ERJ Express. Published on May 30, 2007 as doi: 10.1183/09031936.00004907

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

I. Sophie T. Bos [1,*], Reinoud Gosens [1,2,*], Annet B. Zuidhof [1], Dedmer Schaafsma [1], Andrew J. Halayko [2], Herman Meurs [1] & Johan Zaagsma [1]

* Both authors contributed equally and can be cited in either order

1 Department of Molecular Pharmacology, University of Groningen, Groningen, the Netherlands

2 Department of Physiology, University of Manitoba, Winnipeg, Canada

Author for correspondence:

Reinoud Gosens

Department of Molecular Pharmacology, University of Groningen,

Antonius Deusinglaan 1, 9713 AV Groningen, the Netherlands

Tel: + 31 50 363 3321

Fax: +31 50 363 6908

Email: r.gosens@rug.nl

Running head: tiotropium and airway remodelling

Word count: 2,904

Abstract

Chronic inflammation in asthma and COPD drives pathological structural remodelling of the airways. Using tiotropium bromide, acetylcholine was recently identified by us to play a major regulatory role in airway smooth muscle remodelling in a guinea pig model of ongoing allergic asthma. We now aimed to investigate other aspects of airway remodelling and to compare the effectiveness of tiotropium to the glucocorticosteroid budesonide.

Ovalbumin-sensitized guinea pigs were challenged for twelve weeks with aerosolized ovalbumin, which induced airway smooth muscle thickening, hypercontractility of tracheal smooth muscle, increased pulmonary contractile protein (sm-myosin) abundance, mucus gland hypertrophy, an increase in MUC5AC positive goblet cells and eosinophilia. We reported previously that treatment with tiotropium inhibits airway smooth muscle thickening and contractile protein expression, and prevents tracheal hypercontractility. Our current studies demonstrate that tiotropium also fully prevented allergen-induced mucus gland hypertrophy, and partially reduced the increase in MUC5AC positive goblet cells and infiltrated eosinophils. Treatment with budesonide also prevented airway smooth muscle thickening, contractile protein expression, tracheal hypercontractility and mucus gland hypertrophy, and partially reduced MUC5AC positive goblet cells 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 bronchodilation.

Keywords: airway remodelling, asthma, airway smooth muscle, mucus hypersecretion, anticholinergics, glucocorticosteroids

Introduction

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 COPD [2]. Therefore, muscarinic receptor antagonists such as 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 merely inducing 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, airway epithelial and inflammatory cells [14-17]. These pro-inflammatory actions of acetylcholine may be enhanced in inflammatory airways diseases, as muscarinic M_3 receptor expression and function is increased on neutrophils from COPD patients [18]. Furthermore, in a previous study, we found evidence 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 the airway smooth muscle have not been described thus far. In addition, no studies to date have directly compared the anti-remodelling effects of tiotropium bromide to other treatment strategies. Therefore, in the present study we aimed 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 induction of mucus producing cells.

Materials and methods

Animals

Outbred, male, specified pathogen free Dunkin Hartley guinea pigs (Harlan, Heathfield, United Kingdom) weighing 250-300 g were sensitized to ovalbumin 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.

Provocations were performed by inhalation of aerosolized solutions of ovalbumin (Sigma, St. Louis, MO, U.S.A.) 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 anti-histaminic was needed to prevent anaphylactic shock.

Study design

Guinea pigs were challenged with either ovalbumin 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), 0.5 h prior to each challenge with saline or ovalbumin. For budesonide treatment, animals received a nebulised dose of budesonide (gift from Prof. Dr. H.W. Frijlink, University of Groningen), suspended in saline supplemented with 1% polysorbatum 80 (1 mM suspension, 15 min) 24 hr and 1 hr prior to each challenge. Summary of treatment groups: OA sensitized, saline-challenged (n=9); OA sensitized, OA challenged (n=10); OA sensitized, saline-challenged, tiotropium treated (n=8); OA sensitized, OA challenged, tiotropium treated (n=7); OA sensitized, saline-challenged, budesonide treated

(n=9); OA sensitized, OA challenged, budesonide treated (n=7). For all of the tiotropium treated animals and for part of the OA-sensitized, saline-challenged (n=5) and OA-sensitized, OA-challenged (n=6) animals, lung material stored from our previous study [19] was used for the histological analyses. For OA-sensitized, saline-challenged and OA-sensitized, OA-challenged animals, data generated from previously stored and newly obtained lung material was pooled to produce a single OA-sensitized, saline-challenged control group and a single OA-sensitized, OA-challenged control group. During the 12 week challenge protocol, guinea pig weight was weekly monitored; no differences in weight gain between treatment groups were found.

Tissue acquisition

Twenty-four h after the last challenge, guinea pigs were sacrificed by experimental concussion, followed by rapid exsanguination. 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), of the following composition (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; gassed with 5 % CO_2 and 95 % O_2, pH 7.4.

Histochemistry

Transverse cross-sections of the main bronchi in both right and left lung lobes were used for morphometric analyses. To identify smooth muscle, extracellular matrix or mucus producing cells, sections were stained using, respectively, immunohistochemical staining for sm-myosin heavy chain (sm-MHC; Neomarkers; Fremont, CA, USA), a Masson's Trichrome staining, a Periodic Acid Shiff (PAS) staining or an immunohistochemical staining for MUC5AC (Neomarkers; Fremont, CA, USA). The antibody used for MUC5AC immunohistochemistry was

previously shown to be cross-reactive with guinea pig MUC5AC [22]. Primary antibodies were visualised using HRP-linked secondary antibodies and diaminobenzidine. Eosinophils were identified in hematoxyllin and eosin (H&E)-stained lung sections. Airways within sections were digitally photographed and subclassified as cartilaginous or non-cartilaginous. All immunohistochemical measurements were carried out digitally using quantification software (ImageJ).

Western analyses

Lung homogenates were prepared as described previously [19]. Protein lysates were separated by SDS/PAGE, followed by standard immunoblotting techniques. Antibodies were visualised by enhanced chemiluminescence (Pierce, Rockford, IL, USA). Photographs of blots were analyzed by densitometry (Totallab ^{tm}; Nonlinear Dynamics, Newcastle, U.K.).

Isometric tension measurements

Isometric concentration experiments were performed as described previously [19]. Briefly, the trachea was prepared free of serosal connective tissue. Single open-ring, epithelium-denuded preparations were mounted for isometric recording in organ baths, containing Krebs-Henseleit solution at 37°C, gassed with 5% CO_2 in O_2. After equilibration, resting tension was adjusted to 0.5 g followed by precontractions with 20 mM 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 shown represent means ± s.e.mean. Unless otherwise specified, statistical differences between means were calculated using one-way analysis of variance, followed by a Student-Newman-Keuls multiple comparisons test. Differences between means were considered to be statistically significant when $P < 0.05$.

Results

The airway smooth muscle

In our previous study, we found that repeated allergen inhalations increase airway smooth muscle mass, pulmonary contractile protein expression and 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 not included in Figures 1-3, but summarized in Table 1 instead.

The same types of experiments were planned to investigate the effects of budesonide treatment. Similar to our previous report, repeated ovalbumin challenges increased airway smooth muscle mass in the non-cartilaginous airways, with no changes in airway smooth muscle mass in the large, cartilaginous airways (Figure 1). Airway smooth muscle mass was quantified using an antibody specific for smooth muscle specific myosin heavy chain (sm-MHC), a stringent marker for the contractile airway smooth muscle phenotype [23]. 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 (Figure 1). Budesonide was more effective in reducing smooth muscle thickening than tiotropium, which prevented the increase in sm-MHC positive area partially, by 76% as previously reported by us [19] (Table 1).

In keeping with these observations, and in agreement with our previous report, western analysis demonstrated a marked 4.8 ± 0.7-fold increase in pulmonary sm-MHC expression in the

ovalbumin-challenged animals (Figure 2). As observed for airway smooth muscle thickness, this increase was nearly completely reversed by budesonide treatment. Budesonide did not significantly reduce sm-MHC positive area and pulmonary sm-MHC expression in saline challenged animals. The effects of budesonide on pulmonary sm-MHC expression in the ovalbumin-challenged animals were more pronounced than the inhibitory effects of tiotropium, which reduced the allergen-induced increase partially, by 38% as previously reported [19] (Table 1).

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

The extracellular matrix

Aberrant extracellular matrix deposition is observed in the airways of asthma and COPD patients [24, 25]. Therefore, experiments were aimed at quantifying extracellular matrix deposition within the airway wall of the guinea pig airways. Extracellular matrix, determined using a Masson's trichrome staining, was not different between ovalbumin and saline-challenged animals, neither in the cartilaginous airways nor in the non-cartilaginous airways. Tiotropium

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

Mucus producing cells

Mucus hypersecretion is a pathological feature seen in both asthma and COPD that contributes significantly to airflow limitation [26, 27]. Mucus hypersecretion in these patients is accompanied by mucus gland hypertrophy and goblet cell hyperplasia [26, 27]. Therefore, mucus producing cells in lung sections were quantified using a Periodic Acid Schiff (PAS) staining. Goblet cells within the airway epithelium and submucosal mucus glands were positive for this staining. Mucus glands and goblet cells were predominantly found in the cartilaginous airways; therefore, only cartilaginous airways were measured for these experiments. Repeated ovalbumin exposures induced a marked 45 ± 7 % increase in mucus gland area. Though tiotropium bromide and budesonide had no effect in saline-challenged animals, both treatments completely prevented the allergen-induced mucus gland hypertrophy (Figure 5A). Repeated allergen exposures also tended to induce an increase in total goblet cell number in the guinea pigs; however, this difference did not reach statistical 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 (Figure 5B).

The relatively small effects of ovalbumin 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 at quantifying MUC5AC staining in the airway epithelium. MUC5AC protein is highly

expressed during mucus differentiation in culture [28], and is reported to be almost undetectable in airways of control rats, despite the presence of goblet cells [29]. Based on these studies, we hypothesized that MUC5AC would be a more sensitive marker than PAS to measure mucus differentiation within the airway epithelium. Indeed, histochemistry indicated MUC5AC expression to be almost absent from saline-challenged guinea pig airways, whereas the glycoprotein was highly induced in ovalbumin challenged animals (Figure 6). Budesonide and tiotropium treatment did not change MUC5AC positive goblet cell number in saline-challenged animals, but partially reduced the MUC5AC induction by ovalbumin (Figure 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

Ovalbumin challenge induced eosinophil influx primarily in submucosal (Figure 7) and adventitial compartments of cartilaginous airways, compared to the airway smooth muscle (Table 2). Similar results were obtained for non-cartilaginous airways. Tiotropium had no effect on airway eosinophil number in saline-challenged animals, but partially prevented eosinophilia in submucosal compartments of cartilaginous and non-cartilaginous airways in ovalbumin-challenged animals; budesonide treatment had similar effects (Figure 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 our previous study [19], the results from the present study demonstrate that the long acting anti-cholinergic 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, mucus 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 anti-cholinergics holds some promise in treating asthma-associated pathophysiology.

Our results also show that the anti-remodelling effects of tiotropium bromide are very similar to the anti-remodelling effects of budesonide. For comparison of these drugs, the effects reported in our previous study and the current study are summarized in Table 1. Clearly, both tiotropium bromide and budesonide proprionate are effective in preventing airway smooth muscle remodelling in allergen-challenged guinea pigs, though some small differences exist. Although a 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 our study, budesonide treatment appeared to be more effective in preventing airway smooth muscle thickening in the non-cartilaginous airways; in addition, budesonide treatment abrogated the increase in contractile protein accumulation, whereas the inhibitory effect of tiotropium bromide was partial. On the other hand, tiotropium bromide reduced contractility of tracheal smooth muscle to a larger extent than budesonide. The effects of these drugs on mucus gland hypertrophy and MUC5AC expression were comparable. Collectively, the data indicate that budesonide and tiotropium are

similarly effective in preventing allergen-induced airway remodelling. At present it is unclear, however, whether 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 [30]. This discrepancy may explain why corticosteroids are generally not fully effective in reversing airway strucural changes in human asthma [31]. Clearly, future studies are warranted to investigate the potential effects of tiotropium on established airway remodelling.

The effects of repeated allergen challenge on airway smooth muscle remodelling were dependent on airway size. In the small, non-cartilaginous 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 characterized 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, as the ~65 % increase in airway smooth muscle in the non-cartilaginous airways is not sufficient to account for the ~4-fold increase in total pulmonary sm-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 from this study provide important novel insights into the mechanisms that regulate mucus gland hypertrophy and MUC5AC expression by goblet cells in response to allergen challenge. Our findings that these pathologies are prevented by tiotropium bromide suggest an important regulatory role for muscarinic receptors in remodelling of mucus producing cells. This

is the first study to demonstrate that mucus gland remodelling and MUC5AC expression in response to allergen are regulated by endogenous acetylcholine. Tiotropium had no effect on mucus 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. Possibly acetylcholine promotes airway structural cells to release chemokines and cytokines as reported for airway epithelial cells and airway smooth muscle [14-16] to attract inflammatory cells to the airways and functionally interacts with growth factors or cytokines that are released by these cells to induce direct remodelling effects on structural target cells, similar to what we observed for airway smooth muscle remodelling [2,11]. Though transactivation of the EGF receptor by muscarinic receptors has been reported in conjunctival goblet cells [32], no detailed molecular studies investigating the role of muscarinic receptors in proliferation and hypertrophy of airway mucus glands exist. Future molecular and cellular studies are needed to clarify the intracellular signalling mechanisms that underpin the muscarinic receptor response.

The anti-remodelling effects of tiotropium bromide were most profound for mucus 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 ovalbumin-challenged animals. Innervation of airway epithelial cells, including goblet cells, is variable among species [33]; however these cells are known to express the

synthesizing enzyme for acetylcholine, choline acetyltransferase (ChAT), transporter molecules for the excretion of acetylcholine, and acetylcholine itself [34-37]. It is unclear whether the source of acetylcholine involved in the allergen-induced mucus 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 our own 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 challenges did not induce a measurable change in Masson's trichrome staining within the airway wall, either in the cartilaginous or non-cartilaginous airways. However, since muscarinic receptors mediate proliferation of human lung fibroblasts [13], the possibility exists that this receptor system to some extent modulates matrix production within the airway wall of patients. The potential effects of anti-cholinergic drugs on extracellular matrix deposition within the airway wall therefore require further investigation.

Our results obtained with budesonide confirm earlier *in vivo* and *in vitro* findings that demonstrate preventive effects of glucocorticosteroids on airway smooth muscle thickening (see [38] for review). The mechanisms that explain the anti-proliferative 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 D_1 and stimulation of the cell cycle inhibitory protein $p21^{Waf1/Cip1}$ [38-40]. In addition, our studies provide some novel insights into the actions of glucocorticosteroids as we demonstrate 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 [41]. What mechanisms are targeted by glucocorticosteroids has not yet been investigated *in vitro* and requires future investigation.

Budesonide prevented remodelling of mucus producing cells in our model. Inhibitory effects of budesonide on allergen-induced goblet cell hyperplasia have been described in mice [42]. Although budesonide reduced goblet cell number in the 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 in saline-challenged conditions; 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 the increase in mucus gland area. Since airway inflammation is key to the development of mucus hypersecretion [43], 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* [44]. In combination with the results obtained using tiotropium bromide, these data indicate that glucocorticosteroids and anti-cholinergic drugs may both be effective in preventing remodelling of mucus producing cells in allergic airways disease.

In conclusion, the results from our 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 anti-cholinergic 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.

Acknowledgements

The studies described in this paper were supported by grants from the Netherlands Asthma Foundation (NAF 99.53) and Boehringer Ingelheim. Reinoud Gosens is currently the recipient of a Marie Curie Outgoing International Fellowship from the European Community (008823).

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Table 1 Comparison of the anti-remodelling effects of tiotropium and budesonide

Treatment	None		Tiotropium		Budesonide	
Challenge	Saline	OA	Saline	OA	Saline	OA
Airway smooth muscle mass	0	++++	0	+	0	0
Pulmonary contractile protein expression	0	++++	0	++	0	0
Tracheal contractility	0	++++	-	-	0	0
Mucus gland hypertrophy	0	++++	0	0	0	0
Goblet cell number *	0	++++	-	+++	-	0
MUC5AC expression by goblet cells	0	++++	0	++	0	++
Airway eosinophilia	0	++++	0	++	0	+
Total	0	28	-2	9	-1	3

Maximal allergen-induced remodelling was scored a 4 (++++) and compared with saline challenged animals, which were scored a 0. The effect of treatment was scored 3 (+++; for up to 25% inhibition), 2 (++; for up to 50% inhibition), 1 (+; for up to 75 % inhibition) and 0 (for complete inhibition) of the ovalbumin effect. A negative score (-) was given when the treatment effect was below that of saline challenge. Both tiotropium and budesonide were highly effective in preventing remodelling features in these animals. Of note: data on the effects of tiotropium on airway smooth muscle mass, pulmonary contractile protein expression and tracheal contractility were derived from our previous paper [19].

* Note: the effect of ovalbumin on Goblet cell number in was not statistically significant (see Figure 5B).

Table 2 Infiltration of eosinophils in different compartments of the airway wall

Treatment	None		Tiotropium		Budesonide	
Challenge	Saline	OA	Saline	OA	Saline	OA
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 ***, ##
Non-cartilaginous						
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 ###

Eosinophils were identified using a H&E stain and expressed as number per mm basement membrane. Data shown represent means ± s.e.mean; five to six airways of each classification were analysed for each animal. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to saline challenged animals; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ compared to ovalbumin-challenged

controls.

Figure legends

Figure 1

Effects of repeated allergen challenge and budesonide treatment on airway smooth muscle mass in the guinea pig lung. Intrapulmonary cartilaginous (A) (average basement membrane length 1.82 ± 0.08 mm) and non-cartilaginous airways (B) (average basement membrane length 0.74 ± 0.02 mm) were identified in sm-MHC stained lung sections after which sm-MHC-positive area was analysed morphometrically. Data shown are expressed as mm^2 sm-MHC positive area per mm^2 basement membrane. Six to eight airways of each classification were analysed for each animal. *** $P < 0.001$.

Figure 2

Effects of repeated allergen challenge and budesonide treatment on 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. Average sm-MHC expression in saline-challenged animals was determined from the ratio sm-MHC / β-actin and set to 100 %; error bar represents variation among saline-challenged animals. Sm-MHC expression (corrected for β-actin) in treatment groups was normalized to saline-challenged animals. Data shown are the means ± s.e.mean. ** $P < 0.01$.

Figure 3

Effects of repeated allergen challenge and budesonide treatment on tracheal contractility. The dose response relationship to methacholine of open, single-ring, epithelium-denuded, guinea pig tracheal rings was determined. Data shown represent means ± s.e.mean; three tracheal rings were

analysed per animal. Dose response curves were fitted to a 4-parameter sigmoidal equation using GraphPad Prism software. * $P < 0.05$.

Figure 4

Effects of repeated allergen challenge and tiotropium or budesonide treatment on extracellular matrix protein expression. Intrapulmonary cartilaginous (A) and non-cartilaginous airways (B) were identified in Masson's trichrome stained lung sections after which positive area was analysed morphometrically. Data shown are expressed as μm^2 positive area per μm basement membrane and represent means ± s.e.mean; four to six airways of each classification were analysed for each animal.

Figure 5

Effects of repeated allergen challenge and tiotropium or budesonide treatment on mucus gland area (A) and total goblet cell number (B) in intrapulmonary cartilaginous airways. Mucus producing cells were identified in PAS stained lung sections. For submucosal mucus glands (A), PAS positive area was analysed morphometrically and expressed as μm^2 positive area per μm basement membrane. For total goblet cell number, epithelial cells positive for PAS were counted and expressed per mm basement membrane. Data shown represent means ± s.e.mean; four to eight airways were analysed for each animal. * $P < 0.05$; ** $P < 0.01$; ** $P < 0.001$.

Figure 6

Effects of repeated allergen challenge and tiotropium or budesonide treatment on MUC5AC positive goblet cell number in the intrapulmonary cartilaginous airways. MUC5AC positive cells

were identified immunohistochemically and expressed as number per mm basement membrane. Data shown represent means ± s.e.mean; two to four airways were analysed for each animal. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 7

Effects of repeated allergen challenge and tiotropium or budesonide treatment on eosinophilia in the submucosal compartments of cartialginous (A) and non-cartilaginous (B) guinea pig airways. Eosinophils were identified using a H&E stain and expressed as number per mm basement membrane. Data shown represent means ± s.e.mean; five to six airways of each classification were analysed for each animal. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 1

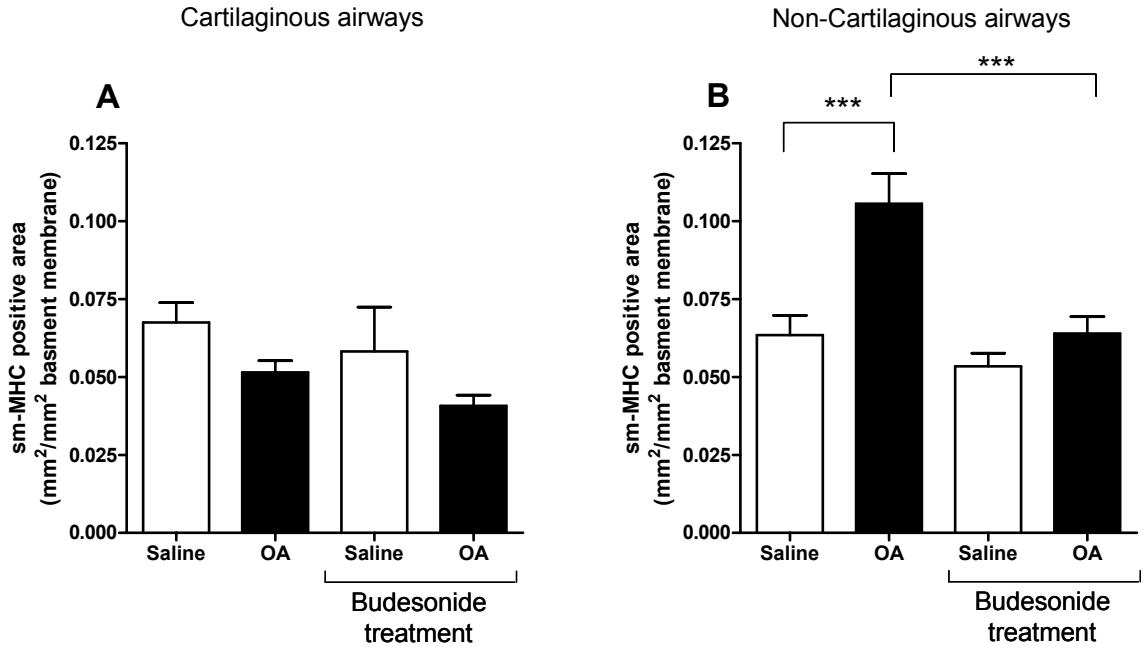

Cartilaginous airways
Non-Cartilaginous airways
A
B
sm-MHC positive area
(mm²/mm² basment membrane)
0.125
0.100
0.075
0.050
0.025
0.000
***
***
Saline
OA
Saline
OA
Budesonide
treatment

Figure 2

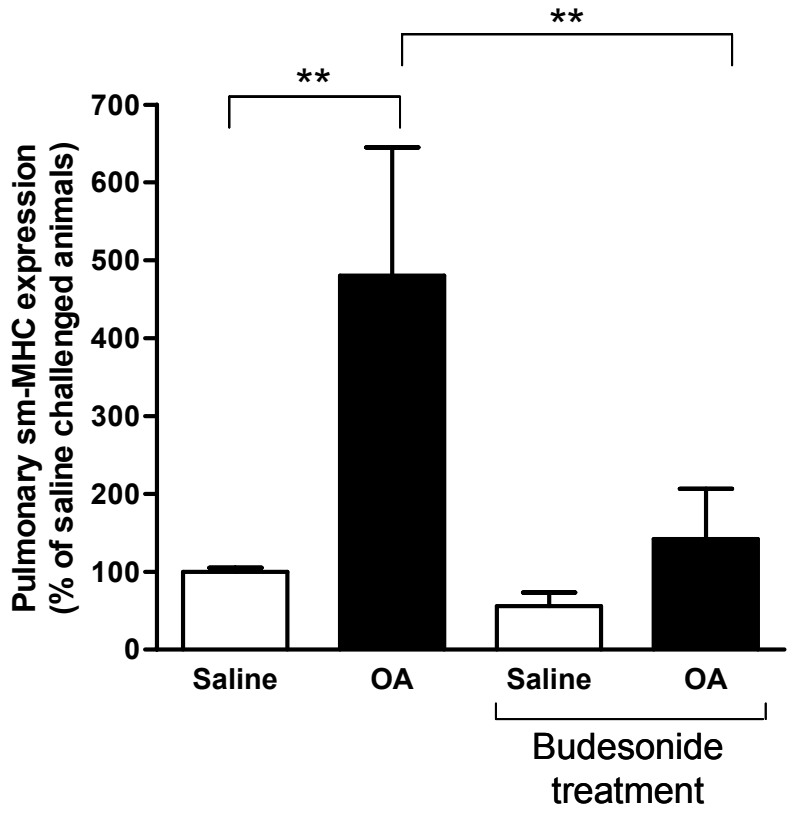

**
**
Pulmonary sm-MHC expression
(% of saline challenged animals)
700
600
500
400
300
200
100
0
Saline
OA
Saline
OA
Budesonide
treatment

Figure 3

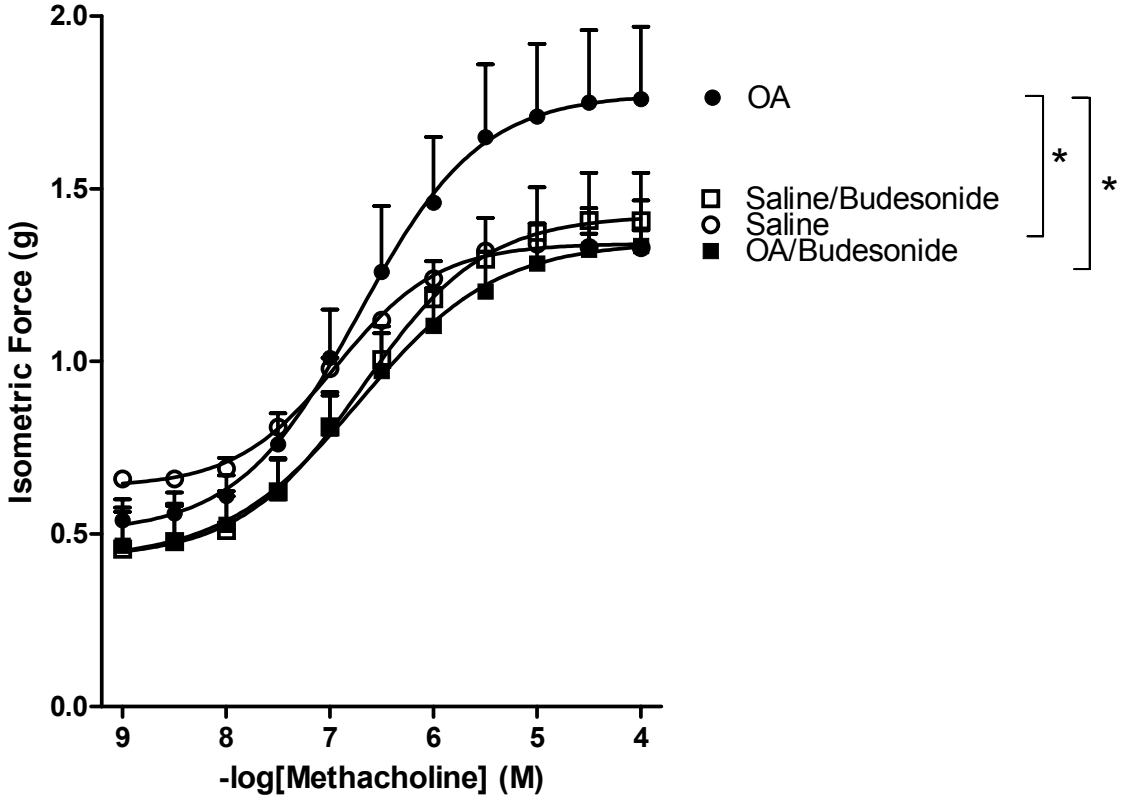

2.0
1.5
1.0
0.5
0.0
Isometric Force (g)
9
8
7
6
5
4
-log[Methacholine] (M)
OA
Saline/Budesonide
Saline
OA/Budesonide
*
*

Figure 4

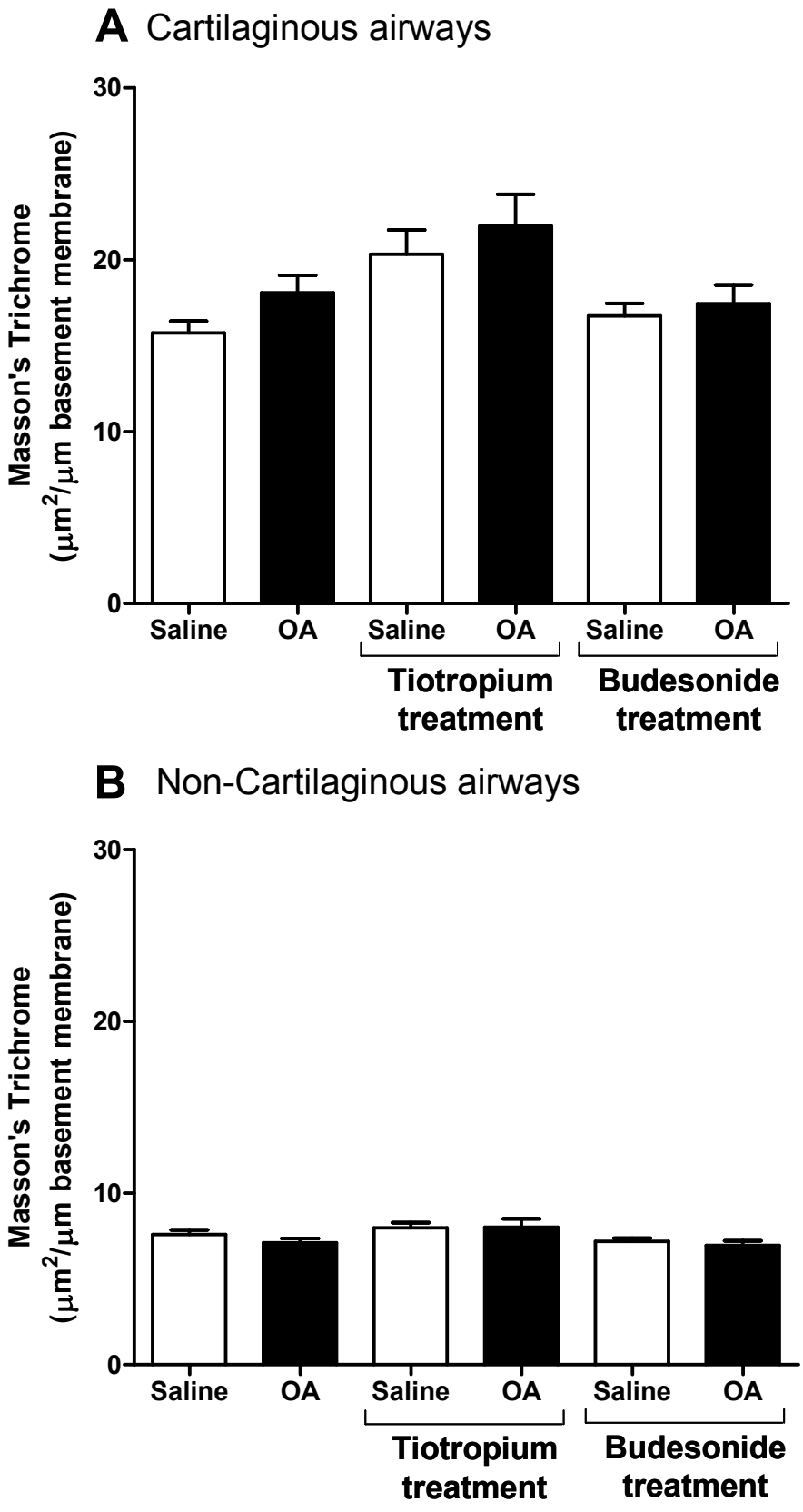

A Cartilaginous airways
Masson's Trichrome
(μm²/μm basement membrane)
30
20
10
0
Saline
OA
Saline
OA
Saline
OA
Tiotropium
treatment
Budesonide
treatment
B Non-Cartilaginous airways
Masson's Trichrome
(μm²/μm basement membrane)
30
20
10
0
Saline
OA
Saline
OA
Saline
OA
Tiotropium
treatment
Budesonide
treatment

Figure 5

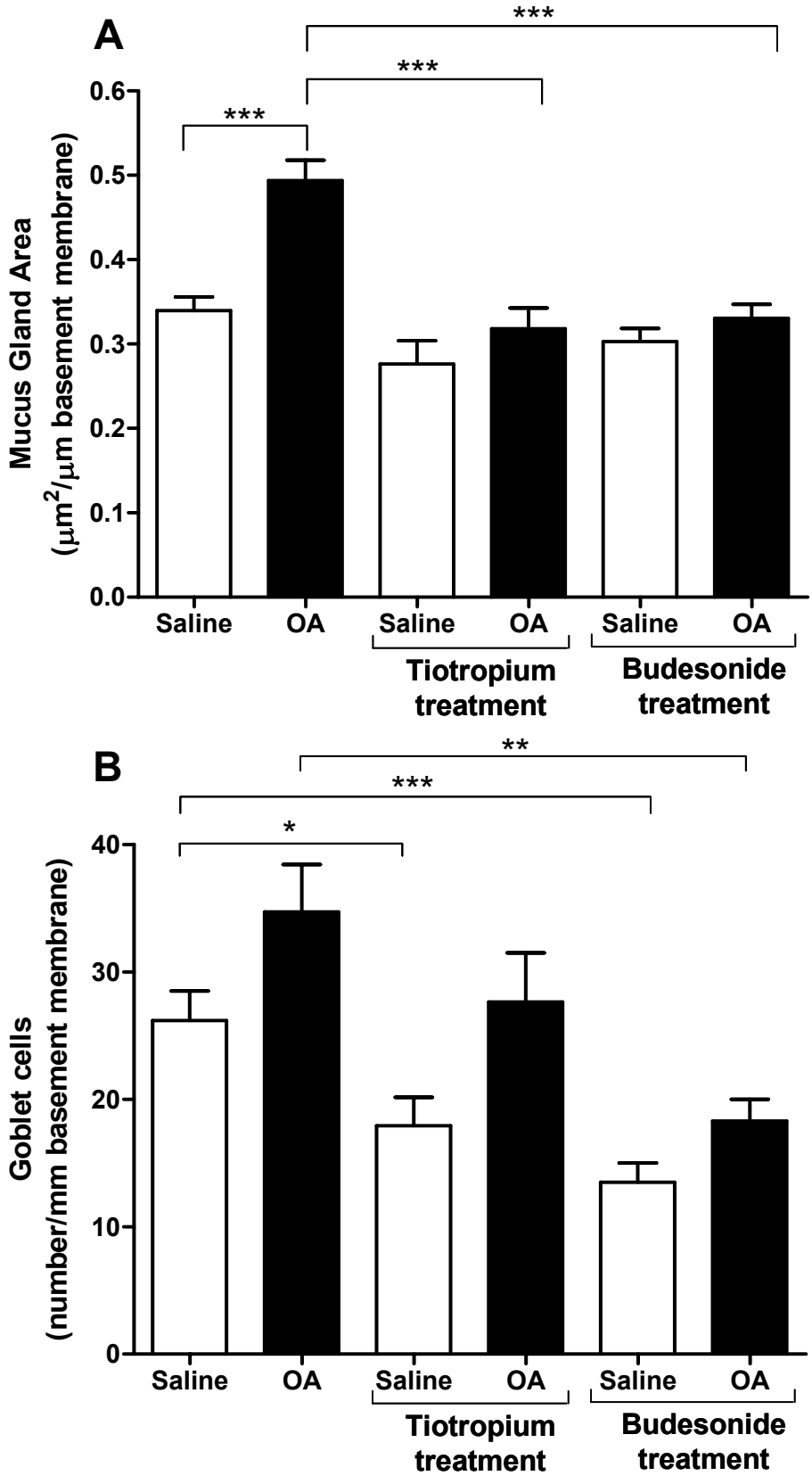

A
***
***
***
Mucus Gland Area
(μm²/μm basement membrane)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Saline
OA
Saline
OA
Saline
OA
Tiotropium
treatment
Budesonide
treatment
B
**
***
*
Goblet cells
(number/mm basement membrane)
40
30
20
10
0
Saline
OA
Saline
OA
Saline
OA
Tiotropium
treatment
Budesonide
treatment

Figure 6

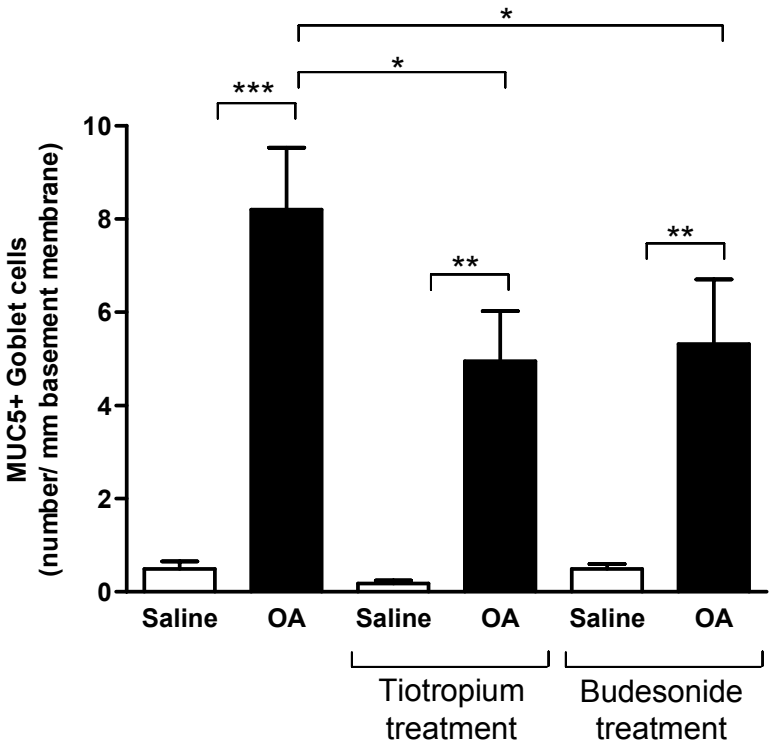
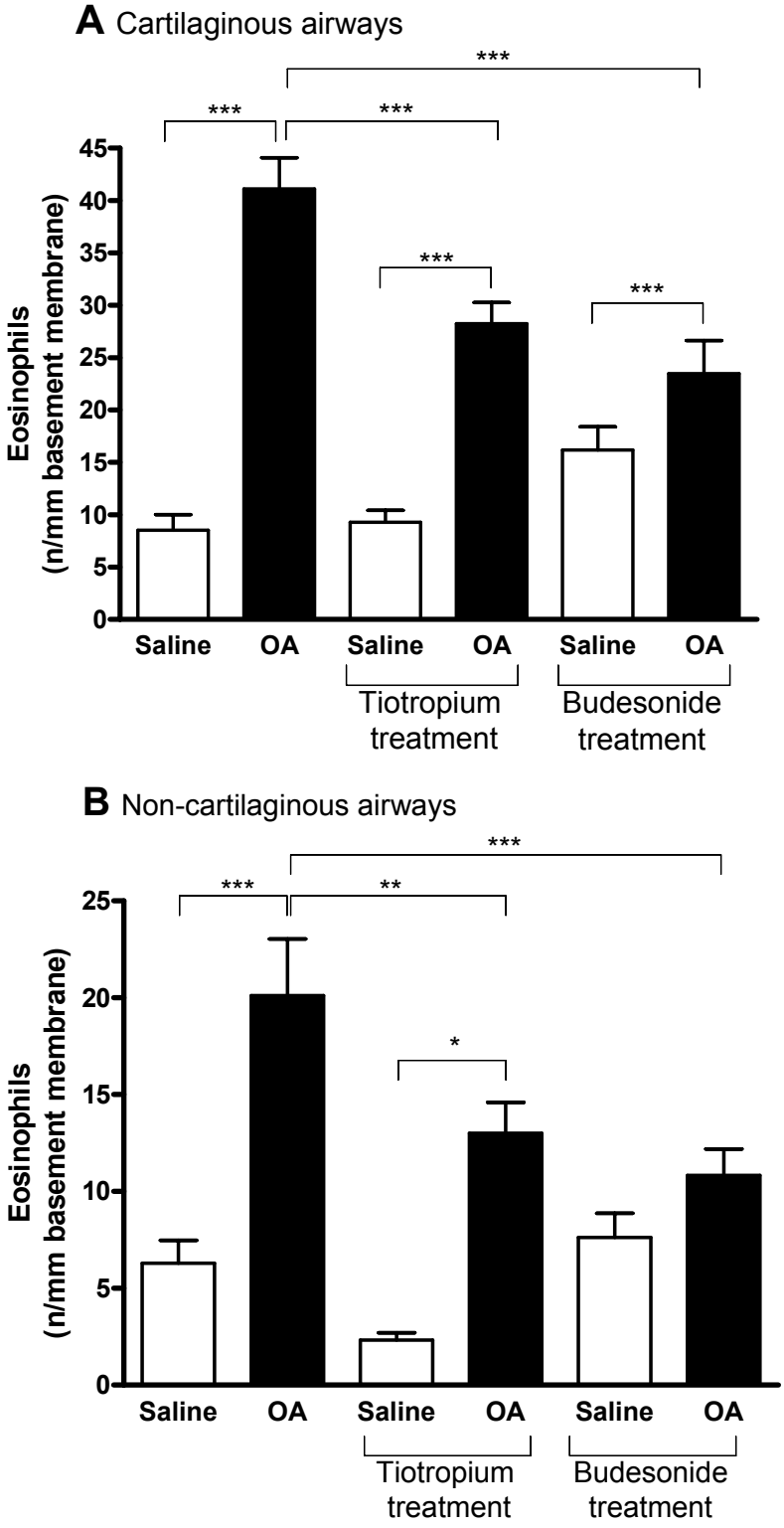

*
*
***
10
8
6
4
2
0
MUC5+ Goblet cells
(number/ mm basement membrane)
**
**
Saline
OA
Saline
OA
Saline
OA
Tiotropium
treatment
Budesonide
treatment

Figure 7


A Cartilaginous airways
***
***
***
***
***
45
40
35
30
25
20
15
10
5
0
Eosinophils
(n/mm basement membrane)
Saline
OA
Saline
OA
Saline
OA
Tiotropium
treatment
Budesonide
treatment
B Non-cartilaginous airways
***
***
**
*
25
20
15
10
5
0
Eosinophils
(n/mm basement membrane)
Saline
OA
Saline
OA
Saline
OA
Tiotropium
treatment
Budesonide
treatment