Chapter 9

Protective effects of tiotropium bromide in the progression of airway smooth muscle remodeling \textit{in vivo}

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Abstract
Recent findings have demonstrated that muscarinic M₃ receptor stimulation enhances airway smooth muscle (ASM) proliferation to peptide growth factors in vitro. Since both peptide growth factor expression and acetylcholine release are known to be augmented in allergic airway inflammation, it may be envisaged that anticholinergics protect against allergen-induced ASM remodeling in vivo. Therefore, we investigated the effects of treatment with the long-acting muscarinic receptor antagonist tiotropium on ASM changes in a guinea pig model of ongoing allergic asthma. Twelve weekly repeated allergen challenges induced an increase in ASM mass in the non-cartilaginous airways. This increase was not accompanied by alterations in ASM cell size, indicating that the allergen-induced changes were entirely due to increased ASM cell number. Morphometric analysis showed no allergen-induced changes in ASM area in cartilaginous airways. However, repeated OA challenge enhanced maximal contraction of open tracheal ring preparations ex vivo. This was associated with an increase in smooth muscle specific myosin expression in the lung, indicating that the ASM cells in the central airways acquired a more contractile phenotype. Treatment with inhaled tiotropium considerably inhibited the increase in ASM mass, myosin expression and contractility. These results indicate a prominent role for acetylcholine in allergen-induced ASM remodeling in vivo, a process that was thus far considered to be primarily caused by growth factors and other mediators of inflammation. Therefore, muscarinic receptor antagonists like the long-acting anticholinergic tiotropium bromide could be beneficial in preventing chronic airway hyperresponsiveness and decline in lung function in allergic asthma.

Introduction
The use of anticholinergics in obstructive airways diseases, like asthma and COPD, is primarily based on their acute bronchodilatory effects. Thus, muscarinic receptor antagonists provide acute relief from the increased levels of acetylcholine (ACh) released in the airways upon reflex vagal nerve stimulation during allergic airway inflammation [1]. Potential effects of anticholinergics on inflammation-induced structural changes in the airways, however, have not been considered thusfar. Nevertheless, it has recently been demonstrated that muscarinic receptor stimulation potentiates the mitogenic response of bovine tracheal smooth muscle (BTSM) cells to platelet-derived growth factor (PDGF), which was mediated by the Gq-coupled muscarinic M₃ receptor [2]. In addition, muscarinic receptor stimulation augmented the mitogenic responses of human airway smooth muscle (ASM) cells to epidermal growth factor (EGF) [3]. These two peptide growth factors can be released from structural cells in the airways, including ASM cells, as well as from infiltrated inflammatory cells [4]. This could indicate that, in addition to ACh, growth factor release may be augmented in allergic airway inflammation. Indeed, the expression levels of some peptide growth factors (eg. EGF and basic fibroblast growth factor, bFGF) have been found elevated in asthma [5,6]. Therefore, it may be envisaged that functional interaction of ACh with growth factors during chronic airway inflammation is involved in the development of ASM thickening, a pathological feature observed in asthmatics as well as in animal models of allergic asthma [7-9].
Airway smooth muscle thickening is considered to be involved in the development of chronic airway hyperresponsiveness in asthma [10].

Both in cell culture [11] and in organ culture [12] settings, growth factor-stimulated ASM growth has been tightly associated with ASM phenotypic plasticity. This phenotypic plasticity allows ASM to adapt to pro-mitogenic environments, resulting in diminished contractility and contractile protein expression but increased proliferative and synthetic properties [13]. Conversely, growth arrest can reconstitute a contractile or even a hypercontractile phenotype [14;15]. Therefore, ASM phenotypic plasticity may contribute to airway inflammation and airway remodeling during periods of allergen exposure and to increased contractility in the periods in between, thereby alternating changes in ASM mass and contractility. Importantly, a recent study showed the occurrence of allergen-induced ASM phenotype switching in Brown-Norway rats, indicating that phenotypic plasticity could indeed accompany ASM thickening in vivo [16]. The role of ACh in allergen-induced ASM phenotypic modulation, however, is presently unknown.

Therefore, in the present study we investigated the contribution of endogenous ACh to allergen-induced remodeling of ASM in vivo. For this purpose, the effects of treatment with the long-acting muscarinic receptor antagonist tiotropium bromide [17] was evaluated on ASM after repeated allergen challenge in a guinea pig model of allergic asthma, characterised by early and late asthmatic reactions, airway hyperresponsiveness after these reactions and airway inflammation [18]. As parameters for ASM remodeling, smooth muscle area, cell number, contractile protein expression and contractility were assessed ex vivo after twelve weekly repeated allergen exposures. It was demonstrated that allergen-induced augmentation of these parameters was considerably reduced by pre-treatment with tiotropium bromide.

Methods

Animals
Outbred, male, specified pathogen free Dunkin Hartley guinea pigs (Harlan, Heathfield, United Kingdom) weighing 250-300 g were sensitized to ovalbumin (OA) as described previously [18]. In short, 0.5 ml of an allergen solution containing 100 µg/ml ovalbumin and 100 mg/ml Al(OH)₃ in saline was injected intraperitoneally, while another 0.5 ml was divided over seven intracutaneous injection sites in the proximity of lymph nodes in the paws, lumbar regions and the neck. The animals were used experimentally from 4 to 8 weeks after sensitization. The animals were group-housed in individual cages in climate controlled animal quarters and given water and food ad libitum, while a 12-h on/12-h off light cycle was maintained. All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation.
Provocation procedures
Four weeks after sensitization, allergen-provocations were performed by inhalation of aerosolized solutions of ovalbumin or saline (control) as described previously [18]. Aerosols were produced by a DeVilbiss nebulizer (type 646, DeVilbiss, Somerset, PA, USA) with an airflow of 8 l/min resulting in an output of 0.33 ml/min. Provocations were carried out in a specially designed perspex cage (internal volume 9 l), in which the guinea pigs could move freely. Before the start of the experimental protocol, the animals were habituated to the provocation procedures on two sequential days. After an adaptation period of at least 30 min, three consecutive provocations with saline were performed, each provocation lasting 3 min, separated by 7 min intervals. Ovalbumin challenges were performed by inhalation of increasing concentrations of 0.05 %, 0.1 % and 0.3 % w/v ovalbumin in saline for 3 min each, with 7 min intervals. Allergen inhalations were discontinued when the first signs of respiratory distress were observed. No anti-histaminic was needed to prevent the development of anaphylactic shock.

Experimental protocol
Guinea pigs were divided into 4 groups: (1) OA-sensitized, saline-challenged controls, (2) OA-sensitized, OA-challenged animals, (3) OA-sensitized, saline-challenged, tiotropium-treated animals and (4) OA-sensitized, OA-challenged, tiotropium-treated animals. Guinea pigs were challenged with antigen once weekly as indicated, for 12 consecutive weeks. For tiotropium treatment animals received a nebulized dose of tiotropium bromide in saline (0.1 mM, 3min), 0.5 h prior to each challenge with saline or ovalbumin. The prolonged treatment with tiotropium bromide and/or ovalbumin did not affect growth of the animals. In week 12, animals weighed 1045 ± 95 g (12 x saline); 1017 ± 40 g (12 x OA); 1012 ± 26 g (12 x tiotropium) and 1029 ± 30 g (12 x OA + tiotropium).

Tissue acquisition
Twenty four h after the last challenge, guinea pigs were sacrificed by a sharp blow on the head, followed by rapid exsanguination. The lungs were immediately resected and kept on ice in a petri dish for further processing. In addition, the trachea was removed and transferred to a Krebs-Henseleit solution (37 °C), pregassed with 5 % CO₂ and 95 % O₂, pH 7.4. The lungs were divided into two equal parts. One part was frozen at −80 °C in isopentane and stored at −80 °C for histological purposes. The remaining part was snap frozen in liquid nitrogen and stored at −80 °C to be used for Western analysis.

Morphometric analysis of airway smooth muscle mass
Smooth muscle area was determined in 8 µm thick cryostat lung sections, with transverse cross-section of the main bronchus. To identify smooth muscle, the sections were stained for smooth muscle α-actin (sm-α-actin) or smooth muscle myosin heavy chain (sm-MHC), each in quadruplicate. Sections were dried for 30 min, fixed with acetone for 10 min and then washed three times in phosphate-buffered saline (PBS, composition (mM) NaCl, 140.0; KCl, 2.6; KH₂PO₄, 1.4; Na₂HPO₄·2H₂O, 8.1; pH 7.4). Subsequently, sections were incubated for 1 h in PBS.
supplemented with 1 % bovine serum albumin (BSA) and primary antibody (diluted 1:200 for sm-α-actin and 1:100 for sm-MHC) at room temperature. Sections were then washed three times with PBS after which endogenous peroxidase activity was blocked by treatment with PBS containing 0.075 % H₂O₂ for 30 min. Sections were washed for another three times with PBS, after which the horseradish peroxidase (HRP)-linked secondary antibody (Rabbit anti-mouse IgG, diluted 1:200) was applied for 30 min at room temperature. After another three washes, sections were incubated with diaminobenzidine (1 mg/ml) for 5 min in the dark, after which sections were washed and stained with hematoxylin. After rinsing with water the sections were embedded in Kaisers glycerol gelatin. Airways within each section were digitally photographed and classified as cartilaginous or non-cartilaginous. For both types of airways, sm-α-actin and sm-MHC positive areas were measured by a single observer in a blinded fashion. In addition, hematoxylin-stained nuclei within the ASM bundle were counted. Generally, 3 to 5 airways of each classification were analyzed per animal. Data were expressed as mm²/mm basement membrane.

**Western analysis of contractile protein expression**

Lung homogenates were prepared by pulverizing tissue under liquid nitrogen and subsequent sonification in homogenization buffer (composition in mM: Tris.HCl 20.0, dithiothreitol (DTT) 0.1, phenyl methyl sulphonyl fluoride (PMSF) 0.2; pH 7.5, supplemented with 2 µg/ml leupeptin, 2 mg/ml aprotinin and 10 µg/ml soybean trypsin inhibitor). Homogenates were stored at −80 °C until further use. Protein content was determined according to Bradford [19]. Protein (50 µg per lane) was separated by SDS/PAGE on 6 % (sm-MHC) or 10 % (sm-α-actin) polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes, which were blocked overnight in blocking buffer (composition: Tris 50.0 mM; NaCl 150.0 mM; dried milk powder 5 %; pH = 7.5). After two washes with washing buffer (composition: Tris 50.0 mM; NaCl 150.0 mM; dried milk powder 2.5 %; pH = 7.5), membranes were incubated at room temperature with primary antibodies (anti-sm-MHC or anti-sm-α-actin, both diluted 1:200 in washing buffer). After three washes with washing buffer supplemented with 0.1 % Tween 20, membranes were incubated in HRP-labelled secondary antibodies (dilution 1:6000 in washing buffer) at room temperature for 1h, followed by another three washes. Antibodies were then visualised by enhanced chemiluminescence. Photographs of blots were analyzed by densitometry (Totallab™; Nonlinear Dynamics, Newcastle, U.K.).

**Isometric tension measurements**

The trachea was prepared free of serosal connective tissue. Single open-ring, epithelium-denuded preparations were mounted for isometric recording in 20 ml water-jacked organ-baths, containing KH-buffer (pH 7.4), continuously gassed with 5 % CO₂ and 95 % O₂ at 37 °C. During a 90-min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 0.5 g. Subsequently, muscle strips were precontracted with 20 mM and 40 mM KCl. Following two wash-outs, basal smooth muscle tone was determined by the addition of 0.1 µM isoprenaline and tension was re-adjusted to 0.5 g, immediately followed by two changes of fresh KH-buffer. After another equilibration period of 30 min, cumulative concentration
response curves (CRCs) were constructed to methacholine ($1.10^{-9}$ M – $1.10^{-4}$ M using 0.5 log increments). When maximal tension was obtained, the strips were washed several times and resting tension was re-established using isoprenaline.

Data analysis
All data represent means ± s.e.mean from $n$ separate experiments. The statistical significance of differences between data was determined by Bonferroni’s Student’s $t$-test or one-way ANOVA, as appropriate. Differences were considered to be statistically significant when $P < 0.05$.

Materials
Methacholine hydrochloride was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Mouse monoclonal anti sm-MHC was from Neomarkers (Fremont, CA, USA). Mouse monoclonal anti sm-α-actin, rabbit anti-mouse IgG (peroxidase conjugate), soybean trypsin inhibitor, dithiothreitol, sodium-dodecyl sulphate, bovine serum albumin, diaminobenzidine, ovalbumin, aprotinin, leupeptin and (-)isoprenaline hydrochloride were from Sigma (St. Louis, MO, U.S.A.). Tiotropium bromide was from Boehringer Ingelheim (Ingelheim, Germany). Enhanced chemiluminescence reagents were from Pierce (Rockford, IL, USA). All other chemicals were of analytical grade.

Results
Effects of ovalbumin challenge and tiotropium treatment on ASM content.
Figure 9.1 shows representative lung sections stained for sm-MHC and sm-α-actin, containing the main bronchus as well as blood vessels. Although the ASM layer stained positively for contractile proteins, sm-α-actin and sm-MHC positive areas were slightly dissimilar, i.e. sm-MHC positive area was more discontinuous and appeared to be somewhat smaller. Indeed, morphometric analysis revealed that in saline challenged controls, sm-α-actin positive area was somewhat larger than sm-MHC positive area, both in cartilaginous (0.100 ± 0.006 vs. 0.073 ± 0.010 mm$^2$/mm basement membrane for sm-α-actin and sm-MHC, respectively; $P = 0.035$) and non-cartilagenous airways (0.064 ± 0.008 vs. 0.042 ± 0.006 mm$^2$/mm basement membrane for sm-α-actin and sm-MHC, respectively; $P = 0.010$; cf. Figures 9.2-9.3).

Repeated ovalbumin challenge did not change ASM content in the larger airways, irrespective of the contractile marker protein used. In non-cartilaginous airways however, OA challenge induced a significant increase in both sm-α-actin and sm-MHC positive area of 0.022 ± 0.006 mm$^2$/mm basement membrane (36 ± 3 % increase) and 0.024 ± 0.006 mm$^2$/mm basement membrane (57 ± 13 % increase), respectively, as compared to saline challenged, age-matched controls (Figures 9.2-9.3). This increase was largely prevented by treatment with tiotropium, for 75 ± 9 % for sm-α-actin positive area and for 76 ± 6 % for sm-MHC positive area. Tiotropium bromide treatment by itself did not induce significant changes in the morphometric parameters analysed, when compared to untreated saline challenged controls (Figures 9.2-9.3).
Cartilaginous airways

Saline OA Tio OA+Tio

α-actin positive area (mm²/mm basement membrane)

0.00 0.02 0.04 0.06 0.08 0.10 0.12 0.14 0.16

Non-cartilaginous airways

Saline OA Tio OA+Tio

α-actin positive area (mm²/mm basement membrane)

0.00 0.02 0.04 0.06 0.08 0.10 0.12 0.14

Figure 9.2 Morphometric analysis of sm-α-actin expression in the guinea pig lung. Cartilaginous (A) and non-cartilaginous airways (B) were identified in sm-α-actin stained sections, after which sm-α-actin-positive area was measured and expressed as mm²/mm basement membrane. Data shown represent means ± s.e.mean of 5 saline-challenged animals (Saline); 6 OA-challenged animals (OA); 8 tiotropium-treated, saline-challenged animals (Tio) and 7 tiotropium-treated, OA-challenged animals (OA+Tio). Three to five airways of each classification were analysed per animal. * P < 0.05.
To determine the nature of the changes in ASM area within the non-cartilaginous airways, the number of nuclei that comprised the airway smooth muscle layer in these airways was counted. To account for differences in ASM content between airways as well as between treatment groups, data were expressed as the number of nuclei per mm$^2$ smooth muscle. With this data, the average apparent volume of the individual ASM cell was also calculated, assuming equal thickness of all sections (8 µm). For saline-challenged animals, 5107 ± 405 nuclei were counted per mm$^2$ smooth muscle, and an apparent volume of 1650 ± 121 µm$^3$ per ASM cell was calculated. No differences in either parameter were found between any of the treatment groups, indicating that the observed differences in ASM content were exclusively caused by changes in cell number, not in cell size (Figure 9.4).
Effects of ovalbumin challenge and tiotropium treatment on contractile protein expression.

Changes in sm-α-actin and sm-MHC positive area could imply changes in contractile protein expression. Therefore, we used Western analysis to determine the relative contents of these contractile proteins in whole lung homogenates. For sm-α-actin expression, differences between treatment groups were small, indicating that the changes in sm-α-actin positive area in non-cartilaginous airways had only little impact on total sm-α-actin expression in the lung (Figure 9.5). Surprisingly therefore, large differences in sm-MHC expression were observed between the treatment groups. Repeated OA challenge strongly increased total sm-MHC expression in the lung to 422 ± 28 % of sm-MHC content in saline challenged controls. Pre-treatment with tiotropium attenuated this increase to 300 ± 22 % (P<0.01), corresponding to 38 ± 6 % inhibition of the OA-induced increase. Tiotropium by itself however, had no effect on sm-MHC expression (Figure 9.6).
Figure 9.5 Effects of repeated allergen challenge and tiotropium treatment on sm-α-actin (upper panel) and sm-MHC (lower panel) expression in the guinea pig lung. Protein matched lung homogenates were Western analysed for sm-α-actin. Data shown represent densitometric means ± s.e.mean of 5 saline-challenged animals (Saline); 6 OA-challenged animals (OA); 8 tiotropium-treated, saline-challenged animals (Tio) and 7 tiotropium-treated, OA-challenged animals (OA+Tio). * P < 0.05; ** P < 0.01; *** P < 0.001. n.s.: not significant. Blots shown are representative. Each lane represents a different animal.
Effects of ovalbumin challenge and tiotropium treatment on tracheal smooth muscle contractility.

Even though OA-challenge-induced changes in ASM content were confined to non-cartilaginous airways, the observed changes in total sm-MHC expression could still allow for changes in contractility in the central airways. Indeed, repeated ovalbumin challenge enhanced methacholine-induced contraction of epithelium-denuded open-ring tracheal preparations from 2.1 ± 0.1 g in saline-challenged animals to 2.7 ± 0.2 g in ovalbumin-challenged guinea pigs (P<0.05; Figure 7). Sensitivity to methacholine, however, was not altered (pEC$_{50}$ = 6.6 ± 0.1 and 6.5 ± 0.1 for saline- and ovalbumin-challenged animals, respectively). Basal smooth muscle tone tended to be somewhat higher in ovalbumin-challenged guinea pigs, but this was not statistically significant. Pre-treatment with tiotropium slightly decreased the sensitivity to methacholine in saline-challenged animals to a pEC$_{50}$ value of 6.3 ± 0.1 (P<0.05), with no significant change in maximal contraction (1.8 ± 0.2 g). However, tiotropium pre-treatment completely prevented the increase in contractility induced by repeated OA challenge (1.6 ± 0.1 g; P<0.001; Figure 9.7).

**Figure 9.7** Effects of repeated allergen challenge and tiotropium treatment on isometric contraction of epithelium-denuded, tracheal open-ring preparations. Data shown represent means ± s.e.mean of 5 saline-challenged animals (Saline); 6 OA-challenged animals (OA); 8 tiotropium-treated, saline-challenged animals (Tio) and 7 tiotropium-treated, OA-challenged animals (OA+Tio). * P < 0.05; *** P < 0.001.

**Discussion**

The most important finding of this study is that changes in airway smooth muscle content, contractile protein expression and contractility induced by repeated allergen exposure can be partially or even fully prevented by tiotropium bromide, a long-acting muscarinic receptor antagonist, used for bronchodilation. In permanently instrumented, conscious and unrestrained guinea pigs, we have previously demonstrated that the tiotropium dose used in this study provides a sustained muscarinic receptor blockade, lasting over 96 h [20], which extends well beyond the
duration of allergen-induced early and late phase asthmatic reactions in this animal model [18]. Therefore, the effects of tiotropium described in this study are likely to represent the cumulative contribution of cholinergic activity to airway smooth muscle remodeling caused by the repeated allergen challenges. This indicates that ACh could have a major impact on the progression of airway remodeling in allergic asthma, a process that has thus far primarily been associated with mediators of inflammation and growth factors [21].

Nevertheless, inflammatory mediators and growth factors are likely to play a crucial part in the observed effects of ACh. Thus, tiotropium bromide was effective only in animals that were challenged with ovalbumin, indicating that ACh release affects structural changes in the airways in combination with allergic airway inflammation. This could, in part, be explained by augmented ACh release after allergen challenge. Thus, eosinophilic inflammation-derived polycations, such as major basic protein (MBP), are known to cause epithelial damage which can expose afferent sensory nerve endings to the airway lumen and increase vagal reflex activity in response to inhaled stimuli [22]. In addition, cholinergic afferents can be stimulated by a variety of mediators involved in allergen-induced airway inflammation (see Undem & Myers for extensive review [1]). Eosinophil-derived MBP can also increase vagally induced ACh release by inhibition of prejunctional auto-inhibitory M₂ receptors [23]. Importantly, both allergen-induced M₂ autoreceptor dysfunction and enhanced cholinergic reflex activity have been demonstrated in the guinea pig model of allergic asthma used in this study [24;25]. An additional mechanism that might contribute to increased levels of ACh after allergen exposure is its release from inflammatory and epithelial cells. This non-neuronal release of ACh may be elevated in conditions of allergic inflammation, as it was found to be increased in skin biopsies from patients with atopic dermatitis, a condition often associated with bronchial asthma [26;27].

Despite the enhanced release of ACh during allergic airway inflammation, it may be envisaged that ACh is ineffective in ASM remodeling by itself and that concerted action with mediators of inflammation and growth factors is required for the effect. This could explain the absence of tiotropium effect in the controls. Indeed, in vitro, muscarinic receptor stimulation does not or only modestly affect ASM proliferation by itself, but effectively augments growth factor-induced responses [2;3]. The latter mechanism may well be responsible for the allergen-induced increase in ASM mass observed in the non-cartilaginous airways, since an increase in cell number rather than cell size was the predominant cause of allergen-induced ASM thickening in these airways.

The mechanism of ACh-induced ASM thickening may also be relevant to airway remodeling in patients with asthma, since an increase in ASM mass in bronchial biopsies of patients with mild to moderate asthma was accompanied by a two-fold increase in cell number without a change in cell volume [28]. Other studies have indicated that hypertrophy may also contribute to increased ASM mass observed in asthmatics [29;30]. However, since no hypertrophy was observed in our model under the applied conditions, a possible role for ACh in this process remains unclear.
ASM content in the large pulmonary airways, including the main bronchi, did not change after repeated allergen challenge. Nevertheless, contractility of tracheal preparations was increased, suggesting a different nature of ASM remodeling in the central airways. This is also indicated by a previous study, demonstrating increased tracheal smooth muscle contraction after repeated allergen-challenge in guinea pigs, without concomitant changes in ASM mass [31]. These observations suggest that ASM cells in the central airways acquire a hypercontractile phenotype upon repeated allergen challenge. Moreover, the inhibitory effects of tiotropium bromide indicate that endogenous ACh contributes to the induction of this hypercontractile phenotype in vivo.

Regulation of contractile protein expression by ACh may be involved in the increased contractility, as our results demonstrate a selective increase in sm-MHC expression in the lung after allergen exposure, which was partially inhibited by tiotropium. Of note, changes in sm-MHC positive area in the non-cartilaginous airways may not be the sole cause of the increase in sm-MHC expression, since a 57% increase in area in these airways as such cannot result in a 322% increase in whole lung myosin expression. Together with the unchanged sm-MHC positive area in the larger airways, increased sm-MHC expression per ASM cell seems to be more likely. However, the localization of this increased sm-MHC expression is as yet unknown.

Sm-α-actin expression did not increase significantly after allergen exposure. The discrepancy between the expression of these contractile proteins may be explained by relatively high sm-α-actin expression by cell types other than ASM cells (e.g. fibroblasts that express sm-α-actin but not sm-MHC [32]), which is supported by the observation that sm-α-actin positive area was larger than sm-MHC positive area even in the ASM layer. In addition, it may be envisaged that sm-α-actin and sm-MHC expression can be regulated independently and to different extents. Indeed, the induction of a hypercontractile canine ASM phenotype in vitro is accompanied by a much greater increase in sm-MHC expression (± 8-fold increase) as compared to sm-α-actin (± 2-fold increase) [15].

The effects of ACh on contractility may at least partially be explained by activation of the RhoA/Rho-kinase pathway, as this pathway has been described to regulate both ASM contractility [33] and smooth muscle specific gene transcription [34]. Moreover, muscarinic M3 receptor-dependent activation of RhoA and Rho-kinase has been reported to induce smooth muscle specific gene transcription in ASM cells in vitro [35]. These RhoA-dependent effects may even be enhanced after repeated allergen exposure, which induces an increase in RhoA expression [36]. Nevertheless, prolonged (8 days) exposure of BTSM strips to high concentrations (≥ 10 µM) of methacholine results in a decline in contractility and contractile protein expression, caused by the prolonged elevation of [Ca^2+], [37]. Therefore, Rho-dependent rather than Ca^2+-dependent mechanisms are likely to regulate ACh-induced alterations in contractility in vivo.
An important consideration is how the effects of tiotropium could relate to the effectiveness of anticholinergics in the long-term treatment of asthma. In asthma, \( \beta_2 \) adrenoceptor-agonists are usually more effective bronchodilators than anticholinergics [38]. Nonetheless, \( \beta_2 \)-agonists appear to be at most modestly effective in inhibiting allergen-induced ASM proliferation \textit{in vivo} [39], despite of their effectiveness in inhibiting ASM proliferation \textit{in vitro} [40]. Moreover, chronic \( \beta_2 \)-agonist exposure have even been reported to increase airway responsiveness to acetylcholine \textit{in vivo} and \textit{ex vivo} [31]. It appears therefore that anticholinergics could be more effective than \( \beta \)-agonists in preventing allergen-induced ASM remodeling. However, the effect of chronic treatment with anticholinergics on airway remodeling, responsiveness and changes in lung function in asthmatic patients is thus far unclear.

Corticosteroids have been reported to inhibit growth factor-induced ASM proliferation, cytokine production and extracellular matrix deposition \textit{in vitro} [41-43], and to inhibit but not to reverse allergen-induced fibronectin deposition in rats \textit{in vivo} [44]. Moreover, the inhibitory effects of corticosteroids on ASM proliferation \textit{in vitro} are strongly inhibited when cells are cultured on collagen type I [45], which is increased in asthma [46]. Surprisingly, however, the effects of corticosteroids on ASM thickening has not yet been investigated in animal models. Future studies using animal models characterised by allergen-induced ASM proliferation could therefore be useful to compare different treatment strategies.

In conclusion, we have demonstrated that tiotropium bromide inhibits allergen-induced airway remodeling in a guinea pig model of ongoing asthma. Therefore, endogenous ACh appears to play an important role in ASM remodeling, a process thusfar primarily associated with mediators of inflammation and growth factors. This could have important implications for the use of anticholinergics in the treatment of allergic asthma, by protecting against the development of chronic airway hyperresponsiveness and decline of lung function in addition to their acute bronchodilating effects.

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