory mechanism for the release of non-neuronal acetylcholine has not been demonstrated until now, thereby suggesting that neuronally released acetylcholine is the main driver of this inflammatory response.

The hypothesis that bronchoconstriction might be the driver of allergen-induced airway remodeling suggests that neuronal acetylcholine also has an important role in allergic asthma [29]. Increasing evidence supports this notion. For example, the late asthmatic response in rats [54] and the development of airway hyper-responsiveness in mice [55] are dependent on neural regulation, presumably by afferent C-fibers controlling efferent bronchoconstrictor responses via the vagal nerve. This is supported by recent findings showing that vagotomy can prevent allergen-induced airway hyper-responsiveness and inflammation in dogs [55], and is in line with earlier findings reporting the contribution of vagal afferents and efferents in bronchoconstriction in response to pro-inflammatory mediators, such as thromboxane A2 [56].

Next to these findings, some evidence supporting a role for non-neuronal acetylcholine does exist. Tiotropium and 4-DAMP have been shown to inhibit alveolar macrophage mediated migration of neutrophils from patients with COPD patients [27]. Moreover, tiotropium inhibited TGF-β-induced matrix metalloproteinase-1 (MMP-1) and MMP-2 expression in human lung fibroblasts [57], and aclidinium has been shown to inhibit TGF-β and cigarette smoke-induced fibroblast to myofibroblast differentiation [37,58]. These studies indicate that non-neuronal acetylcholine might contribute to airway inflammation and remodeling of airway cells in an autocrine or paracrine manner. Furthermore, a role for non-neuronal acetylcholine has been observed in human airway epithelial cells. Epithelial cells are a source of non-neuronal acetylcholine and might be important contributors to the effects of non-neuronal acetylcholine [59,60]. For example, airway epithelial cells secrete pro-inflammatory cytokines in response to muscarinic receptor stimulation [25] and it was demonstrated that tiotropium significantly inhibits IL-13-induced goblet cell metaplasia by inhibiting the increase in MUC5AC-positive cells and goblet cells [31]. This indicates that non-neuronal acetylcholine contributes to goblet cell metaplasia by a direct effect on epithelial cells (Figure 1). In the airways, mucus is secreted by goblet cells and submucosal glands. Submucosal glands are innervated by airway nerves and the release of mucus from glands is under cholinergic neural control [61]. It is still a matter of debate whether goblet cells can also release mucus in response to neuronal acetylcholine, but data from this study suggest at least a role for non-neuronal acetylcholine in this response.

Based on the evidence there is so far, we propose that the effects of acetylcholine on airway inflammation and remodeling in asthma and COPD are mediated by both neuronal and non-neuronal acetylcholine. However, the relative contribution of neuronal and non-neuronal acetylcholine *in vivo* is still uncertain and is likely dependent on the type of exposure (allergen or smoke). Studies applying vagotomy on animals under controlled experimental conditions might definitively establish the contribution of neuronal versus non-neuronal acetylcholine to inflammation and remodeling in disease models. Moreover, targeted lung denervation in patients with asthma might answer whether neuronal acetylcholine is also a contributor to airway inflammation in asthma (Box 2).

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. We provided the first evidence that acetylcholine might act as a pro-inflammatory mediator in patients with COPD, because airway inflammation is attenuated after TLD [62]. From different trials, including the Understanding Potential Long-term Impacts on Function with Tiotropium (UPLIFT) trial, it is known that tiotropium reduces the number of exacerbations [1]. Treatment with glycopyrrolate or aclidinium, albeit for a shorter period, was also shown to affect exacerbations, because the time to the first exacerbation was increased [63,64]. This

Box 2. Outstanding questions

- Does anticholinergic therapy affect inflammation and remodeling in patients with COPD or asthma?
- If so, does acetylcholine mainly regulate this via direct effects on inflammation and remodeling, or indirectly sequential to bronchoconstriction?
- Is it possible to selectively inhibit M_3 receptors and does this lead to better outcomes for patients with COPD or asthma?
- Does vagotomy and/or ablation of airway nerves prevent airway inflammation and remodeling?

suggests an anti-inflammatory effect of anticholinergic therapy, because the inflammatory response is enhanced during exacerbations, with increased expression of pro-inflammatory cytokines such as IL-8 [65,66]. Moreover, patients who have more exacerbations demonstrate increased levels of inflammatory markers at stable state [67,68]. 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 [69,70].

There is no strong clinical evidence to suggest that acetylcholine promotes remodeling in patients with COPD. In the Lung Health Study, treatment with the short-acting anticholinergic ipratropium did not influence long-term decline in FEV_1 [71]. Initially, it was thought that the long-acting tiotropium did affect rate of decline in FEV_1, because a retrospective study suggested that the use of tiotropium was associated with a significant reduction in decline of lung function after 1 year [72]. However, tiotropium did not affect the rate of decline in lung function in the overall study population of the prospective UPLIFT trial [1]. Tiotropium did inhibit the accelerated decline in lung function in specific subgroups of the trial, including young patients and patients with moderate disease [73,74]. 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, because it is known from the ECLIPSE study that this is variable between patients with COPD, and lung function might even increase in a subset of patients, irrespective of treatment [75]. Instead, more direct measurements of remodeling parameters, using airway biopsies from patients, taken before and after long-term anticholinergic therapy might help to elucidate whether a role of acetylcholine in airway remodeling in patients with COPD exists (Box 2).

Clinical implications: asthma

Anticholinergics are currently not included in guidelines as controller therapy for the treatment of asthma and are not commonly used for this indication. However, recent trials suggest that patients with asthma would benefit from anticholinergic controller therapy, because tiotropium has been shown to induce bronchodilation in patients with moderate to severe asthma [19,20]. In patients with severe asthma, addition of tiotropium to standard therapy with inhaled glucocorticosteroids and long-acting β_2-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 [19]. 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 review. Repeated challenges with methacholine in patients with mild asthma did demonstrate that muscarinic receptor stimulation can induce airway remodeling [40]. Moreover, methacholine challenge induced epithelial cell proliferation and goblet cell metaplasia, suggesting a role for acetylcholine in mucus hypersecretion. This is supported by a study demonstrating that tiotropium inhibits goblet cell metaplasia of human airway epithelial cells [31]. Tiotropium has been shown to reduce sputum levels in patients with chronic mucus hypersecretion [76]. 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 data to support that anticholinergics are beneficial for patients with mucus hypersecretion [77]. 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 [78]. In addition, and relevant for both asthma and COPD, tiotropium might affect cough. It was shown in guinea pig and human tissue that tiotropium can directly inhibit the transient receptor potential V1 (TRPV1) and thereby inhibit the cough reflex via a reduction in airway sensory nerve activity [79].

Concluding remarks

In conclusion, recent studies have revealed that acetylcholine contributes to airway inflammation and remodeling in animal models of COPD and asthma, primarily via M_3 receptors, which involves neuronal and non-neuronal acetylcholine (Figure 1). As bronchodilators, anticholinergics might also affect airway remodeling by preventing mechanical stress. This suggests that patients with COPD or asthma would benefit from anticholinergic therapy to a larger extent than previously appreciated. Furthermore, recent studies demonstrating that kinetic M_3 selectivity is a beneficial property of currently available long-acting anticholinergics, advocating for even more selective compounds, not inhibiting M_1 receptors. Taken together, we believe that the combined effects of anticholinergic therapy on bronchoconstriction, mucus secretion, inflammation, and remodeling may account for the positive outcome of treatment with these drugs for patients with COPD or asthma.

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