Long-acting muscarinic antagonists and small airways: which link?

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Abstract

Involvement of small airways, those of less than 2 mm in internal diameter, is present in all stages of asthma and contributes substantially to the pathophysiologic expression of asthma. Therefore, small airways are increasingly viewed as a potential target in optimal asthma control. Airway tone, which is increased in asthma, is mainly controlled by the vagus nerve that releases acetylcholine (ACh) and activates muscarinic ACh receptors (mAChRs) post-synaptically on airway smooth muscle (ASM). In small airways, M_3 mAChRs are expressed, but there is no vagal innervation. Non-neuronal ACh released from the epithelial cells that may express choline acetyltransferase (ChAT) in response to inflammatory stimuli, as well as from other structural cells in the airways, including fibroblasts and mast cells, can activate these receptors. By antagonizing M_3 mAChR, the contraction of the ASM is prevented and, potentially, local inflammation can be reduced and the progression of remodeling may be affected. In fact, ACh also contributes to inflammation and remodeling of the airways and regulates the growth of ASM. Several experimental studies have demonstrated the potential benefit derived from the use of mAChR antagonists, mainly long-acting mAChR antagonists (LAMAs), on small airways in asthma. However, there are several confounding factors that may cause a wrong estimation of the relationship between LAMAs and small airways in asthma.

Abbreviations: ACh, acetylcholine; ASM, airway smooth muscle; BAL, bronchoalveolar lavage; β_2-ARs, β_2-adrenoceptors; cAMP, cyclic adenosine monophosphate; ChAT, choline acetyltransferase; ERK, extracellular signal-regulated kinase; IL, interleukin; ICS, inhaled corticosteroid; LAMAs, long-acting mAChR antagonists; mAChRs, muscarinic ACh receptors; OVA, ovalbumin; TGF-β1, transforming growth factor-β1

Introduction

Small airways are usually defined as those airways of less than 2 mm in internal diameter and located beyond the 7^{th}-8^{th} generation of airways [1]. They represent more than 98% of the cross-sectional area of the lung but only less than 10% of the total lung volume [2], and end with the respiratory acinar zone (generations 17^{th}-23^{rd}) involved in gas exchange [1]. Small airways differ from large airways, having no cartilage, a relatively greater proportion of smooth muscle and fewer mucus-secreting goblet cells in the epithelial layer [3]. Because of the lack of cartilage that could support their structure, they are easily collapsible during forced expiration or smooth muscle contraction [1]. Physiological features of small airway obstruction include premature closure of airways with air trapping and regional heterogeneity and exaggerated volume dependence of airflow limitation [4].

Small airways in asthma

Small airway involvement is present in all stages of asthmatic disease [2] and affects 50-60% of individuals [5]. Several specific abnormalities of small airways in asthma such as cellular infiltration of bronchiolar walls, alveoli, and perivascular spaces [6, 7], goblet cell hyperplasia of the epithelium [7], collections of mucus and inflammatory cells obstructing the airway lumen [8], smooth muscle thickening, and submucosal remodeling [10] have been described.

The small airways exhibit greater resistance in moderate-to-severe asthma. However, their dilatation in deep breathing is not affected in the absence of bronchial stimulation, with amplified response in the presence of spirometry defined airflow obstruction [11]. This suggests that effective small airway targeting is needed to achieve functional improvements in moderate to severe asthma patients.

Indeed, as highlighted by an Interasma (Global Asthma Association - GAA) and World Allergy Organization (WAO) document endorsed by Allergic Rhinitis and its Impact on Asthma (ARIA) and Global Allergy and Asthma European Network (GA^2LEN), the role of the small airways in asthma is increasingly viewed as a potential target in optimal disease control [12]. Actually, dynamic hyperinflation resulting from small airway inflammation, which is highly prevalent in moderate-to-severe asthma and is related to important patient outcomes associated to his or her daily life, is an important goal for asthma treatment [13].

Acetylcholine and parasympathetic nerves in asthmatic small airways

The parasympathetic nerves carried in the vagus nerve are tonically active at rest, producing a stable and easily reversible basic tone of the airway smooth muscle (ASM) [14]. Their responses can be modulated by activation of vagal afferents after a mechanical or chemical stimulus in the airway [15]. There is no direct sympathetic innervation of ASM, although the airway vascular system receives sympathetic innervation. However, sympathetic input to the parasympathetic ganglia has been documented and, furthermore, there are β_2-adrenoceptors (ARs) on ASM [14, 16]. Acetylcholine (ACh) is the neurotransmitter of the parasympathetic nervous system [14,16, 17]. It is released from the vagus and induces the contraction of the ASM primarily via stimulation of G_q-coupled M_3 muscarinic ACh receptors (mAChRs) expressed on ASM cells.

The M_3 mAChR is found predominantly in the bronchus, but its density decreases from segmental to subsegmental bronchi, and it is absent in the lung parenchyma [18]. β_2-ARs increase in number along the airways and their density in the subsegmental bronchi and lung parenchyma is approximately twice that of the M_3 mAChRs in the same region [18]. Using human tissues, it was observed that mAChR antagonists were more effective in central airways whereas β_2-AR agonists worked better on peripheral airways, but both classes of bronchodilators were shown to be able to induce relaxation of the smooth muscle of the entire bronchial tree [19, 20].

Actually, in the proximal airways, ACh is released from the vagus and activates the M_3 mAChRs present on ASM. In the peripheral airways there is no cholinergic innervation, but M_3 mAChRs are expressed. The so-called non-neuronal ACh, which is released by epithelial cells that can express choline acetyltransferase (ChAT) in response to inflammatory stimuli and are not innervated by extrinsic or intrinsic cholinergic neurons, can activate these receptors [21]. In detail [22], the high affinity choline transporter 1 mediates the uptake of choline into cells. ACh is synthesized from choline and acetyl-coenzyme A by ChAT. Newly synthesized ACh is transported by the vesicular ACh transporter into the vesicles, stored there and then released into the extracellular space. Cigarette smoking or inflammatory stimuli induce the release of ACh from the epithelial cells of the airways. Released ACh stimulates mAChRs on epithelial cells and surrounding cells in an autocrine or paracrine manner (figure).

Also inflammatory cells such as macrophages, lymphocytes, and granulocytes, as well as structural cells in the airways, including fibroblasts and mast cells are other non-neuronal ACh sources [23].

Nevertheless, there is a growing evidence of the presence of a cholinergic innervation in human peripheral airways. Atropine significantly reduced human small airway contractions following repeated electrical field stimulation of precision-cut lung slices [24]. Furthermore, recent imaging studies have suggested that small airways are innervated to an extent similar to that of larger airways [25, 26].

As already mentioned, ACh increases bronchoconstriction acting on the M_3 mAChRs on ASM, but it also contributes to inflammation and remodeling of the airways and regulates the growth of ASM [27].

ACh is a key molecule of asthma pathophysiology [23]. In a mouse model of asthma, the lungs had significantly more ACh than controls [28]. ACh was secreted to the entire lung regardless of its central or peripheral location. The increase in ACh was accompanied by an intense infiltration of eosinophils even in the absence of an acetylcholinesterase deficiency. The quantitative distribution of ACh in the lung tissue was strongly associated with asthma severity, which implies that inflammatory cell levels potentially affect ACh levels in asthma pathology.

Experimental evidence suggests that exposure to allergens in the first years of life causes an increase in ASM parasympathetic innervation and ACh release [29]. This involves an altered regulation of M_3 mAChRs with consequent dysregulation of the expression of the genes involved in the functional maturation of ASM which, in turn, causes the hypercontractile phenotype of ASM.

The increased neuronal activity may be the consequence of neuronal plasticity and remodeling likely driven by immune/inflammatory cells, such as eosinophils and mast cells, or by proteins that induce and promote impaired nerve growth and function, such as neurotrophins [30, 31]. Plasticity is manifested by an increase in the density of the parasympathetic ganglia, which results in an additional release of ACh. The increased signal activated by ACh causes a further increase in the density of the cholinergic nerves, thus creating a vicious circle.

Airway remodeling is a pathological feature observed in asthma [32]. It can affect both large and small airways and pathology limited to the small airways is uncommon [33]. Cycles of bronchoconstriction and mechanotransduction are one plausible mechanism for its origin [34].

Both carbachol and transforming growth factor-β1 (TGF-β1) increased the expression of M_1 and M_3 mAChRs proteins and reduced M_2 mAChRs expression on fibroblasts after 48

hours of exposure [35]. In human lung fibroblasts, mAChRs exert stimulating effects on collagen synthesis [36]. Furthermore, activation of mAChRs induces the transition from fibroblasts to myofibroblasts through an increase in extracellular signal-regulated kinase (ERK)1/2 phosphorylation, the formation of Ras homolog family member A-guanosine-5'-triphosphate and a decrease in cyclic adenosine monophosphate (cAMP) levels [35]. The cholinergic and TGF-β1 pathways interact to induce the transition of myofibroblasts. The distal lung is an important site of extracellular matrix remodeling in fatal asthma, with an imbalance between collagen I and III (increased collagen I and decreased collagen III content) and an increase in the fibronectin and matrix metalloproteinase content [37].

Exposure of wild-type mice to ovalbumin (OVA) induced goblet cell metaplasia, thickening of airway smooth muscle, remodeling of pulmonary vascular smooth muscle, and deposition of collagen I and fibronectin in the airway wall [38]. These effects were absent or significantly less pronounced in mice with M_3 mAChR subtype deficiency (M_3 mAChR$^{-/-}$), while M_1 mAChR$^{-/-}$ and M_2 mAChR$^{-/-}$ mice responded similarly to wild-type mice. In wild type and M_3 mAChR$^{-/-}$ mice, challenge with OVA induced a 10-fold increase in the number of eosinophils. The number of eosinophils was also increased in M_1 mAChR$^{-/-}$ and M_2 mAChR$^{-/-}$ mice after stimulation with OVA. Stimulation with OVA induced an increase in IL-4, IL-5 and IL-17 in protein level mice, which was even greater in M_3 mAChR$^{-/-}$ mice. Similar results were obtained at the level of gene expression for IL-13. These data indicate that ACh contributes to allergen-induced remodeling via the M_3 mAChR and not via the M_1 or M_2 mAChRs. No active role in inflammation was observed for individual muscarinic receptors.

All these findings suggest that the role of ACh in remodeling is independent of the allergic inflammatory response and may involve bronchoconstriction. In fact, even in the absence of inflammatory stimuli, mechanical compression alone is sufficient to induce an asthma-like molecular pattern in non-asthmatic airway epithelial cells [39]. Bronchoconstriction without further inflammation induces remodeling of the airways in patients with asthma [40].

This conclusion is of paramount importance when setting up treatment for asthmatic patients. In fact, although treatment of the inflammatory component of asthma is the first-line approach to disease control, bronchial hyperreactivity is often not normalized by ICS therapy, particularly in patients with more severe asthma, and additional therapy is required [40].

Rationale for using M_3 mAChR antagonists in asthma

To this end, the concept that stresses the importance of airway tone alteration in patients with asthma is becoming increasingly accepted. Airway tone is altered due to an increase in vagal activity caused by several mechanisms that focus primarily on the amplified expression and enhanced function of signal pathway molecules essential for the ASM contraction mediated by M_3 mAChRs and the dysfunction of M_2 mAChRs which act as autoreceptors [31].

Targeting M_3 mAChRs has the potential to prevent harmful changes in gene expression and thus reverse the hypercontractility of ASM triggered by exposure to allergens in the first years of life [29]. However, this therapeutic approach has never been tested and, therefore, it remains only a hypothesis. Instead, the use of mAChR antagonists in asthmatic patients is realistic although the mechanisms of small airway contraction are still partially unknown.

An experimental study that examined small airway relaxation using lungs obtained from subjects undergoing resection showed that a pre-treatment of pulmonary slices with tiotropium, 4-DAMP, which a selective M_3 mAChR antagonist, or AF-DX116, which is a selective M_2 mAChR antagonist, inhibited carbachol-induced contraction of small airways in a concentration-dependent manner [41]. AF-DX116, gallamine, which is another selective M_2 mAChR antagonist, and pertussis toxin, which is an inhibitor of the G_i protein, significantly increased isoproterenol-induced relaxation of small airways.

Collectively, these data suggest that mAChRs blockade in human small airways inhibits airway contraction via direct inhibition of contraction via M_3 mAChRs and inhibition of M_2 mAChRs-mediated inhibition of the relaxation response [41]. MAChR antagonists, by inhibiting the M_2 mAChRs coupled to the post-synaptic G_i protein, maintain the β_2-AR agonist-induced relaxation of ASM and support the activity of adenylyl cyclase, although inhibition of the presynaptic M_2 mAChRs may increase the release of ACh into the synaptic space [42]. Neural regulation of pulmonary function occurs within the central nervous system and extends into the conducting airways, making differentiating between central and peripheral effects difficult [43], but the prevailing opinion is that there is no cholinergic innervation in the peripheral airways [21].

Pharmacological effects of LAMAs in asthma also considering small airways

Several experimental studies have demonstrated the potential benefit derived from the use of mAChR antagonists, mainly long-acting mAChR antagonists (LAMAs), in asthma. In particular, they highlighted the effects of this class of bronchodilators on small airways.

Glycopyrronium was found to be significantly more effective in relaxing human bronchial rings passively sensitized with atopic serum than non-sensitized tissues [44]. The relaxing effect of glycopyrronium was also significantly more evident in the peripheral airways when they were passively sensitized.

In a guinea-pig model of asthma, antigen-induced hyperreactivity was completely blocked by tiotropium pre-treatment but only partially blocked by atropine pre-treatment [45]. Tiotropium blocked i.v. ACh-induced bronchoconstriction, but did not inhibit vagus-mediated bronchoconstriction in sensitized controls. This finding suggested that tiotropium does not inhibit hyperreactivity by blocking receptors for ACh released by the vagus. Rather, tiotropium may have acted through an anti-inflammatory mechanism, since it inhibited the antigen-induced accumulation of eosinophils in the lungs and around the nerves or, alternatively prevented airway hyperreactivity by blocking ACh released from non-neuronal sources such as epithelial cells or macrophages. There is experimental documentation that LAMAs, such as glycopyrronium and tiotropium, reduced the release of ACh from isolated human bronchi when tested at low concentrations [20, 46]. Removal of the epithelium resulted in inhibition of this effect of LAMAs.

In a murine acute model of asthma, in which the mice were immunized on days 0 and 7 by subcutaneous administration of OVA and challenged with intranasal OVA on days 21, 23, 25, and 27 and tiotropium was administered via the intranasal route 0.5 hour before the OVA challenge, the number of total cells, macrophages and eosinophils in bronchoalveolar lavage (BAL) fluids was significantly increased in the tiotropium-treated group compared to the control group [47]. M_3 mAChRs were more expressed and M_2 mAChRs less expressed in the OVA group than in the naive group. In the chronic asthma model, in which the mice were immunized with OVA once a week for 1 month and then underwent intranasal OVA challenges that started on day 31 and repeated twice a week for 3 months and tiotropium was administered twice a week for 3 months, the tiotropium group had significantly lower total cell counts, macrophages, eosinophils, and lymphocytes in BAL than the control group. Furthermore, in the acute asthma model, the tiotropium group showed increased levels of interleukin (IL)-4, IL-5, and IL-13 compared to the control group, whereas in the chronic asthma model, compared to the control group the IL-5 concentration in the BAL

fluids was significantly lower and concentrations of IL-4 and IL-13 showed a trend towards a decrease in the tiotropium group. It was also observed that the tiotropium treatment for 3 months had a protective effect against airway remodeling parameters, including peribronchial fibrosis and smooth muscle thickening, by differential regulation of M_2 mAChRs and M_3 mAChRs expression. In fact, tiotropium treatment significantly reduced expression of the M_3 mAChRs and increased that of the M_2 mAChRs compared to the control group.

Tiotropium inhibited allergen-induced airway remodeling in a guinea pig model of ongoing asthma [48]. ASM, contractility and contractile protein expression were all partially or completely reduced. It has been suggested that the relaxation of ASM cells induced by tiotropium can prevent the production of extracellular matrix via the β-catenin signal [49]. β-Catenin is a dual-function protein involved in the regulation and coordination of cell-cell adhesion. The activation of the β-catenin signal has a regulatory function in the remodeling of the airways [50]. It is involved in the regulation of proliferation of ASM cells, epithelial-to-mesenchymal transition, differentiation of myofibroblasts, and production of extracellular matrix.

Aclidinium, a M_3 mAChR antagonist, dose-dependently inhibited the transition of human lung fibroblast to a myofibroblast contractile phenotype caused by carbachol and TGF-β1 stimulation and also reduced fibroblast proliferation and migration [51]. Furthermore, aclidinium pre-treatment prevented the upregulation of M_1 mAChRs and M_3 mAChRs, but not M_2 mAChRs downregulation induced by carbachol or TGF-β1.

In addition to allergens, also cigarette smoke may induce inflammatory responses in human bronchial epithelial cells [52] and participate in lung remodeling [32]. In particular, cigarette smoke activates mAChRs in human bronchial fibroblasts by a non-neuronal cholinergic system, a mechanism that is involved in the upregulation of the myofibroblasts markers [53]. Glycopyrronium exerted a potent suppressive effect on lung inflammation and small airway remodeling induced by subchronic exposure to cigarette smoke in mice [54]. Furthermore, treatment with glycopyrronium suppressed cigarette smoke extract-induced inflammatory responses in human bronchial epithelial cells and proliferation and collagen production in human lung fibroblasts. It has been reported that another LAMA, aclidinium reversed the cigarette smoke-induced myofibroblast markers through inhibition of reactive oxygen species, generation, cAMP depletion, ERK1/2 phosphorylation and ChAT overexpression induced by cigarette smoke [52].

All this information generated in in vitro and in vivo studies using various experimental models suggests that the combination of a LAMA with a corticosteroid, which is still the key element in the treatment of asthma, may be pharmacologically useful inducing an additive or even synergistic effect.

When comparing the ability of tiotropium and budesonide to inhibit allergen-induced airway remodeling, it was possible to document that the anti-remodeling effects of tiotropium were not substantially dissimilar to those of budesonide [55]. Similar to budesonide, tiotropium partially reduced the accumulation of eosinophils in the submucosal compartments of the cartilaginous and non-cartilaginous airways in guinea pigs sensitized to OVA and stimulated with OVA for 12 weeks. However, budesonide was more effective in preventing ASM thickening in the noncartilaginous airways and abrogated the increase in contractile protein accumulation, whereas the inhibitory effect of tiotropium was partial but tiotropium reduced the contractility of tracheal smooth muscle to a greater extent than did budesonide.

However, in another chronic asthma model, ASM thickening in noncartilaginous airways was reduced by both tiotropium and ciclesonide treatment [56]. No remodeling of the pulmonary microcirculation was observed. OVA-induced airway eosinophilia was reduced in a dose-dependent manner by both tiotropium and ciclesonide treatment, but this inhibitory effect was most marked in the submucosa of cartilaginous airways. Nevertheless, combined treatment with tiotropium and ciclesonide had profound anti-inflammatory effects compared to OVA-challenged animals, significantly inhibiting airway eosinophilia in both cartilaginous and noncartilaginous airways. Furthermore, it inhibited OVA-induced ASM mass by 81%.

A study that pharmacologically investigated the interaction between beclomethasone and the glycopyrronium on the human ASM tone was unable to detect synergistic interaction in non-sensitized airways, but the beclomethasone/glycopyrronium combination synergistically enhanced the relaxation of passively sensitized medium and small airways [44]. The synergistic interaction between beclomethasone and glycopyrronium was associated with an increase of cAMP concentrations.

Conclusion

A substantial amount of experimental evidence of LAMAs efficacy in asthmatic patients is currently available. It allows us to believe that there is a role for this class of

bronchodilators in asthma and also a link between small airways and potential benefits from LAMA in this disease. However, as appropriately highlighted by Gosens and Gross [23], further studies are needed to clarify in more detail the role of cholinergic control in the pathophysiology of asthma, and we add in the control of small airways. In particular, the areas that require further investigation are neuronal plasticity in asthma and its contribution to hyperreactivity and remodeling of the airways, the anti-inflammatory effects of LAMAs in asthmatic patients, the mechanisms underlying the cholinergic control of inflammation, in particular T2-type inflammation, and airway remodeling, and remodeling induced by bronchoconstriction.

It difficult to give an answer to still unanswered questions because there are several confounding factors that may cause a wrong estimation of the relationship between LAMAs and small airways in asthma. In particular, in the asthmatic subject, airway obstruction and bronchial hyperreactivity usually affect the central parts of the lungs while the inflammation can be distributed more peripherally [57]. This implies the absolute need to be able to differentiate the broncholytic effects of LAMAs from the anti-inflammatory one and, therefore, better understand the interaction between LAMAs and corticosteroids at different levels of the bronchial tree. Furthermore, prolonged treatment and, in addition, a chronic pathological condition can modify the response of mAChRs and also the access of the drug to mAChRs.

In our opinion, the possibility that there is a different response to LAMAs related to the sex of the patient is a potential important issue that need for a rapid elucidation. It has been shown that small airway involvement in asthma, as reflected by methacholine-induced air trapping on CT scan, was significantly greater in males than females while bronchial exhaled nitric oxide, which may reflect small airway inflammation, was greater in females than males [58]. This indicates that males show a small airway involvement that is due to reduced small airway patency whereas in females it is mainly due to small airway inflammation. Nevertheless, a greater pulmonary gene expression for M_3 mAChRs compared to M_2 mAChRs has been found in the female lungs than male ones and it has been suggested that the sexual dimorphism in the expression of these receptors in the airways may modulate therapeutic responses to LAMAs [59].

These two findings indicate that a therapeutic approach that considers the simultaneous administration of a LAMA and a corticosteroid may be the most appropriate approach to overcome the sexual differences in the prevailing pathological impact of asthma on small

airways and expression for M_3 mAChRs. Moreover, this approach, which is still empirical, would allow us to overcome, though only on a clinical level, the mentioned confounding factors. In particular, considering what we have described above, it seems essential for the treatment of smokers with asthma who have more severe obstructive impairments than light and never smokers with similar inhaled corticosteroid (ICS) dose [60], also because of the greater presence of small airway involvement [61]. Obviously, LAMAs must always be included in the context of a triple therapy because there is scarce evidence showing that ICS/long-acting β_2-AR agonist can be replaced by ICS/LAMA [31].

Conflict of interest statement

MC has participated as a speaker and/or advisor in scientific meetings and courses under the sponsorship of Abdi Ibrahim, Almirall, AstraZeneca, Biofutura, Boehringer Ingelheim, Chiesi Farmaceutici, Cipla, GlaxoSmithKline, Lallemand, Menarini Group, Mundipharma, Novartis, Pfizer, Verona Pharma, and Zambon and has been a consultant to ABC Farmaceutici, Chiesi Farmaceutici, Edmond Pharma, Lallemand, Novartis, Ockham Biotech, Verona Pharma, and Zambon.

LC has participated as advisor in scientific meetings under the sponsorship of Boehringer Ingelheim and Novartis, received nonfinancial support from AstraZeneca, a research grant partially funded by Chiesi Farmaceutici, Boehringer Ingelheim, Novartis, and Almirall, and is or has been a consultant to ABC Farmaceutici, Edmond Pharma, Ockham Biotech, Verona Pharma, and Zambon.

MGM has participated as a speaker and/or advisor in scientific meetings and courses under the sponsorship of ABC Farmaceutici, Almirall, AstraZeneca, Boehringer Ingelheim, Chiesi Farmaceutici, GlaxoSmithKline, and Novartis and has been a consultant to ABC Farmaceutici, GlaxoSmithKline, and Chiesi Farmaceutici.

Author contributions

All authors coordinated the work, designed the structure of the review, and reviewed the literature. MC wrote the manuscript, and prepared the figure. LC and MGM critically reviewed and finalized the manuscript.

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Figure – Potential effects of ACh on asthmatic small airways. ACh, acetylcholine; ChAT, choline acetyltransferase.