Eur Respir J 2007; 30: 653–661
DOI: 10.1183/09031936.00004907

Inhibition of allergen-induced airway remodelling by tiotropium and budesonide: a comparison

I.S.T. Bos*,¶, R. Gosens*,#,¶, A.B. Zuidhof*, D. Schaafsma*, A.J. Halayko#, H. Meurs* and J. Zaagsma*

ABSTRACT: Chronic inflammation in asthma and chronic obstructive pulmonary disease drives pathological structural remodelling of the airways. Using tiotropium bromide, acetylcholine was recently identified as playing a major regulatory role in airway smooth muscle remodelling in a guinea pig model of ongoing allergic asthma. The aim of the present study was to investigate other aspects of airway remodelling and to compare the effectiveness of tiotropium to the glucocorticosteroid budesonide.

Ovalbumin-sensitised guinea pigs were challenged for 12 weeks with aerosolised ovalbumin.

The ovalbumin induced airway smooth muscle thickening, hypercontractility of tracheal smooth muscle, increased pulmonary contractile protein (smooth-muscle myosin) abundance, mucous gland hypertrophy, an increase in mucin 5 subtypes A and C (MUC5AC)-positive goblet cell numbers and eosinophilia. It was reported previously that treatment with tiotropium inhibits airway smooth muscle thickening and contractile protein expression, and prevents tracheal hypercontractility. This study demonstrates that tiotropium also fully prevented allergen-induced mucous gland hypertrophy, and partially reduced the increase in MUC5AC-positive goblet cell numbers and eosinophil infiltration. Treatment with budesonide also prevented airway smooth muscle thickening, contractile protein expression, tracheal hypercontractility and mucous gland hypertrophy, and partially reduced MUC5AC-positive goblet cell numbers and eosinophilia.

This study demonstrates that tiotropium and budesonide are similarly effective in inhibiting several aspects of airway remodelling, providing further evidence that the beneficial effects of tiotropium bromide might exceed those of bronchodilation.

KEYWORDS: Airway remodelling, airway smooth muscle, anticholinergics, asthma, glucocorticosteroids, mucus hypersecretion

Acetylcholine is the primary parasympathetic neurotransmitter in the airways and an autocrine or paracrine hormone that is secreted from non-neuronal origins, including the airway epithelium and inflammatory cells [1–4]. In the respiratory system, acetylcholine is traditionally associated with inducing airway smooth muscle contraction and mucus secretion [5]; both of these effects are mediated by muscarinic receptors, with a predominant involvement of the muscarinic M_3 receptor subtype [6–9]. Parasympathetic activity is increased in obstructive airways diseases, including asthma and chronic obstructive pulmonary disease (COPD) [2]. Therefore, muscarinic receptor antagonists, such as the short-acting ipratropium bromide and long-acting tiotropium bromide, are frequently used bronchodilators for these diseases, particularly in COPD, in which vagal tone appears to be the major reversible component of airways obstruction [10].

Recent evidence indicates that the functional role of acetylcholine in the respiratory system exceeds mere induction of airway smooth muscle contraction and mucus secretion. *In vitro* studies have revealed that prolonged stimulation of muscarinic receptors enhances airway smooth muscle contractile protein expression, pro-mitogenic signalling and cell proliferation [1, 11, 12]. Moreover, muscarinic receptor stimulation induces cell proliferation of primary cultured pulmonary fibroblasts [13], and triggers the release of pro-inflammatory mediators, including leukotriene B_4, from airway smooth muscle and airway epithelial and inflammatory cells [14–17]. These

AFFILIATIONS
*Dept of Molecular Pharmacology, University of Groningen, Groningen, The Netherlands.
#Dept of Physiology, University of Manitoba, Winnipeg, MB, Canada.
¶Both authors contributed equally to this article.

CORRESPONDENCE
R. Gosens
Dept of Molecular Pharmacology
University of Groningen
Antonius Deusinglaan 1
9713 AV Groningen
The Netherlands
Fax: 31 503636908
E-mail: r.gosens@rug.nl

Received:
January 13 2007
Accepted after revision:
May 18 2007

SUPPORT STATEMENT
This study was supported by a grant (NAF 99.53) from the Netherlands Asthma Foundation (Leusden, the Netherlands). R. Gosens is currently the recipient of a Marie Curie Outgoing International Fellowship from the European Community (008823; Brussels, Belgium).

STATEMENT OF INTEREST
Statements of interest for R. Gosens and J. Zaagsma, and for the study itself can be found at www.erj.ersjournals.com/misc/statements.shtml

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

pro-inflammatory actions of acetylcholine may be enhanced in inflammatory airways diseases, since M_3 expression and function are increased on neutrophils from COPD patients [18]. Furthermore, in a previous study, evidence was found that airway smooth muscle mass, contractility and contractile protein expression were all increased in repeatedly allergen-challenged guinea pigs and that each of these features of airway smooth muscle remodelling was (partially or completely) reduced by treatment with the long-acting muscarinic receptor antagonist tiotropium bromide [19]. This indicates that acetylcholine, acting on muscarinic receptors, may contribute to the pathophysiology and pathogenesis of asthma and COPD to a much larger extent than is currently appreciated [2].

The potential anti-remodelling effects of anticholinergics on features of allergen-induced airway remodelling, other than in airway smooth muscle, have not been described to date. In addition, no studies have directly compared the anti-remodelling effects of tiotropium bromide to other treatment strategies. Therefore, the aim of the present study was to compare the effectiveness of tiotropium bromide with that of the glucocorticosteroid budesonide proprionate, and to investigate the effects of these treatment strategies on various aspects of airway remodelling, including airway smooth muscle remodelling, extracellular matrix deposition and the induction of mucus-producing cells.

MATERIALS AND METHODS

Animals

Outbred male specific-pathogen-free Dunkin–Hartley guinea pigs (Harlan, Heathfield, UK) weighing 250–300 g were sensitised to ovalbumin (OVA) using $Al(OH)_3$ as adjuvant. The animals were used experimentally 4 weeks later. All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation (University of Groningen, Groningen, the Netherlands).

Provocations were performed by inhalation of aerosolised solutions of OVA (Sigma, St Louis, MO, USA) or saline under conscious and unrestrained conditions, as described previously [20]. Allergen inhalations were discontinued when the first signs of respiratory distress were observed. No antihistaminic was needed to prevent anaphylactic shock.

Study design

Guinea pigs were challenged with either OVA or saline once weekly, for 12 consecutive weeks, to induce airway remodelling as described previously [19, 21]. For tiotropium treatment, animals received a nebulised dose of tiotropium bromide (Boehringer Ingelheim, Ingelheim, Germany) in saline (0.1 mM solution; 3min) 30 min prior to each challenge with saline or OVA. For budesonide treatment, animals received a nebulised dose of budesonide (gift of H.W. Frijlink, University of Groningen) suspended in saline supplemented with 1% Tween 80 (1 mM suspension; 15 min) 24 and 1 h prior to each challenge. Treatment groups were as follows: OVA-sensitised/saline-challenged (n=9); OVA-sensitised/OVA-challenged (n=10); OVA-sensitised/saline-challenged/tiotropium-treated (n=8); OVA-sensitised/OVA-challenged/tiotropium-treated (n=7); OVA-sensitised/saline-challenged/budesonide-treated (n=9); and OVA-sensitised/OVA-challenged/budesonide-treated (n=7). For all of the tiotropium-treated animals and for some of the OVA-sensitised/saline-challenged (n=5) and OVA-sensitised/OVA-challenged (n=6) animals, lung material stored from a previous study [19] was used for the histological analyses. For OVA-sensitised/saline-challenged and OVA-sensitised/OVA-challenged animals, data generated from previously stored and newly obtained lung material was pooled to produce a single OVA-sensitised/saline-challenged control group and a single OVA-sensitised/OVA-challenged control group. During the 12-week challenge protocol, guinea pig weight was monitored weekly; no differences in weight gain between treatment groups were found.

Tissue acquisition

Guinea pigs were sacrificed by experimental concussion followed by rapid exsanguination 24 h after the last challenge. The lungs were immediately resected and kept on ice for further processing. The trachea was removed and transferred to a Krebs–Henseleit solution (37°C) saturated with 5% carbon dioxide/95% oxygen, composed as follows (mM): NaCl 117.5; KCl 5.6; $MgSO_4$ 1.18; $CaCl_2$ 2.5; NaH_2PO_4 1.28; $NaHCO_3$ 25.0; and glucose 5.5, (pH 7.4).

Histochemistry

Transverse cross-sections of the main bronchi from both right and left lung lobes were used for morphometric analyses; the sections were stained in order to identify their various components. Smooth muscle was identified using immunohistochemical staining for smooth-muscle-specific myosin heavy chain (SM-MHC; Neomarkers, Fremont, CA, USA), extracellular matrix was identified using Masson trichrome staining, and mucus-producing cells were identified using periodic acid–Schiff (PAS) staining or immunohistochemical staining for mucin 5 subtypes A and C (MUC5AC; Neomarkers). The antibody used for MUC5AC immunohistochemistry had previously been shown to be cross-reactive with guinea pig MUC5AC [22]. Primary antibodies were visualised using horseradish-peroxidase-linked secondary antibodies and diaminobenzidine. Eosinophils were identified in haematoxylin-and-eosin-stained lung sections. Airways within sections were digitally photographed and subclassified as cartilaginous or noncartilaginous. All immunohistochemical measurements were carried out digitally using quantification software [23].

Western analyses

Lung homogenates were prepared as described previously [19]. Protein lysates were separated by sodium dodecylsulphate-polyacrylamide gel electophoresis, followed by standard immunoblotting techniques. Antibodies were visualised using enhanced chemiluminescence (Pierce, Rockford, IL, USA). Photographs of blots were analysed densitometrically (Totallab^{TM}; Nonlinear Dynamics, Newcastle, UK).

Isometric tension measurements

Isometric concentration experiments were performed as described previously [19]. Briefly, the trachea was prepared free of serous connective tissue. Single open-ring epithelium-denuded preparations were mounted for isometric recording in organ baths containing Krebs–Henseleit solution at 37°C and saturated with 5% carbon dioxide/95% oxygen. After equilibration, the resting tension was adjusted to 0.5 g; this was

followed by pre-contractions using 20 and 40 mM KCl. Following wash-outs and another equilibration period of 30 min, cumulative concentration–response curves were constructed using methacholine.

Data analysis

All data are presented as mean±SEM. Unless otherwise specified, statistical differences between means were calculated using one-way ANOVA, followed by a Newman–Keuls multiple comparisons test. Differences between means were considered to be significant at a p-value of <0.05.

RESULTS

Airway smooth muscle

In a previous study, it was found that repeated allergen inhalation increased airway smooth muscle mass, pulmonary contractile protein expression and the contractility of tracheal smooth muscle, all indicative of airway smooth muscle remodelling [19]. These changes were partially to fully prevented by treatment with tiotropium bromide. Since the data on the effects of tiotropium on airway smooth muscle remodelling have already been published [19], these data are summarised in table 1.

The same types of experiment were planned in order to investigate the effects of budesonide treatment. Similar to the previous report, repeated OVA challenges increased airway smooth muscle mass in the noncartilaginous airways, with no changes in airway smooth muscle mass in the large cartilaginous airways (fig. 1). Airway smooth muscle mass was quantified using an antibody specific to SM-MHC, a stringent marker for the contractile airway smooth muscle phenotype [24]. Compared to saline-challenged controls, the SM-MHC-positive area increased by 66±15% after repeated allergen exposure ($p<0.001$). This increase was completely prevented by treatment with budesonide (fig. 1). Budesonide was more effective in reducing smooth muscle thickening than tiotropium, which partially prevented the increase in SM-MHC-positive area, by 76%, as previously reported (table 1) [19].

In keeping with these observations, and in agreement with the previous report [19], Western analysis demonstrated a marked 4.8±0.7-fold increase in pulmonary SM-MHC expression in the OVA-challenged animals (fig. 2). As observed for airway smooth muscle thickness, this increase was almost completely reversed by budesonide treatment. Budesonide did not significantly reduce the SM-MHC-positive area and pulmonary SM-MHC expression in saline-challenged animals. The effects of budesonide on pulmonary SM-MHC expression in the OVA-challenged animals were more pronounced than the inhibitory effects of tiotropium, which partially reduced the allergen-induced increase, by 38%, as previously reported (table 1) [19].

Furthermore, repeated OVA exposure increased the contractility of tracheal smooth muscle by 32±1% compared to saline-challenged animals (fig. 3). Although budesonide treatment alone had no effect compared to saline-treated animals, it fully reversed the OVA-induced increase in contractility (fig. 3). Tiotropium treatment also fully reversed the OVA-induced increase in tracheal contractility and had additional beneficial effects, since it reduced maximal contraction, even in saline-challenged animals, as previously reported (table 1) [19]. Collectively, these results indicate that budesonide and tiotropium are both effective in reducing allergen-induced airway smooth muscle remodelling in guinea pigs.

Extracellular matrix

Aberrant extracellular matrix deposition is observed in the airways of asthma and COPD patients [25, 26]. Therefore, experiments aimed at quantifying extracellular matrix deposition within the guinea pig airway wall were performed. Extracellular matrix levels, determined using Masson trichrome staining, did not differ between OVA- and saline-challenged animals, in either cartilaginous or noncartilaginous

TABLE 1 Comparison of the anti-remodelling effects of no treatment, tiotropium and budesonide following saline or ovalbumin (OVA) challenge in guinea pigs

	No treatment		Tiotropium		Budesonide	
	Saline	OVA	Saline	OVA	Saline	OVA
Airway smooth muscle mass	0	++++	0	+	0	0
Pulmonary contractile protein expression	0	++++	0	++	0	0
Tracheal contractility	0	++++	-	-	0	0
Mucous gland hypertrophy	0	++++	0	0	0	0
Goblet cell number#	0	++++	-	+++	-	0
Goblet cell MUC5AC expression	0	++++	0	++	0	++
Airway eosinophilia	0	++++	0	++	0	+
Total	0	28	-2	9	-1	3

Both tiotropium and budesonide were highly effective at preventing remodelling features in these animals. Data on the effects of tiotropium on airway smooth muscle mass, pulmonary contractile protein expression and tracheal contractility were from [19]. MUC5AC: mucin 5 subtypes A and C. -: treatment effect less than that of saline-challenge; 0: complete inhibition of OVA effect (equivalent to saline challenge); +/scored 1: ⩽75% inhibition; ++/scored 2: ⩽50% inhibition; +++/scored 3: ⩽25% inhibition; ++++/scored 4: maximal allergen-induced remodelling. #: the effect of OVA on goblet cell number was not significant (see Mucus-producing cells section).

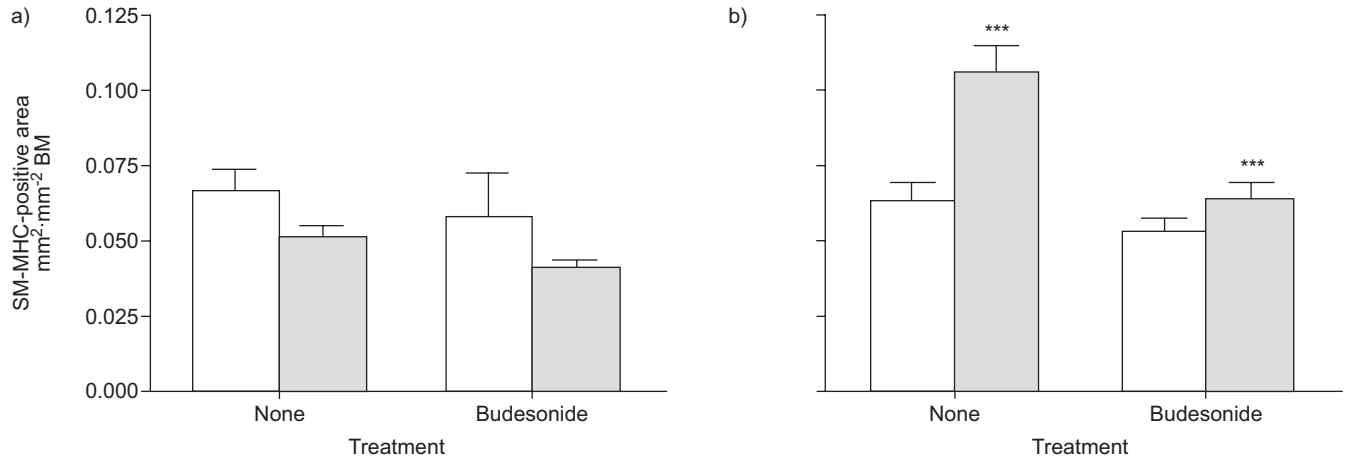

FIGURE 1. Effects of repeated allergen challenge and budesonide treatment on airway smooth muscle mass in the guinea pig lung in: a) cartilaginous airways (basement membrane (BM) length 1.82±0.08 mm); and b) noncartilaginous airways (BM length 0.74±0.02 mm). Airways were identified in smooth-muscle-specific myosin heavy chain (SM-MHC)-stained lung sections, and the SM-MHC-positive area was analysed morphometrically. □: saline; ■: ovalbumin. Data are presented as mean±SEM. Six to eight airways of each classification were analysed for each animal (animal numbers as detailed in Study design section). ***: $p<0.001$ *versus* saline challenge.

airways. Tiotropium and budesonide treatment were also without effect on matrix deposition in the airway wall (fig. 4).

Mucus-producing cells

Mucus hypersecretion is a pathological feature, seen in both asthma and COPD, which contributes significantly to airflow limitation [27, 28]. Mucus hypersecretion, in these patients, is accompanied by mucous gland hypertrophy and goblet cell hyperplasia [27, 28]. Therefore, mucus-producing cells in lung sections were quantified using PAS staining. Goblet cells within the airway epithelium and submucosal mucous glands were positive for this staining. Mucous glands and goblet cells were predominantly found in the cartilaginous airways; therefore, only cartilaginous airways were assessed in these experiments. Repeated OVA exposure induced a marked 45±7% increase in mucous gland area. Although tiotropium bromide and budesonide had no effect in saline-challenged animals, both treatments completely prevented allergen-induced mucous gland hypertrophy (fig. 5a). Repeated allergen exposure also tended to induce an increase in total goblet cell number in the guinea pigs; however, this difference did not reach significance ($p=0.06$). Interestingly, budesonide treatment partially reduced total goblet cell number, irrespective of subsequent allergen challenge; tiotropium bromide also reduced goblet cell number in saline-challenged animals (fig. 5b).

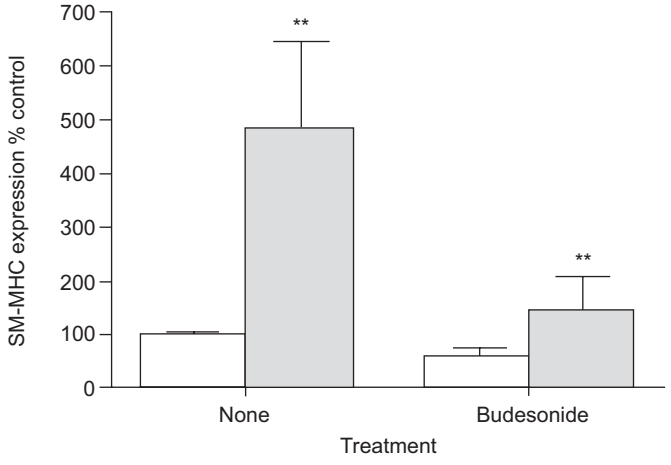

FIGURE 2. Effects of repeated allergen challenge and budesonide treatment on smooth-muscle-specific myosin heavy chain (SM-MHC) expression in the guinea pig lung. Lung tissue homogenates were analysed by Western blotting for SM-MHC and β-actin (loading control) expression. Mean SM-MHC expression was normalised to β-actin and that in untreated saline-challenged animals (control) set to 100%. □: saline; ■: ovalbumin. Data are presented as mean±SEM. **: $p<0.01$ *versus* saline challenge.

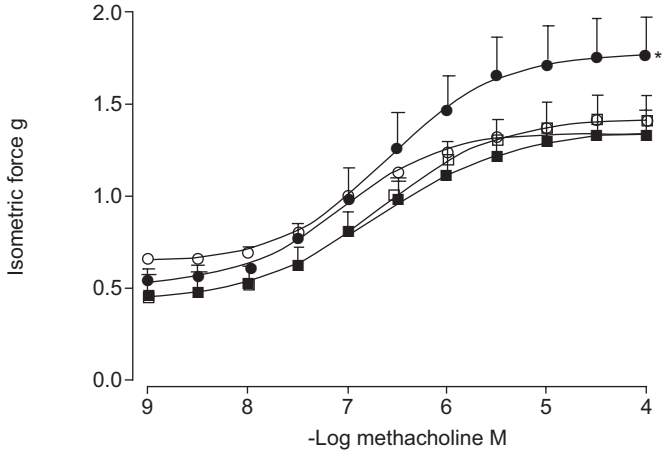

FIGURE 3. Effects of repeated allergen challenge (○ and □: saline; ● and ■: ovalbumin) and budesonide treatment (○ and ●: no treatment; □ and ■: budesonide treatment) on tracheal contractility. The methacholine dose–response relationship of open single-ring epithelium-denuded guinea-pig tracheal rings was determined and fitted to a four-parameter sigmoidal equation. Data are presented as mean±SEM. Three tracheal rings were analysed per animal (animal numbers as detailed in Study design section). *: $p<0.05$ *versus* saline challenge and *versus* ovalbumin/budesonide.

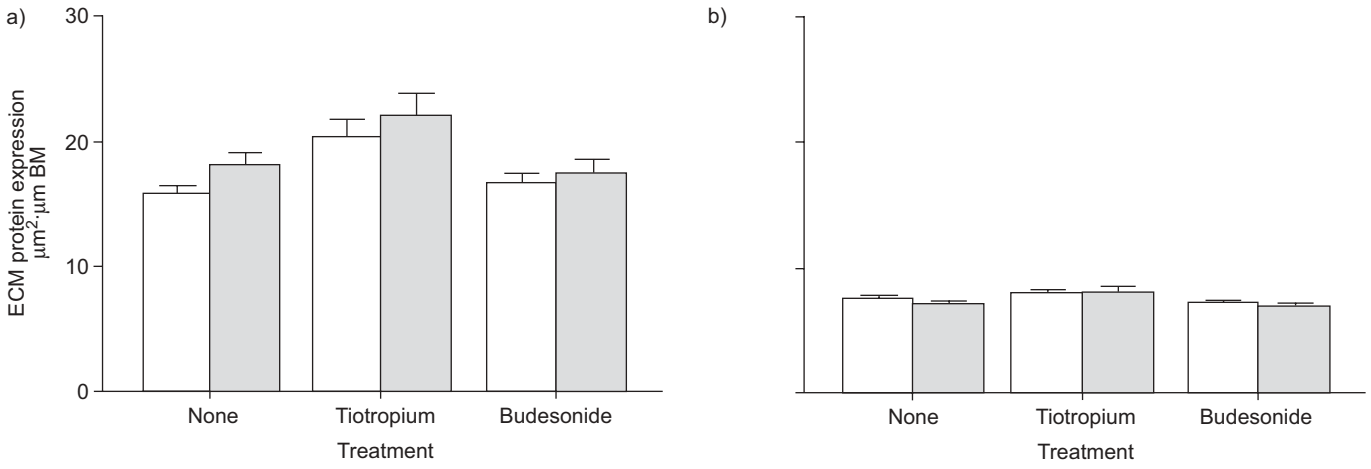

FIGURE 4. Effects of repeated allergen challenge and tiotropium or budesonide treatment on extracellular matrix (ECM) protein expression in the guinea pig lung in: a) cartilaginous; and b) noncartilaginous airways. ECM was identified in Masson-trichrome-stained lung sections, and the Masson-positive area was analysed morphometrically. □: saline; ■: ovalbumin. BM: basement membrane. Data are presented as mean±SEM. Four to six airways of each classification were analysed for each animal (animal numbers as detailed in Study design section).

The relatively small effects of OVA on goblet cell hyperplasia could relate to the large number of goblet cells that are present in guinea pig airways, even in control saline-challenged animals, possibly masking further induction by allergen. Therefore, additional experiments were directed towards quantification of MUC5AC staining in the airway epithelium. MUC5AC is highly expressed during mucous differentiation in culture [29], and is reported to be almost undetectable in the airways of control rats, despite the presence of goblet cells [30]. Based on these studies, it was hypothesised that MUC5AC would be a more sensitive marker than PAS stain for measuring mucous differentiation within the airway epithelium. Indeed, histochemistry indicated MUC5AC expression to be almost absent in saline-challenged guinea pig airways, whereas this glycoprotein was highly induced in OVA-challenged animals (fig. 6). Budesonide and tiotropium treatment did not change MUC5AC-positive goblet cell number in saline-challenged animals, but partially reduced MUC5AC induction by OVA (fig. 6b). Collectively, these data indicate that budesonide and tiotropium are equally effective in reducing allergen-induced remodelling of mucus-producing cells in the airways.

Airway eosinophilia

OVA challenge induced eosinophil influx primarily in the submucosal (fig. 7) and adventitial compartments of the cartilaginous airways, compared to airway smooth muscle (table 2). Similar results were obtained for noncartilaginous airways. Tiotropium had no effect on airway eosinophil number in saline-challenged animals, but partially prevented eosinophilia in submucosal compartments of cartilaginous and noncartilaginous airways in OVA-challenged animals; budesonide treatment had similar effects (fig. 7). Comparable inhibitory effects on eosinophilia for tiotropium and budesonide were obtained for the airway smooth muscle and adventitial compartments of both airway classifications (table 2).

DISCUSSION

In combination with the previous study [19], the results of the present study demonstrate that the long-acting anticholinergic agent tiotropium bromide prevents several aspects of allergen-induced airway remodelling in guinea pigs, including airway smooth muscle thickening, increased pulmonary contractile protein expression, hypercontractility of tracheal smooth muscle, mucous gland hypertrophy, MUC5AC expression by goblet cells and airway eosinophilia. Collectively, it appears that endogenous acetylcholine, acting on muscarinic receptors, plays a broad role in the chronic pathology of allergic airways disease, and that targeting these effects with anticholinergics holds some promise in the treatment of asthma-associated pathophysiology.

The present results also show that the anti-remodelling effects of tiotropium bromide are very similar to those of budesonide. For comparison of these drugs, the effects reported in the previous and current studies are summarised in table 1. Clearly, both tiotropium bromide and budesonide proprionate are effective in preventing airway smooth muscle remodelling in allergen-challenged guinea pigs, although some small differences exist. Although the potential for quantitative comparison of the two drugs is limited in the absence of detailed dose–response relationships, at the concentrations of tiotropium and budesonide used in the present study, budesonide treatment appeared to be more effective in preventing airway smooth muscle thickening in the noncartilaginous airways; in addition, budesonide treatment abrogated the increase in contractile protein accumulation, whereas the inhibitory effect of tiotropium bromide was partial. Conversely, tiotropium bromide reduced the contractility of tracheal smooth muscle to a greater extent than did budesonide. The effects of these drugs on mucous gland hypertrophy and MUC5AC expression were comparable. Collectively, the data indicate that budesonide and tiotropium are similarly effective in preventing allergen-induced airway remodelling. It is currently unclear, however, whether or not

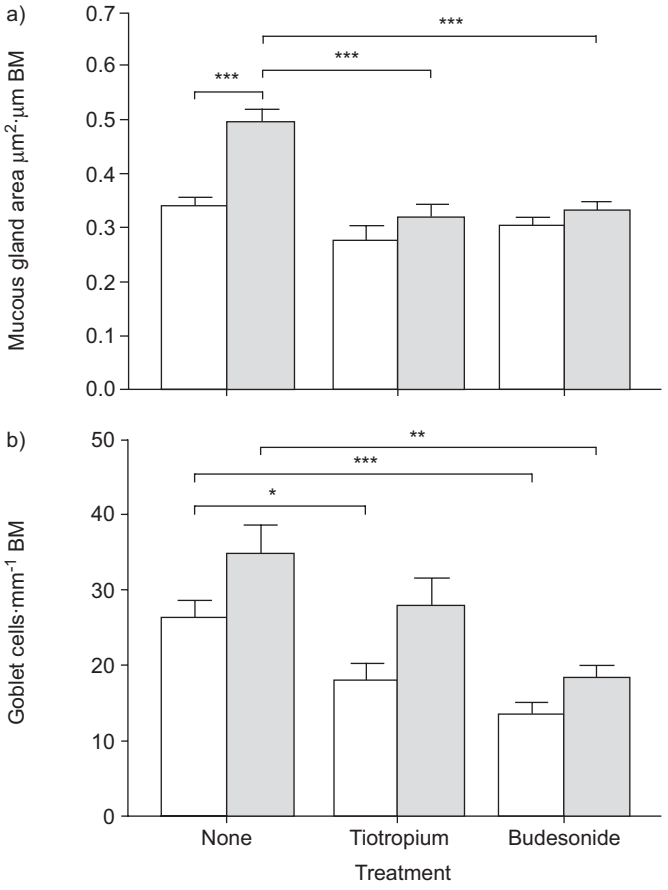

FIGURE 5. Effects of repeated allergen challenge and tiotropium or budesonide treatment on: a) mucous gland area; and b) total goblet cell number in intrapulmonary cartilaginous airways. Mucus-producing cells were identified in periodic acid–Schiff (PAS)-stained lung sections. For submucosal mucous glands, the PAS-positive area was analysed morphometrically; for goblet cell number, epithelial cells positive for PAS staining were counted. □: saline; ■: ovalbumin. BM: basement membrane. Data are presented as mean±SEM. Four to eight airways were analysed for each animal (animal numbers as detailed in Study design section). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

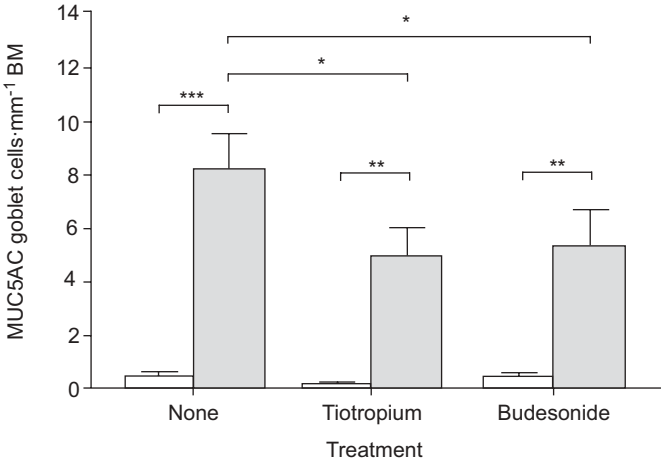

FIGURE 6. Effects of repeated allergen challenge and tiotropium or budesonide treatment on mucin 5 subtypes A and C (MUC5AC)-positive goblet cell number in the intrapulmonary cartilaginous airways. MUC5AC-positive cells were identified immunohistochemically. □: saline; ■: ovalbumin. BM: basement membrane. Data are presented as mean±SEM. Two to four airways were analysed for each airways (animal numbers as detailed in Study design section). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

tiotropium is also effective in reversing established airway structural changes. The corticosteroid fluticasone effectively prevents airway remodelling in Brown Norway rats, but does not adequately reverse established airway structural changes in the same animal model [31]. This discrepancy may explain why corticosteroids are generally not fully effective in reversing airway structural changes in human asthma [32]. Clearly, future studies are warranted in order to investigate the potential effects of tiotropium on established airway remodelling.

The effects of repeated allergen challenge on airway smooth muscle remodelling were dependent upon airway size. In the small noncartilaginous airways, airway smooth muscle thickening was observed, which was not seen in the large cartilaginous airways. Nonetheless, tracheal smooth muscle contractility was increased, suggesting that airway smooth muscle remodelling in this model is characterised by an airway-generation-dependent combination of airway smooth muscle thickening and phenotype maturation. The allergen-induced changes in pulmonary SM-MHC expression support this contention, since the ~65% increase in airway smooth muscle in the noncartilaginous airways is not sufficient to account for the approximately four-fold increase in total pulmonary smooth-myosin. This indicates that the expression of SM-MHC per smooth muscle cell must have increased, hence explaining the tracheal hypercontractility at similar smooth muscle mass.

The results of the present study provide important novel insights into the mechanisms that regulate mucous gland hypertrophy and MUC5AC expression by goblet cells in response to allergen challenge. The present finding that these pathologies are prevented by tiotropium bromide suggest an important regulatory role for muscarinic receptors in the remodelling of mucus-producing cells. This is the first study to demonstrate that mucous gland remodelling and MUC5AC expression in response to allergen are regulated by endogenous acetylcholine. Tiotropium had no effect on mucous glands or MUC5AC expression in healthy airways, suggesting that the pathophysiological remodelling induced by acetylcholine is conditional on the presence of airway inflammation. This is supported by the results obtained using the anti-inflammatory drug budesonide, which produced strikingly similar effects. Furthermore, tiotropium bromide prevented airway eosinophilia in allergen-challenged animals. It is possible that acetylcholine promotes the release of chemokines and cytokines from airway structural cells, as reported for airway epithelial cells and airway smooth muscle [14–16], in order to attract inflammatory cells to the airways, and functionally interacts with the growth factors or cytokines that are released by these cells to induce direct remodelling effects on structural target cells, similar to what has been observed for airway smooth muscle remodelling [2, 11]. Although transactivation of the epidermal growth factor receptor by muscarinic receptors has been reported in conjunctival goblet cells [33], no detailed

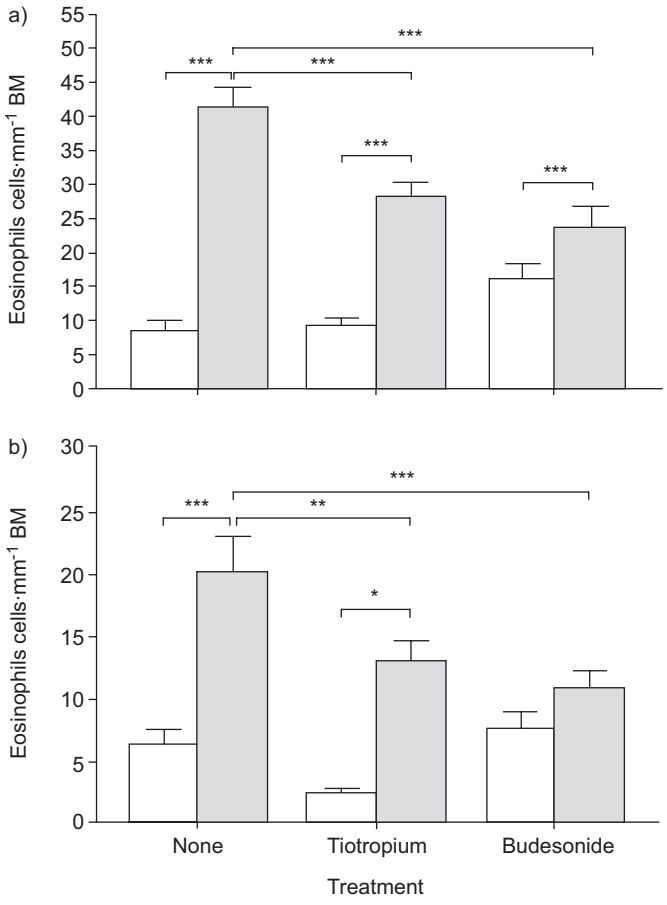

FIGURE 7. Effects of repeated allergen challenge and tiotropium or budesonide treatment on eosinophilia in the submucosal compartments of: a) cartilaginous; and b) noncartilaginous guinea pig airways. Eosinophils were identified using haematoxylin–eosin stain. □: saline; ■: ovalbumin. BM: basement membrane. Data are presented as mean±SEM. Five to six airways of each classification were analysed for each animal (animal numbers as detailed in Study design section). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

molecular studies investigating the role of muscarinic receptors in the proliferation and hypertrophy of airway mucous glands exist. Future molecular and cellular studies are needed in order to clarify the intracellular signalling mechanisms underpinning the muscarinic receptor response.

The anti-remodelling effects of tiotropium bromide were most profound for mucous glands and airway smooth muscle. Both are innervated by the vagal nerve, particularly in the proximal airways, and express muscarinic receptors [2]. In addition, tiotropium bromide also partially prevented MUC5AC expression by airway goblet cells, and tended to reduce goblet cell number in saline- and OVA-challenged animals. Innervation of airway epithelial cells, including goblet cells, varies between species [34]; however, these cells are known to express the acetylcholine-synthesising enzyme choline acetyltransferase, transporter molecules for the excretion of acetylcholine and acetylcholine itself [35–38]. It is unclear whether the acetylcholine involved in the allergen-induced mucous differentiation of the airway epithelium is neuronal or non-neuronal in origin. However, *in vitro* and *ex vivo* studies that demonstrate effects of muscarinic receptors on lung fibroblast proliferation [13] and airway inflammation [15–18] and the present observations that tiotropium reduced airway eosinophilia raise the real possibility that non-neuronal acetylcholine in the airways may be a determinant of airway remodelling in asthma and COPD; future studies are clearly indicated in this area.

Repeated allergen challenge did not induce a measurable change in Masson trichrome staining within the airway wall, either in the cartilaginous or noncartilaginous airways. However, since muscarinic receptors mediate proliferation of human lung fibroblasts [13], the possibility exists that this receptor system modulates matrix production within the airway wall of patients to some extent. The potential effects of anticholinergic drugs on extracellular matrix deposition within the airway wall, therefore, require further investigation.

The present results obtained with budesonide confirm earlier *in vivo* and *in vitro* findings that demonstrate preventive effects of

TABLE 2 Effects on infiltration of eosinophils into different compartments of the airway wall of no treatment, tiotropium and budesonide following saline or ovalbumin (OVA) challenge

	No treatment cells·mm^{-1} BM		Tiotropium cells·mm^{-1} BM		Budesonide cells·mm^{-1} BM	
	Saline	OVA	Saline	OVA	Saline	OVA
Cartilaginous						
Submucosa	8.5±1.5	41.1±3.0***	9.3±1.1	28.2±2.0***,###	16.2±2.2	23.5±3.2***,###
Airway smooth muscle	0.1±0.1	0.9±0.2***	0.3±0.1	0.7±0.1*	0.5±0.1	0.5±0.1
Adventitia	10.0±0.9	45.0±3.5***	7.9±0.9	31.4±3.8***,##	20.8±4.7	32.0±3.1***,##
Noncartilaginous						
Submucosa	6.3±1.2	20.1±2.9***	2.3±0.4	13.0±1.6*,##	7.6±1.2	10.8±1.4###
Airway smooth muscle	0.0±0.0	1.7±0.5***	0.1±0.1	0.5±0.2###	0.0±0.0	0.3±0.2###
Adventitia	24.2±3.2	59.9±6.4***	10.6±0.7	51.0±4.2***	21.7±3.9	33.7±2.8###

Data are presented as mean±SEM (five or six airways of each classification per animal were analysed). Eosinophils were identified using haematoxylin–eosin stain. BM: basement membrane. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ *versus* saline-challenged animals; #: $p<0.05$; +: $p<0.01$; ###: $p<0.001$ *versus* OVA-challenged controls.

glucocorticosteroids on airway smooth muscle thickening [39]. The mechanisms that explain the antiproliferative effects of glucocorticosteroids on airway smooth muscle have not yet been completely elucidated, but appear to involve inhibition of the cell cycle regulatory protein cyclin D1 and stimulation of the cell cycle inhibitory protein $p21^{Waf1/Cip1}$ [39–41]. In addition, the present study provides some novel insights into the actions of glucocorticosteroids, as it has been demonstrated that budesonide is effective in preventing contractile protein expression and hypercontractility of tracheal smooth muscle. This suggests that glucocorticosteroid therapy prevents airway smooth muscle phenotype maturation *in vivo*, which fits with recent studies using cultured human airway smooth muscle cells, showing that glucocorticosteroids inhibit contractile protein accumulation [42]. The mechanisms that are targeted by glucocorticosteroids have not yet been investigated *in vitro* and require investigation.

Budesonide prevented remodelling of mucus-producing cells in the present model. Inhibitory effects of budesonide on allergen-induced goblet cell hyperplasia have been described in mice [43]. Although budesonide reduced goblet cell number in guinea pigs, this effect appeared independent of subsequent allergen challenge. This was also observed for tiotropium, which reduced goblet cell number in saline-challenged animals. Guinea pigs express high numbers of goblet cells, even under conditions of saline-challenge; apparently, basal goblet cell number can be modulated by targeting glucocorticoid and muscarinic receptors, suggesting the involvement of constitutive chemokine/cytokine and acetylcholine release in maintaining goblet cell number in guinea pigs. Budesonide also prevented the allergen-induced increase in MUC5AC-positive goblet cells and mucous gland area. Since airway inflammation is key to the development of mucus hypersecretion [44], the inhibitory actions of budesonide may not be entirely unexpected. In addition, direct effects of glucocorticosteroids on mucin gene expression have been reported *in vitro* [45]. In combination with the results obtained using tiotropium bromide, these data indicate that glucocorticosteroids and anticholinergic drugs may both be effective in preventing remodelling of mucus-producing cells in allergic airways disease.

In conclusion, the results of the present study demonstrate that tiotropium effectively prevents multiple features of airway remodelling in repeatedly allergen-challenged guinea pigs. This indicates an important regulatory role for acetylcholine, acting through muscarinic receptors, in the pathophysiology of allergic airways disease. The anti-remodelling effects of tiotropium are comparable to those of the glucocorticosteroid budesonide. Thus the beneficial therapeutic effects of anticholinergic drugs, such as tiotropium bromide, may exceed their bronchodilatory effects, and might reduce airway remodelling and lung function decline in patients suffering from chronic airways diseases.

REFERENCES

1 Gosens R, Zaagsma J, Grootte Bromhaar M, Nelemans A, Meurs H. Acetylcholine: a novel regulator of airway smooth muscle remodelling? *Eur J Pharmacol* 2004; 500: 193–201.
2 Gosens R, Zaagsma J, Meurs H, Halayko AJ. Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respir Res* 2006; 7: 73.
3 Racke K, Matthiesen S. The airway cholinergic system: physiology and pharmacology. *Pulm Pharmacol Ther* 2004; 17: 181–198.
4 Racke K, Juergens UR, Matthiesen S. Control by cholinergic mechanisms. *Eur J Pharmacol* 2006; 533: 57–68.
5 Belmonte KE. Cholinergic pathways in the lungs and anticholinergic therapy for chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005; 2: 297–304; discussion 311–312.
6 Roffel AF, Elzinga CR, Van Amsterdam RG, De Zeeuw RA, Zaagsma J. Muscarinic M_2 receptors in bovine tracheal smooth muscle: discrepancies between binding and function. *Eur J Pharmacol* 1988; 153: 73–82.
7 Roffel AF, Elzinga CR, Zaagsma J. Muscarinic M_3 receptors mediate contraction of human central and peripheral airway smooth muscle. *Pulm Pharmacol* 1990; 3: 47–51.
8 Ramnarine SI, Haddad EB, Khawaja AM, Mak JC, Rogers DF. On muscarinic control of neurogenic mucus secretion in ferret trachea. *J Physiol* 1996; 494: 577–586.
9 Ishihara H, Shimura S, Satoh M, *et al.* Muscarinic receptor subtypes in feline tracheal submucosal gland secretion. *Am J Physiol* 1992; 262: L223–L228.
10 Gross NJ, Skorodin MS. Role of the parasympathetic system in airway obstruction due to emphysema. *N Engl J Med* 1984; 311: 421–425.
11 Gosens R, Nelemans SA, Grootte Bromhaar MM, McKay S, Zaagsma J, Meurs H. Muscarinic M_3-receptors mediate cholinergic synergism of mitogenesis in airway smooth muscle. *Am J Respir Cell Mol Biol* 2003; 28: 257–262.
12 Liu HW, Kassiri K, Voros A, *et al.* Gaq-receptor coupled signaling induces RHO-dependent transcription of smooth muscle specific genes in cultured canine airway myocytes. *Am J Respir Crit Care Med* 2002; 165: A670.
13 Matthiesen S, Bahulayan A, Kempkens S, *et al.* Muscarinic receptors mediate stimulation of human lung fibroblast proliferation. *Am J Respir Cell Mol Biol* 2006; 35: 621–627.
14 Kanefsky J, Lenburg M, Hai CM. Cholinergic receptor and cyclic stretch-mediated inflammatory gene expression in intact ASM. *Am J Respir Cell Mol Biol* 2006; 34: 417–425.
15 Koyama S, Rennard SI, Robbins RA. Acetylcholine stimulates bronchial epithelial cells to release neutrophil and monocyte chemotactic activity. *Am J Physiol* 1992; 262: L466–L471.
16 Koyama S, Sato E, Nomura H, Kubo K, Nagai S, Izumi T. Acetylcholine and substance P stimulate bronchial epithelial cells to release eosinophil chemotactic activity. *J Appl Physiol* 1998; 84: 1528–1534.
17 Sato E, Koyama S, Okubo Y, Kubo K, Sekiguchi M. Acetylcholine stimulates alveolar macrophages to release inflammatory cell chemotactic activity. *Am J Physiol* 1998; 274: L970–L979.
18 Profita M, Giorgi RD, Sala A, *et al.* Muscarinic receptors, leukotriene B_4 production and neutrophilic inflammation in COPD patients. *Allergy* 2005; 60: 1361–1369.
19 Gosens R, Bos IS, Zaagsma J, Meurs H. Protective effects of tiotropium bromide in the progression of airway smooth muscle remodeling. *Am J Respir Crit Care Med* 2005; 171: 1096–1102.

20 Meurs H, Santing RE, Remie R, *et al.* A guinea pig model of acute and chronic asthma using permanently instrumented and unrestrained animals. *Nat Protoc* 2006; 1: 840–847.

21 Wang ZL, Walker BA, Weir TD, *et al.* Effect of chronic antigen and β_2 agonist exposure on airway remodeling in guinea pigs. *Am J Respir Crit Care Med* 1995; 152: 2097–2104.

22 Chorley BN, Crews AL, Li Y, Adler KB, Minnicozzi M, Martin LD. Differential Muc2 and Muc5ac secretion by stimulated guinea pig tracheal epithelial cells *in vitro*. *Respir Res* 2006; 7: 35.

23 National Institutes of Health, ImageJ. Image Processing and Analysis in Java. http://rsb.info.nih.gov/ij/index.html Date last updated: July 23, 2007. Date last accessed: August 7, 2007.

24 Halayko AJ, Stelmack GL, Yamasaki A, McNeill K, Unruh H, Rector E. Distribution of phenotypically disparate myocyte subpopulations in airway smooth muscle. *Can J Physiol Pharmacol* 2005; 83: 104–116.

25 Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. *Am J Respir Crit Care Med* 2001; 164: S28–S38.

26 Postma DS, Timens W. Remodeling in asthma and chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2006; 3: 434–439.

27 Rogers DF. Mucus hypersecretion in chronic obstructive pulmonary disease. *Novartis Found Symp* 2001; 234: 65–83.

28 Rogers DF. Airway mucus hypersecretion in asthma: an undervalued pathology? *Curr Opin Pharmacol* 2004; 4: 241–250.

29 Guzman K, Bader T, Nettesheim P. Regulation of MUC5 and MUC1 gene expression: correlation with airway mucous differentiation. *Am J Physiol* 1996; 270: L846–L853.

30 Lou YP, Takeyama K, Grattan KM, *et al.* Platelet-activating factor induces goblet cell hyperplasia and mucin gene expression in airways. *Am J Respir Crit Care Med* 1998; 157: 1927–1934.

31 Vanacker NJ, Palmans E, Kips JC, Pauwels RA. Fluticasone inhibits but does not reverse allergen-induced structural airway changes. *Am J Respir Crit Care Med* 2001; 163: 674–679.

32 Ward C, Walters H. Airway wall remodelling: the influence of corticosteroids. *Curr Opin Allergy Clin Immunol* 2005; 5: 43–48.

33 Kanno H, Horikawa Y, Hodges RR, *et al.* Cholinergic agonists transactivate EGFR and stimulate MAPK to induce goblet cell secretion. *Am J Physiol Cell Physiol* 2003; 284: C988–C998.

34 Rogers DF. Motor control of airway goblet cells and glands. *Respir Physiol* 2001; 125: 129–144.

35 Wessler IK, Kirkpatrick CJ. The non-neuronal cholinergic system: an emerging drug target in the airways. *Pulm Pharmacol Ther* 2001; 14: 423–434.

36 Proskocil BJ, Sekhon HS, Jia Y, *et al.* Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. *Endocrinology* 2004; 145: 2498–2506.

37 Lips KS, Volk C, Schmitt BM, *et al.* Polyspecific cation transporters mediate luminal release of acetylcholine from bronchial epithelium. *Am J Respir Cell Mol Biol* 2005; 33: 79–88.

38 Kummer W, Wiegand S, Akinci S, *et al.* Role of acetylcholine and polyspecific cation transporters in serotonin-induced bronchoconstriction in the mouse. *Respir Res* 2006; 7: 65.

39 Halayko AJ, Tran T, Ji SY, Yamasaki A, Gosens R. Airway smooth muscle phenotype and function: interactions with current asthma therapies. *Curr Drug Targets* 2006; 7: 525–540.

40 Fernandes D, Guida E, Koutsoubos V, *et al.* Glucocorticoids inhibit proliferation, cyclin D1 expression, and retinoblastoma protein phosphorylation, but not activity of the extracellular-regulated kinases in human cultured airway smooth muscle. *Am J Respir Cell Mol Biol* 1999; 21: 77–88.

41 Roth M, Johnson PR, Borger P, *et al.* Dysfunctional interaction of C/EBPα and the glucocorticoid receptor in asthmatic bronchial smooth-muscle cells. *N Engl J Med* 2004; 351: 560–574.

42 Goldsmith A, Hershenson MB, Wolbert MP, Bentley JK. Regulation of airway smooth muscle α-actin expression by glucocorticoids. *Am J Physiol Lung Cell Mol Physiol* 2006; 292: L99–L106.

43 McMillan SJ, Xanthou G, Lloyd CM. Therapeutic administration of budesonide ameliorates allergen-induced airway remodelling. *Clin Exp Allergy* 2005; 35: 388–396.

44 Rogers DF, Barnes PJ. Treatment of airway mucus hypersecretion. *Ann Med* 2006; 38: 116–125.

45 Kai H, Yoshitake K, Hisatsune A, *et al.* Dexamethasone suppresses mucus production and MUC-2 and MUC-5AC gene expression by NCI-H292 cells. *Am J Physiol* 1996; 271: L484–L488.