Acetylcholine beyond bronchoconstriction: a regulator of inflammation and remodeling
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Preface

The objective of this thesis is to establish the role of acetylcholine and individual muscarinic receptor subtypes in inflammation and remodeling of the airways. Inflammation and remodeling are two important pathophysiological processes in asthma and chronic obstructive pulmonary disease (COPD), affecting the decline in lung function and severity of the disease. In the airways, acetylcholine acts as a parasympathetic neurotransmitter, but also as an autocrine and/or paracrine hormone. Muscarinic receptors are target receptors for acetylcholine and muscarinic receptor antagonists are used as a therapy for COPD, and to a lesser extent also for asthma. Consequently, a potential role for acetylcholine in inflammation and remodeling in COPD and asthma could have important therapeutic implications.

Acetylcholine, a neurotransmitter and a hormone

Acetylcholine – a neurotransmitter

Acetylcholine is the primary parasympathetic neurotransmitter of the airways. Acetylcholine was detected by Otto Loewi and Sir Henry Dale in the early 1920s, for which they received the Nobel prize in 1936. As a neurotransmitter, acetylcholine is synthesized in nerve endings from the substrates choline and acetyl-CoA by the enzyme choline acetyl transferase (ChAT) (1). The uptake of choline from the extracellular space is the rate-limiting step in this process (2). Synthesized acetylcholine is translocated into synaptic vesicles via the vesicular acetylcholine transporter (VACHT), and released by exocytosis. Exocytosis is triggered by a depolarizing stimulus and modulated by several regulatory mechanisms in the neuroeffector junction (1). This results in the release of acetylcholine from airway parasympathetic nerve endings in the extracellular space, where it interacts with postsynaptic target receptors, but also with presynaptic receptors on cholinergic nerve terminals themselves. Target receptors of acetylcholine include muscarinic receptors and nicotinic receptors, which will be introduced in more detail below. Acetylcholine in the synaptic cleft is rapidly degraded into acetate and choline by the highly expressed enzyme acetylcholine esterase (AChE). Alternatively, acetylcholine can be degraded by butyrylcholinesterase (BChE) (1). Choline can be taken up again by the nerve for acetylcholine synthesis. The acetylcholine synthesis pathway in neurons is summarized in figure 1A, together with the non-neuronal synthesis pathway, which will be elaborated on later.
**Figure 1.** Schematic representation of acetylcholine synthesis, release, action and breakdown in neuronal cells at a cholinergic nerve terminal (A) and in non-neuronal cells, such as epithelial cells (B). In neurons, there is an efficient metabolism of acetylcholine (ACh), involving the high-affinity choline transporter-1 (CHT1), choline acetyltransferase (ChAT), the vesicular ACh transporter (VACHT) and ACh release via exocytosis. Although some non-neuronal cells also express these components, they are not common to all cell types and probably alternative, less efficient, mechanisms predominate. Perhaps as a consequence, acetylcholine content is lower. In non-neuronal cells, choline is taken up not only via CHT1, but also via choline transporter-like proteins (CTL), including CTL1, CTL2 and CTL4. Carnitine acetyltransferase (CarAT) has been identified as an alternative synthesizing enzyme of ACh. Organic cation transporters (OCT), including OCT1 and OCT2, have been shown to transport ACh in and out of cells (3). MR: muscarinic receptor, NR: nicotinic receptor, AChE: acetylcholinesterase, BCHE: butyrylcholinesterase.

**Airway nerves**

Airway nerves regulate many aspects of airway function. One of the most prominent effects is the regulation of airway smooth muscle tone, the neural component of which is almost solely controlled by the parasympathetic nervous system (4, 5). Although airways also express sympathetic and non-adrenergic, non-cholinergic (NANC) neural pathways, focus of this thesis is on the parasympathetic neural pathway, which is the dominant neural pathway in the airways and uses acetylcholine as a neurotransmitter. Airway vagal nerve fibers can be divided into three major components: the primary afferent nerve fibers, the integrating centers in the brain and the parasympathetic efferent nerve fibers (see figure 2) (6). Afferent nerve fibers in the airways include stretch-sensitive myelinated nerve fibers or A-fibers, and unmyelinated C-fibers (7). A-fibers conduct action potentials...
at a relatively high velocity and consist of rapidly adapting receptors (RARs) in the mucosal layer, which respond to the dynamic phase of inspiration, and slowly adapting receptors (SARs) in the smooth muscle layer, which respond to maintained inflation (7-9). However, most of the airway afferent nerves are unmyelinated C-fibers, which are present throughout the airways and conduct action potentials at a much slower velocity compared to A-fibers (6, 10). C-fibers respond to noxious stimuli such as heat, cold or mechanical forces, but also to mediators released upon tissue damage and inflammation (7). Activation of these C-fibers results in bronchoconstriction, mucus secretion and cough (11). This can be a local reflex in the airways at the level of the parasympathetic ganglia, which involves the release of peptide neurotransmitters, including substance P and other tachykinins, directly from the nerve terminals (12). Next to this local reflex, a central reflex arch via the brain stem exists (9). Parasympathetic efferent nerve fibers consist of preganglionic neurons, which innervate the parasympathetic ganglia in or near the airway wall of larger airways, and postganglionic neurons (6). Postganglionic neurons innervate airway smooth muscle, mucus glands and the microvasculature throughout the airway tree (figure 2). In addition, postganglionic parasympathetic neurons, which do not use acetylcholine as a transmitter, exist. Instead, these NANC pathways use nitric oxide and/or neuropeptides such as vasoactive intestinal peptide as a transmitter, and are in fact bronchodilatory (6). However, the parasympathetic cholinergic nervous system is the major neural regulator of airway smooth muscle tone (4, 9, 13).

![Figure 2](image-url)

**Figure 2.** The vagal nervous system in the airways. Mechanical forces, inhaled irritants and endogenous inflammatory mediators activate afferent nerve fibres, which send signals to the central nervous system (CNS). This results in the release of acetylcholine (ACh) in the airways. Parasympathetic, post-ganglionic neurons are located within the airway wall and innervate airway smooth muscle and submucosal glands to induce contraction and mucus secretion, respectively. Blue: afferent nerve fibers; red: efferent nerve fibers. RAR: rapidly adapting receptors; SAR: slowly adapting receptors.
**Acetylcholine - a hormone**

During the last decades, it has become clear that acetylcholine production in the airways is not only restricted to the nerves. Bacteria, algae, protozoa, tubellariae and primitive plants, all lacking a nervous system, express acetylcholine. This suggests an early and widely distributed role of acetylcholine (14). Indeed, cells of higher organisms, including cells of the human airway, have been shown to produce acetylcholine (14, 15). Thus, acetylcholine can act as a local signaling molecule in an autocrine or paracrine fashion, which is referred to as non-neuronal acetylcholine. RNA expression of ChAT has been detected in almost all cell types of the airways, including epithelial cells, airway smooth muscle cells and inflammatory cells (16). Additionally, carnitine acetyltransferase (CarAT) has been detected as an alternative, albeit less efficient, route for acetylcholine synthesis in airway cells (2). In particular epithelial cells have been shown to express relatively high levels of acetylcholine (17, 18), and the epithelial non-cholinergic system has been characterized in detail (2). Evidence for the actual release of non-neuronal acetylcholine from other airway cell types is still limited (15). Part of the biosynthesis pathway of non-neuronal acetylcholine overlaps with that used by the neuronal system; however, clear differences exist, the former being less efficient (2). For example, neurons use the highly efficient CHT1 transporter for choline uptake, whereas non-neuronal cells mainly use choline transporter-like proteins (CTLs) and organic cation transporters (OCTs) for choline uptake (2). In addition, in neurons, acetylcholine is stored and subsequently released from vesicles, whereas in non-neuronal cells acetylcholine is mainly released directly via OCTs (2, 3) (figure 1B). As a consequence, acetylcholine levels seem to be lower in non-neuronal cells compared to neuronal cells. The functional relevance of non-neuronal acetylcholine in the airways still has to be established and it is unclear whether non-neuronal acetylcholine contributes to the classical cholinergic driven effects as described above (3). However, the concept that acetylcholine is not only a neurotransmitter but also a hormone has dramatically broadened the view on the role of acetylcholine in the airways, and holds therapeutic promises for the future, which will be further elaborated on in chapter 2 (19-21).

**Targets of acetylcholine**

Acetylcholine can act via two different classes of receptors, namely muscarinic and nicotinic receptors. Muscarinic receptors are G-protein coupled receptors, characterized by a seven transmembrane domain (1). Five different subtypes have been identified (M1-M5), of which M1-M3 receptors are expressed abundantly in the airways (1, 16, 21). Almost all cell types in the airways express muscarinic receptors, as discussed in detail below. Activation of muscarinic receptors in the airways leads to bronchoconstriction and mucus secretion (21). Nicotinic receptors are ligand gated ion channels, which are comprised of...
one to five different types of subunits (α, β, γ, δ and ε) (1). Multiple different nicotinic receptor isotypes exist and at least ten different α-subunits and four different β-subunits have been identified. In the airways, almost all cell types express nicotinic receptors (1, 15). Nicotinic receptors are involved in neurotransmission in the airways, and activation of nicotinic receptors causes an influx of positively charged ions, leading to membrane depolarization (1, 22). The nicotinic α7 receptor is highly expressed on multiple cell types in the airways and plays an immunomodulatory role (15). Moreover, the nicotinic α7 receptor is thought to play a regulatory role in lung cancer (23). Current therapy for obstructive airway diseases, however, is selectively directed to muscarinic receptors. Therefore, the focus of this thesis is on muscarinic receptors, and nicotinic receptors will not be discussed in more detail here.

Muscarinic receptors

Expression of muscarinic receptors

Expression of muscarinic M1, M2 and M3 receptors has been detected in the airways. Although muscarinic receptor antibodies have been proven not to be very useful in the detection of subtype specific expression of muscarinic receptors because of lack of specificity (24, 25), other techniques, including binding studies and gene expression analyses, have elucidated the expression pattern of muscarinic receptors throughout the airways. From these studies, it is clear that M1 receptors appear to be expressed particularly in peripheral lung tissue, M2 receptors are expressed throughout the airways, whereas expression of M3 receptors is highest in the larger airways (1, 26). Almost all cells of the airways express muscarinic receptors, with high expression on airway smooth muscle cells, epithelial cells and submucosal glands. Moreover, in parasympathetic ganglia, M2 receptors are expressed, as well as autoinhibitory prejunctional M2 receptors (21, 22). Expression of muscarinic receptors per cell type, and their major functional effects, are summarized in table 1.

Signal transduction of muscarinic receptors

Muscarinic receptors couple to heterotrimeric G proteins. G proteins are composed of an α-, β- and γ-subunit, and are classified according to the α-subunit. Four different families have been identified: Goα, Go1/2, Goα11, and Go12/13 (30, 31). M2 receptors couple primarily to Goq and M3 and M2 receptors couple primarily to Goq (32). Receptor activation promotes the exchange of guanosine diphosphate (GDP), bound to the α-subunit, with guanosine triphosphate (GTP). This will lead to dissociation of the α-subunit from the βγ-heterodimer and subsequent activation of second messengers (30). Activation of Goq, via M2 receptors results in inhibition of adenylyl cyclase (AC), which in turn inhibits the second
messenger cyclic AMP (cAMP), and Gβγ mediated inhibition of potassium channels (33, 34). Activation of Gαq via M1 and M3 receptors results in activation of phospholipase C (PLC). PLC converts phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 induces the release of calcium from internal endoplasmic reticulum stores, whereas DAG activates protein kinase C (PKC). The initial calcium release is followed by a more sustained calcium release mediated via ryanodine receptors on the endoplasmic reticulum, and via an increase in the open probability of calcium channels in the cell membrane, mediated via PKC (35).

Functional roles in the airways

Neurotransmission

M1 receptors expressed in parasympathetic ganglia contribute to neurotransmission, by facilitating depolarization via inhibition of potassium currents. The magnitude of muscarinic depolarization may not be sufficient to initiate an action potential by itself, but rather enhances or facilitates nicotinic-induced depolarization (1). It should be noted therefore that there is still debate about the functional relevance of M1-mediated neurotransmission (1). Evidence for the auto-inhibitory role of prejunctial M2 receptors is more convincing, since M2 selective antagonists enhance electrical field stimulation-induced acetylcholine release from guinea pig and human trachea (36, 37). The autoinhibitory M2 receptor is dysfunctional in asthma, which contributes to the enhanced cholinergic tone in this disease (16). No such mechanism has been observed in COPD (38).

Table 1. Expression of muscarinic receptors on airway cells and their major effects (16, 27-29).

<table>
<thead>
<tr>
<th>Cell</th>
<th>Muscarinic receptor expression</th>
<th>Functional effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuron</td>
<td>M1, M2</td>
<td>Neurotransmission</td>
</tr>
<tr>
<td>Airway smooth muscle cell</td>
<td>M2, M3</td>
<td>Bronchoconstriction via M3</td>
</tr>
<tr>
<td>Epithelial cell</td>
<td>M1, M2, M3</td>
<td>Mucus secretion via M3</td>
</tr>
<tr>
<td>Submucosal gland</td>
<td>M1, M2, M3</td>
<td>Mucus secretion via M3</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>M1, M2, M3</td>
<td>Proliferation, ECM production</td>
</tr>
<tr>
<td>Mast cell</td>
<td>M1, M2</td>
<td>Inhibition of histamine release</td>
</tr>
<tr>
<td>Macrophage</td>
<td>M1, M2, M3</td>
<td>Cytokine production</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>M1, M2, M3</td>
<td>Cytokine production</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>M1, M2, M3</td>
<td>Cytokine production</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>M1, M2, M3</td>
<td>?</td>
</tr>
</tbody>
</table>
Airway smooth muscle contraction
Muscarinic receptors on airway smooth muscle mediate airway smooth muscle contraction, and therefore play an important role in bronchoconstriction associated with COPD and asthma. Although M2 receptors represent the majority of muscarinic receptors expressed on airway smooth muscle, airway smooth muscle contraction is primarily mediated by M3 receptors (39). This is clear from affinity profiles of selective muscarinic antagonists in different species, including humans (16). Moreover, the introduction of specific muscarinic receptor subtype deficient animals confirmed that this is an M3- mediated effect, as M3 receptor deficient mice (M3R⁻/⁻), and not M2R⁻/⁻ mice, lack methacholine and vagally-induced bronchoconstriction in vivo (40).

Mucus secretion
Muscarinic receptors regulate mucus secretion in the airways. Mucus secretion is enhanced in COPD and asthma, which contributes to airflow obstruction of the airways (41). Mucus is secreted by airway submucosal glands, which are in connection to the airway lumen, and by goblet cells, which are embedded in the airway epithelium. Submucosal glands are innervated by parasympathetic nerves (see also figure 2), and mucus secretion from glands is under cholinergic control, mediated via M3 receptors. Thus, electrical field stimulation increases mucus secretion from airway preparations, which can be inhibited by the M3 antagonist 4-DAMP (1,1-Dimethyl-4-diphenylacetoxy-piperidinium iodide) (42, 43). Acetylcholine can also induce mucus secretion from goblet cells, again mediated primarily via M3 receptors (21, 41). There is still debate whether acetylcholine-induced mucus secretion from goblet cells is mediated by neuronal or non-neuronal acetylcholine, or by a combination of both. M1 receptors are also expressed on epithelial cells and submucosal glands, and mediate electrolyte and water secretion in cooperation with M3 receptors (21, 42).

Vagal dysregulation
Activity of the neuronal system is altered in disease, including asthma and COPD, via several mechanisms, leading to exaggerated acetylcholine release and airway hyperresponsiveness (9, 13, 44). This can be via [1] changes in activity of afferent nerves, [2] changes in synaptic transmission and [3] changes in neurotransmitter content in the synaptic cleft. First, changes in activity of afferent nerves have been observed in response to inflammatory mediators, present in the airways of patients with COPD or asthma. This results in enhanced release of acetylcholine from vagal nerve endings and an increase in cholinergic tone (6). This effect can be mediated via several inflammatory mediators, including histamine, tachykinins, prostaglandins and thromboxane A2, which stimulate
sensory nerve fibers directly (6, 45). Moreover, epithelial damage will expose afferent sensory nerve endings in the subepithelial layer to the airway lumen (46). Second, inflammation can also change synaptic transmission, by increasing synaptic efficacy and by increasing acetylcholine release, via the release of mediators including tachykinins, which interact with receptors on nerve terminals (6, 12). Moreover, autoinhibitory prejunctional M₂ receptors, which limit acetylcholine release under healthy conditions, are dysfunctional in asthmatic airways. This has been shown in animal models of allergen exposure, viral infection and ozone exposure (47), but also in patients with asthma (16, 48). M₂ receptor dysfunction is probably mediated via several mechanisms, but convincing evidence exists for the involvement of major basis protein. Major basic protein is secreted by eosinophils that are recruited to airway nerves, and acts as an allosteric antagonist for the M₂ receptor (49). In support, treatment with an antibody against major basic protein prevents M₂ receptor dysfunction in guinea pigs (50). Third, acetylcholine content in the synaptic cleft is altered. In part this is due to dysfunctional M₂ receptors as described above, but it has also been shown that activity of AChE is reduced in tracheal smooth muscle homogenates obtained from ragweed pollen-sensitized dogs compared to sham controls (51). Together, this results in enhanced acetylcholine concentrations in the synaptic cleft and prolonged effects on postjunctional target receptors.

There is little evidence for altered muscarinic receptor expression in asthma or COPD. Using radioligand binding studies, no significant changes were observed in affinity or density of muscarinic receptors in the central or peripheral airways of patients compared to healthy controls (52, 53). Moreover, until now, there is no evidence for genetic polymorphisms of muscarinic receptors, as at least M₂ and M₃ receptors seem to be highly conserved, and no significant differences between healthy and asthmatic subjects were detected (54).

Besides these acute effects on vagal nerves, there is also evidence for neural remodeling. Growth and development of airway nerves continues well into adolescence, and exposure to allergens might alter airway nerve structure (55). Pan et al. demonstrated that allergen exposure in guinea pigs results in a shift from a NANC phenotype, which does not use acetylcholine as a neurotransmitter, towards a cholinergic phenotype (56). In addition, alterations in airway nerve structure might start very early in life, which may affect the incidence of airway diseases later in life (57). A recent paper demonstrated that early-life exposure of mouse neonates to ovalbumin leads to a two-fold increase in airway smooth muscle innervation in adulthood (58). Furthermore, exposure of infant rhesus monkeys to ozone and/or house dust mite results in changes in airway innervation in the epithelial region (59). Thus, exposure to allergen or environmental stress, either early in life or in
adulthood, may lead to dysregulated vagal innervation, and might thereby affect airways diseases.

Although most of these studies focus on asthmatic airways, evidence suggests that innervation is also altered in the airways of COPD patients (7). The inflammatory response in the airways of patients with COPD might trigger acetylcholine release via above described mechanisms. Moreover, altered breathing patterns and changes in gas tensions in patients with COPD activate RAR-fibers and SAR-fibers (7), and the ability of SARs to respond to lung inflation seems to be be altered in patients with COPD (13). Therefore, dysregulation of vagal nerves, leading to an increased cholinergic tone, largely contributes to both COPD and asthma pathophysiology. Strikingly, increased cholinergic tone is the major reversible component of airflow limitation in COPD (60, 61).

Muscarinic receptors in the treatment of COPD and asthma

Therapeutic targets in COPD and asthma
As described above, muscarinic receptors play a key role in regulating bronchoconstriction and mucus secretion, and cholinergic tone is increased in patients with COPD and asthma. For these reasons, muscarinic receptors represent key therapeutic targets for COPD and asthma. Muscarinic receptor antagonists, referred to as anticholinergics, have already been used for centuries for the treatment of obstructive airway diseases. This started with the use of naturally occurring medicinal plants containing anticholinergic alkaloids such as atropine. Fumes of these medicinal plants were inhaled, and later on even smoked via cigarettes, until the middle of the 20th century (62, 63). Since then, more safe and effective synthetic anticholinergics have been introduced to the market.

Anticholinergics
Ipratropium was the first synthetic anticholinergic introduced to the market. Because of the quaternary ammonium structure, ipratropium is much safer compared to the natural occurring anticholinergic atropine. Atropine can easily pass the blood-brain barrier leading to numerous side effects, which is prevented by the quaternary structure of ipratropium. The safety profile of ipratropium is further enhanced by applying the drug via inhalation, thereby limiting systemic exposure. Besides dry mouth, ipratropium, but also other anticholinergics, have limited side effects (64). Thereafter, oxitropium was introduced. Both drugs have relatively short durations of action (4-8 hours), and there was a need for long-acting anticholinergics. Tiotropium is the first long-acting anticholinergic introduced to the market in the early 2000s. Tiotropium has been shown to be a potent muscarinic
Table 2. Binding affinity and half-life time at the $M_1$, $M_2$ and $M_3$ receptor for the different anticholinergics against human $M_1$, $M_2$ and $M_3$ receptors.

<table>
<thead>
<tr>
<th></th>
<th>$M_1$R</th>
<th>$M_2$R</th>
<th>$M_3$R</th>
<th>$M_1$R</th>
<th>$M_2$R</th>
<th>$M_3$R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipratropium</td>
<td>9.40</td>
<td>9.53</td>
<td>9.58</td>
<td>0.1</td>
<td>0.03</td>
<td>0.22</td>
</tr>
<tr>
<td>Tiotropium</td>
<td>10.80</td>
<td>10.69</td>
<td>11.02</td>
<td>10.5</td>
<td>2.6</td>
<td>27</td>
</tr>
<tr>
<td>Aclidinium</td>
<td>10.78</td>
<td>10.68</td>
<td>10.74</td>
<td>6.4</td>
<td>1.8</td>
<td>10.7</td>
</tr>
<tr>
<td>Glycopyrronium</td>
<td>10.09</td>
<td>9.67</td>
<td>10.04</td>
<td>2.0</td>
<td>0.37</td>
<td>6.1</td>
</tr>
</tbody>
</table>

Binding affinity was determined in heterologous competition experiments against $[^3H]$NMS. Data represent $pK_a$ 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 $[^3H]$NMS and different concentrations of antagonist. At least three independent experiments were performed in triplicate. Data from Casarosa et al (66).

Receptor antagonist; the affinity of tiotropium for muscarinic receptors is around 10-fold higher compared to ipratropium. However, onset of action is slower compared to ipratropium (65). 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 much slower compared to the other receptor subtypes, in particular compared to $M_2$ receptors (table 2) (66, 67). Used as a bronchodilator, this is a desired property, since inhibition of $M_3$ receptors inhibits airway smooth muscle contraction, whereas antagonizing $M_2$ receptors 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 (68). Recently, inhaled glycopyrronium and aclidinium were also introduced to the market. As becomes clear from table 2, like tiotropium, glycopyrronium and aclidinium are kinetically selective for the $M_3$ receptor, and similarly, there is no difference in affinity of both drugs for individual muscarinic receptors. However, the dissociation half-life from the $M_3$ receptor of these compounds is smaller compared to tiotropium (66). Glycopyrronium is given once daily, whereas aclidinium is given twice daily. Several new anticholinergics have recently reached the market, as well as combinations of long-acting anticholinergics with long-acting $\beta_2$-agonists and/or corticosteroids (69).

**Use of anticholinergics in COPD**

In COPD, anticholinergics represent a first-line of treatment according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (70). Long-acting anticholinergics and long-acting $\beta_2$-agonists are the most frequently prescribed bronchodilators for COPD treatment. Anticholinergics have a particular value in the
treatment of COPD because they block the increased cholinergic tone, which is the major reversible component of the disease (60, 61). The first randomized controlled clinical trial into the efficacy and safety of tiotropium in patients with COPD was published in 2000 (71). In this study, tiotropium was compared to ipratropium. Both tiotropium and ipratropium induced an increase in lung function over a 13 week period, and the long-acting tiotropium was more effective compared to the short-acting ipratropium. The safety profile of both drugs was similar (71). Since then, a number of studies have been undertaken, of which the UPLIFT (Understanding Potential Long-term Impacts on Function with Tiotropium) trial was the most extensive study. In this study, a total of 5993 patients were randomly assigned to placebo or tiotropium, and followed for 4 years. Tiotropium significantly improved lung function and quality of life, and reduced the number of exacerbations compared to placebo. Tiotropium did not alter the rate of decline in forced expiratory volume in 1 second (FEV1) (72). More recently, new anticholinergics were introduced, including glycopyrronium and aclidinium. Glycopyrronium (NVA237) was shown to improve lung function, quality of life and dyspnoea, and to reduce the risk of exacerbations compared to placebo in patients with moderate to severe COPD (73), with efficacy comparable to tiotropium (74). Similarly, aclidinium was shown to improve lung function, quality of life and dyspnoea compared to placebo in moderate to severe COPD patients (75, 76), which was comparable to the effects of tiotropium over a 15 day period (77). Fixed-dose combinations of several long-acting anticholinergics and long-acting β2-agonists are currently under development or have recently reached the market, and large trials are evaluating the efficacy and safety of fixed-dose combinations (35, 78). First evidence suggests that the fixed-dose combinations of tiotropium and olodaterol (79), glycopyrronium and indacaterol (80, 81), aclidinium and formoterol (82, 83), and umecilidinium and vilanterol (84, 85) are more effective than monotherapy.

**Use of anticholinergics in asthma**

In asthma, the use of anticholinergics is limited to the treatment of exacerbations, and anticholinergics are not approved for chronic treatment (86). Short-acting anticholinergics have a significant but small effect on peak expiratory flow (PEF) compared to placebo in patients with stable asthma (87). The effects of short-acting anticholinergics are smaller than the effects of short-acting β2-agonists in patients with asthma (64, 87). According to the Global Initiative for Asthma (GINA) guidelines, ipratropium can be used in stable state as an alternative bronchodilator to short-acting β2-agonists, but is usually less effective. In case of an exacerbation, the addition of a short-acting anticholinergic to the short-acting β2-agonists is recommended (86). More recently, clinical trials into the effects of the long-acting anticholinergic tiotropium on lung function in patients with asthma have started. In one of the first studies, Peters et al. demonstrated that the addition of tiotropium to
glucocorticoid therapy in patients with mild to moderate asthma was more effective than doubling of the glucocorticoid dose by means of morning peak expiratory flow, the proportion of asthma-control days, FEV₁ and daily symptom scores, and very similar to the effects of adding salmeterol (88). Moreover, it was shown in a phase 2 study in patients with severe uncontrolled asthma that addition of tiotropium to standard therapy with inhaled corticosteroids and long-acting β₂-agonists improved lung function over an 8 week period (89). These findings were confirmed by two replicate phase 3 randomized controlled trials involving 912 severe asthma patients over a period of 48 weeks. Tiotropium induced bronchodilation on top of the use of inhaled corticosteroids and long-acting β₂-agonists. Furthermore, tiotropium reduced exacerbations and episodes of worsening of asthma (90). Two phase 2 studies in patients with moderate persistent asthma demonstrated that tiotropium can also induce bronchodilation in this patient group, on top of the use of corticosteroids (91, 92). These results have been confirmed in two large replicate phase 3 trials in 2100 patients with uncontrolled asthma on medium dose corticosteroids, of which the results have only been presented as abstracts (93). Tiotropium induced sustained bronchodilation after 24 weeks, accompanied by improvements in asthma control, all very similar to salmeterol (93). Together these data indicate that asthma patients might benefit from addition of tiotropium to standard therapy.

**Regulation of inflammation and remodeling by muscarinic receptors**
COPD and asthma are associated with airway inflammation and remodeling. Increasing evidence suggests a role for acetylcholine in inflammation and remodeling in both obstructive airway diseases (16, 21). This suggests that the role of acetylcholine in patients with COPD or asthma might be much broader than previously appreciated.

**Inflammation and remodeling in COPD**
COPD is associated with persistent inflammation and remodeling of the airways. Inflammation is present throughout the airways (94), and the degree of inflammation correlates to the severity of the disease (95). Upon inhalation of cigarette smoke, which is the main risk factor for COPD, epithelial cells are activated and macrophages are attracted, resulting in the release of chemotactic factors. Macrophages are thought to orchestrate the inflammatory response in COPD (96). Amongst others, macrophages release CC-chemokine ligand 2 (CCL2), also known as monocyte chemotactic protein (MCP1), to attract more monocytes to the lung, and CXC-chemokine ligand (CXCL)-1 and CXCL-8, to attract neutrophils (94, 96). Moreover, both macrophages and epithelial cells release CXCL9, CXCL10 and CXCL11, which attract T-cells to the lung (94, 97). In COPD, T-
cells are mainly T-helper type 1 (T\(_h1\)) and type 1 cytotoxic T (T\(_c1\)) CD8\(^+\) cells (94). The inflammatory response is thought to contribute to the remodeling of the airways, by the release of TGF-\(\beta\) and proteases such as matrix metalloproteinase 9 (MMP9) (94, 98). Fibrosis, by means of peribroncholar deposition of extracellular matrix proteins, mainly occurs around the small airways, and is an important factor contributing to the irreversible airway narrowing observed in COPD (99). Moreover, alveolar walls in the lung parenchyma are destructed due to the chronic inflammation, which is called emphysema. Emphysema results in enlargement of parenchymal airspaces and loss of elastic recoil, and is a major contributor to morbidity and mortality in COPD (100, 101). Remodeling of the epithelium also occurs, leading to squamous and mucous metaplasia (100). Together with mucus gland hypertrophy, this leads to mucus hypersecretion in patients with COPD (102, 103). Remodeling of the airways starts later in life in patients with COPD, but is progressive and irreversible, and is the major contributor to the decline in lung function (100).

**Inflammation and remodeling in asthma**

Like COPD, asthma is characterized by airway inflammation and remodeling. However, there are marked differences between the pattern of inflammation and remodeling in asthma compared to COPD. In asthma, inflammation and remodeling is mainly present in the larger conducting airways. Depending on the severity of disease, small airways can also be affected, however, the lung parenchyma is generally not affected in most patients with asthma (94). The nature of the inflammation and the cell types involved in the inflammatory response in asthma are different compared to COPD. Upon allergen challenge, which is the main trigger for an inflammatory response in allergic asthma, mast cells are activated. Mast cells release bronchoconstricting agents, including histamine and lipid mediators. Moreover, CD4\(^+\) T-helper type 2 (T\(_h2\)) cytokines IL-4, IL-5 and IL-13 are released by mast cells (94, 97). In addition to mast cells, TH2 cells also release these cytokines, and T\(_h2\) cells play a central role in orchestrating the inflammatory response in asthma (94). IL-4 release stimulates B cells to synthesize IgE, the driver of allergic inflammation that binds to high-affinity Fc receptors for IgE (FceRI) on mast cells. The release of IL-5 attracts eosinophils, whereas the release of IL-13 contributes to an enhancement of airway hyperresponsiveness and an increase in goblet cell number (104, 105). There is also an increase in the size of submucosal glands (106), and together this leads to excessive mucus production. Enhanced mucus secretion significantly contributes to airflow limitation in asthma by obstructing the airways (41, 107). Furthermore, there is thickening of the basement-membrane, as a result of collagen deposition under the epithelium, and thickening of the airway smooth muscle layer, as a result of airway smooth muscle hyperplasia and hypertrophy (100). The former is observed in all patients with asthma, whereas the latter is mainly observed in patients with severe asthma (108).
Airway smooth muscle thickness is related to severity of the disease, but not to its duration, since airway smooth muscle thickening is already present in children with asthma (109, 110). Pulmonary vascular remodeling and increased angiogenesis are also observed, which is mediated by various angiogenic proteins, including several growth factors, like vascular endothelial growth factor (VEGF) (100, 111). Structural changes in the airways of patients with asthma are considered an important component of airflow limitation and may accelerate the decline in lung function (112, 113).

Muscarinic receptor regulation of inflammation and remodeling

Increasing evidence suggests a role for acetylcholine in regulating airway inflammation and remodeling. From in vitro studies it is known that muscarinic receptor stimulation can enhance the release of cytokines from inflammatory cells and structural cells of the airways, either alone or in concerted action with stimuli including cigarette smoke and growth factors. Moreover, acetylcholine has been shown to enhance remodeling parameters in vitro. Thus, muscarinic receptor stimulation of airway smooth muscle cells and fibroblast enhances proliferation and the production of extracellular matrix proteins (16, 21). This is further supported by evidence from in vivo studies, demonstrating that airway inflammation and remodeling can be inhibited by anticholinergic intervention. It has been shown that cigarette smoke-induced inflammation in mice, and LPS-induced inflammation and remodeling in guinea pigs can be inhibited by tiotropium, indicating the potential relevance of anticholinergic treatment for patients with COPD (114, 115). In addition, allergen-induced inflammation and remodeling can be inhibited by tiotropium in both mice and guinea pigs, indicating the potential relevance of anticholinergic treatment for patients with asthma (116-118). The role of acetylcholine in inflammation and remodeling is extensively reviewed in chapter 2, and will therefore not be elaborated on in more detail here.

Clinical implications

As briefly described above and reviewed in chapter 2, there is convincing evidence from in vitro and in vivo animal studies that acetylcholine contributes to airway inflammation and remodeling. This might be relevant for patients with COPD and asthma, especially since acetylcholine release is enhanced in these disease states. However, this hypothesis still needs to be proven by clinical studies investigating the effect of acetylcholine or anticholinergic therapy on airway inflammation and remodeling. In the UPLIFT study, COPD patients treated with tiotropium for 4 years showed improvements in lung function, quality of life and exacerbation frequency (72). The latter might suggest an anti-inflammatory effect of tiotropium, as the inflammatory response is enhanced during exacerbations, with increased expression of pro-inflammatory cytokines, including IL-8.
(119, 120). However, Powrie et al. were not able to demonstrate a reduction in sputum IL-6 or IL-8 levels after tiotropium use for one year, even though the exacerbation frequency was reduced (121). In this study, tiotropium reduced the amount of sputum, which might result in a concentration of cytokines in the sputum of patients treated with tiotropium, as proposed by the authors to explain this discrepancy. Therefore, further studies are needed to elucidate the mechanism by which tiotropium reduces the number of exacerbations in patients with COPD and whether a direct anti-inflammatory effect of tiotropium is involved. Structural changes in the airways of COPD patients may accelerate the decline of lung function. Initially, it was thought that tiotropium might affect this process, since a retrospective study suggested that the use of tiotropium was associated with a reduction in decline of lung function after 1 year (122). However, this was not replicated in the large, prospective UPLIFT trial, in which no significant effect on the rate of decline in lung function was observed in the overall study population (72). Pre-specified post-hoc subgroup analysis revealed that tiotropium did inhibit the accelerated decline in lung function in young patients and in patients with moderate disease (123, 124). Future studies are needed to understand the effects of tiotropium on lung function decline in patients with COPD in these subgroups.

Recently, clinical studies into the effects of tiotropium in patients with asthma have started. Addition of tiotropium to standard therapy for severe asthma patients with long-acting β-agonists and corticosteroids increased the time to the first exacerbation and reduced the risk of a severe exacerbation (90). Moreover, it has been shown that repeated inhalation of the muscarinic receptor agonist methacholine induces airway remodeling in mild asthma patients (125). This suggests that acetylcholine might also affect airway inflammation and remodeling in patients with asthma, but clearly more studies are needed to further elucidate the effects of acetylcholine or anticholinergic therapy on airway inflammation and remodeling in patients with asthma.

**Scope of the thesis**

The above mentioned findings suggest that acetylcholine is not only a neurotransmitter involved in bronchoconstriction and mucus secretion in the airways, but might also be a mediator of airway inflammation and remodeling, which possibly involves non-neuronal acetylcholine. This will have implications for therapy of obstructive airways diseases like COPD and asthma, in which muscarinic antagonists are frequently used. This thesis will further elucidate the role of acetylcholine in inflammation and remodeling in COPD and asthma. Specifically, the aim of this thesis is to investigate the role of individual muscarinic
receptor subtypes, as well as the contribution of neuronal versus non-neuronal acetylcholine, to airway inflammation and remodeling in COPD and asthma.

Chapter 2 provides a comprehensive review on the regulation of airway inflammation and remodeling in COPD and asthma by muscarinic receptors. In addition, therapeutic implications of muscarinic receptor regulation of inflammation and remodeling are discussed.

The following chapters deal with the role of acetylcholine in inflammation in COPD. As stated above, there is evidence for a pro-inflammatory role of acetylcholine in inflammation. However, it is not known which individual muscarinic receptor subtypes are involved in this response. There is a focus for anticholinergics on M3 receptor selectivity, because of involvement of this receptor subtype in bronchoconstriction, however, there is no evidence that the pro-inflammatory effects of acetylcholine are also mediated via this receptor subtype. Therefore, in chapter 3, we investigated the contribution of individual muscarinic receptor subtypes to cigarette smoke-induced inflammation. Because of limited selectivity of available muscarinic receptor antagonists, muscarinic receptor subtype specific knock-out animals were used. In this way, the contribution of individual muscarinic receptors can be investigated in detail. Knock-out animals were exposed to cigarette smoke for four days and the inflammatory response in the airways was analyzed.

The contribution of the M3 receptor to cigarette smoke-induced inflammation was investigated in more detail in chapter 4. As discussed above, practically all cells in the airways express muscarinic receptors. It is not known which cell types contribute to the pro-inflammatory effect of the M3 receptor as described in chapter 3. To distinguish between effects of structural cells and inflammatory cells, bone marrow chimeric animals were generated and exposed to cigarette smoke, using a similar protocol as described in chapter 3. In this way, the contribution of structural cells versus inflammatory cells to the pro-inflammatory effect of the M3 receptor can be defined.

In chapter 5, we further elucidated the role of acetylcholine in inflammation in patients with COPD. In this chapter, we investigated the effects 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 radio-frequency energy in the main bronchi using bronchoscopy, to inhibit acetylcholine-induced bronchoconstriction. We hypothesized that TLD would also inhibit airway inflammation. Specifically, we studied whether TLD affects inflammatory cell number and pro-inflammatory cytokine expression in bronchial wash fluid and gene expression of pro-inflammatory cytokines in bronchial brush specimen.
The second half of the thesis is focused on the role of acetylcholine in inflammation and remodeling in asthma. As is described above for COPD, it is not known which muscarinic receptor subtypes mediate inflammation and remodeling in asthma. Therefore, in chapter 6, we investigated the effects of individual muscarinic receptor subtypes on allergen-induced inflammation and remodeling. To this aim, muscarinic receptor subtype specific knock-out animals were exposed to ovalbumin, and lungs were collected for analysis of inflammation and remodeling.

Increasing evidence suggests that airway remodeling might not only be a consequence of inflammation, but might also or alternatively be a consequence of bronchoconstriction, which triggers TGF-β release via biomechanical activation. In chapter 7, we aimed to investigate the effect of TGF-β and bronchoconstriction on remodeling, and the involvement of the M₃ receptor in this response, by using murine precision cut lung slices (PCLS). TGF-β was used to induce remodeling in PCLS from wild-type animals. In addition, PCLS were stimulated with methacholine, to investigate the effect of bronchoconstriction on remodeling. Moreover, PCLS from M₃R⁻/⁻ mice, in which bronchoconstriction is abolished, were exposed to TGF-β and methacholine, to investigate the involvement of the M₃ receptor in this response. After two days, expression of remodeling parameters, as well as the functional impact on bronchoconstriction, was analyzed.

In chapter 8, we investigated the effects of endogenous non-neuronal acetylcholine on epithelial cell differentiation. There is evidence from in vivo studies which suggests an indirect role for acetylcholine in epithelial cell differentiation and goblet cell metaplasia. Here, we aimed to investigate direct effects of endogenous non-neuronal acetylcholine on epithelial cell differentiation and possible mechanisms involved. Human airway epithelial cells were isolated from healthy donors and cultured at an air-liquid interface. During differentiation at the air-liquid interface, cells were exposed to tiotropium, to investigate the effects of acetylcholine on epithelial cell differentiation following air exposure, and to IL-13 and tiotropium, to investigate the effects of acetylcholine on IL-13-induced goblet cell metaplasia.

Finally, in chapter 9, the results of this thesis are summarized and discussed. Perspectives for future studies are provided.
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