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RESEARCH PAPER

Non-bronchodilating mechanisms of tiotropium prevent airway hyperreactivity in a guinea-pig model of allergic asthma

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BACKGROUND AND PURPOSE

Asthma is characterized by reversible bronchoconstriction and airway hyperreactivity. Although M_3 muscarinic receptors mediate bronchoconstriction, non-selective muscarinic receptor antagonists are not currently recommended for chronic control of asthma. We tested whether selective blockade of M_3 receptors, at the time of antigen challenge, blocks subsequent development of airway hyperreactivity in antigen-challenged guinea-pigs.

EXPERIMENTAL APPROACH

Ovalbumin-sensitized guinea-pigs were pretreated with 1 $\mu g \cdot kg^{-1}$ of a kinetically selective M_3 receptor antagonist, tiotropium, or 1 $mg \cdot kg^{-1}$ of a non-selective muscarinic receptor antagonist, atropine, and challenged with inhaled ovalbumin. Animals were anaesthetized, paralyzed, ventilated and vagotomized 24 h later. We measured vagally mediated bronchoconstriction and i.v. ACh-induced bronchoconstriction.

KEY RESULTS

Electrical stimulation of both vagus nerves induced frequency-dependent bronchoconstriction in sensitized animals that was significantly increased after antigen challenge. Antigen-induced hyperreactivity was completely blocked by tiotropium pretreatment but only partially blocked by atropine pretreatment. Surprisingly, although tiotropium blocked bronchoconstriction induced by i.v. ACh, it did not inhibit vagally-induced bronchoconstriction in sensitized controls, suggesting that tiotropium does not block hyperreactivity by blocking receptors for vagally released ACh. Rather, tiotropium may have worked through an anti-inflammatory mechanism, since it inhibited eosinophil accumulation in the lungs and around nerves.

CONCLUSIONS AND IMPLICATIONS

These data confirm that testing M_3 receptor blockade with exogenous ACh does not predict vagal blockade. Our data also suggest that selective blockade of M_3 receptors may be effective in asthma via mechanisms that are separate from inhibition of bronchoconstriction.

Abbreviations

i.s., insufflated

Introduction

In the lungs, airway smooth muscle tone is controlled by ACh released from parasympathetic nerves. ACh stimulates M_3 muscarinic receptors on airway smooth muscle to induce smooth muscle contraction, resulting in bronchoconstriction. Parasympathetic nerves not only maintain airway tone, but they also mediate reflex bronchoconstriction (Canning, 2006). Diverse chemical and physical stimuli, including inhaled antigens, histamine and cold air, activate afferent sensory nerves in the lungs to initiate a reflex that increases ACh release from parasympathetic nerves and

bronchoconstriction (Gold *et al.*, 1972; Sheppard *et al.*, 1982; Canning, 2006).

Humans with asthma are hyperreactive to bronchoconstrictor stimuli. Exposure to antigens (antigen challenge) causes inflammation and airway hyperreactivity that develop within 24 h and persist for up to a week in both humans with asthma and sensitized animals (Cockcroft *et al.*, 1977; Chung *et al.*, 1985; Brown *et al.*, 1998). In antigen-challenged animals, airway hyperreactivity is mediated by increased ACh release from the vagus nerves (Evans *et al.*, 1997, 2001; Verbout *et al.*, 2007). Thus, anticholinergics, which block the action of ACh at M_3 muscarinic receptors, would be expected to be beneficial in asthma. However, anticholinergics are not currently recommended for long-term control of asthma (EPR-3, 2007). This is because many studies that used the anticholinergics atropine or ipratropium in humans with asthma found they were unable to effectively block bronchoconstriction (Fryer and Jacoby, 2008). This may be due, in part, to the low doses of anticholinergics used, since benefits were found in studies with higher doses (Ward *et al.*, 1981; Sheppard *et al.*, 1982).

Another possible explanation is that atropine and ipratropium are not selective for M_3 muscarinic receptors over other muscarinic receptor subtypes. M_1–M_3 muscarinic receptors are expressed on structural cells in the lungs, including smooth muscle, nerves, mucous glands, epithelial cells, fibroblasts and endothelial cells, and all five muscarinic receptors (M_1–M_5) are found on inflammatory cells (Wessler and Kirkpatrick, 2008). Non-selective blockade of all muscarinic receptor subtypes could counteract the inhibition of bronchoconstriction through M_3 muscarinic receptor blockade in asthma. For example, M_2 muscarinic receptors found on prejunctional parasympathetic nerves are stimulated by ACh to limit further release of ACh from the nerves onto airway smooth muscle (Fryer and Jacoby, 2008). Blocking these inhibitory M_2 receptors can increase ACh enough to overcome M_3 receptor blockade and increase bronchoconstriction, as has been shown in guinea-pigs treated with atropine and ipratropium (Fryer and Maclagan, 1987).

Experiments in this paper were designed to test whether selectively blocking M_3 muscarinic receptors at the time of antigen challenge reduces subsequent airway hyperreactivity. Tiotropium bromide, a kinetically selective muscarinic receptor antagonist, was used to block M_3 muscarinic receptors in sensitized guinea-pigs at the time of antigen challenge, and airway hyperreactivity was measured 24 h later. The results show that selective blockade of M_3 muscarinic receptors prevents airway hyperreactivity through a mechanism that is separate from direct inhibition of bronchoconstriction, and that this blockade is associated with inhibition of the inflammatory response to antigen challenge.

Methods

Animals

Specific pathogen-free female Hartley guinea-pigs (Elm Hill Labs; Chelmsford, MA, USA) were housed in high-efficiency particulate-filtered air with *ad libitum* access to food and water. All animal care and experimental procedures were in accordance with the National Institutes of Health (NIH) guidelines, and were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee.

Sensitization and challenge with antigen

All guinea-pigs (150–200 g) were sensitized to Grade II ovalbumin (20 mg·kg^{-1}, i.p., Sigma-Aldrich, St. Louis, MO, USA) on days 1, 3 and 6. Treatments and challenge were given 21 days after the last injection. Some animals were challenged with an aerosol of 5% ovalbumin containing 0.2% antifoam Y-30 emulsion (Sigma-Aldrich) in sterile PBS for 10 min or until signs of respiratory distress appeared, in which case antigen challenge was immediately stopped (three of 27 animals).

Treatment with insufflated tiotropium and lactose

Tiotropium is a kinetically selective M_3 receptor antagonist that dissociates more slowly from M_3 (human $t_{1/2}$ = 27–34.7 h) muscarinic receptors than M_2 (human $t_{1/2}$ = 2.6–3.6 h) or M_1 (human $t_{1/2}$ = 10.5–14.6 h) muscarinic receptors (Disse *et al.*, 1993; Casarosa *et al.*, 2009). Tiotropium is the active ingredient in Spiriva (HandiHaler capsules, Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, CT, USA), which contains α lactose monohydrate filler. Tiotropium was administered as a powder to some sensitized animals using a DP-4 dry powder insufflator (Penn-Century Inc., Philadelphia, PA, USA) to mimic the method of delivery in humans. Since 2–5 mg of powder must be loaded in this insufflator, Spiriva was mixed with additional D-(+)-lactose monohydrate (Mallinckrodt Baker Inc., Phillipsburg, NJ, USA). Guinea-pigs were anaesthetized with ketamine (30 mg·kg^{-1}, i.m., JHP Pharmaceuticals LLC, Rochester, MI, USA) and xylazine (5 mg·kg^{-1}, i.m., Vedco Inc., St. Joseph, MO, USA) and supported vertically while the insufflator delivery tube was inserted into the trachea with the aid of a small animal laryngoscope (WelchAllyn, Skaneateles Falls, NY, USA). Tiotropium bromide 0.2 μg·kg^{-1} (0.05 mg Spiriva·kg^{-1}) or 1 μg·kg^{-1} (0.25 mg Spiriva·kg^{-1}) was then insufflated (i.s.) into the lungs. Vehicle control animals for these experiments received an equivalent amount of lactose powder. The actual dose administered was confirmed by weighing the insufflator sample chamber before and after delivery.

Treatment with atropine

Some sensitized animals were treated with the non-selective muscarinic receptor antagonist, atropine (1 mg·kg^{-1}, i.p.) 1 h before challenge and again 6 h after challenge as previously described (Verbout *et al.*, 2007).

Experimental groups

To determine the effect of selectively blocking M_3 receptors with tiotropium at the time of challenge on airway hyperresponsiveness, physiological measurements were made in seven groups of animals. Three groups of animals were sensitized: (i) sensitized controls; (ii) sensitized animals treated with lactose; and (iii) sensitized animals treated with 1 μg·kg^{-1} tiotropium. *In vivo* physiology was measured 48 h after tiotropium or lactose administration in these animals. Four groups

of animals were sensitized and challenged: (i) sensitized and challenged animals; (ii) sensitized animals treated with lactose as a vehicle control and challenged 24 h later; (iii) sensitized animals treated with 1 $\mu g \cdot kg^{-1}$ tiotropium and challenged 24 h later; and (iv) sensitized animals treated with atropine and challenged 1 h later. *In vivo* physiology was measured 24 h after challenge with inhaled ovalbumin in these groups, which corresponds to 48 h after tiotropium or lactose administration and 25 h after the first injection of atropine.

Physiological measurements were also made at the time of challenge (24 h after treatment with lactose or tiotropium) in four groups of animals: (i) sensitized controls (anaesthetized with ketamine and xylazine); (ii) sensitized animals treated with lactose (vehicle control); (iii) sensitized animals treated with 0.2 $\mu g \cdot kg^{-1}$ tiotropium; and (iv) sensitized animals treated with 1 $\mu g \cdot kg^{-1}$ tiotropium.

Measurement of pulmonary inflation pressure and vagal reactivity

Guinea-pigs were anaesthetized with urethane (1.7 $g \cdot kg^{-1}$ i.p., Sigma-Aldrich Chemical Co.), and temperature was maintained at 37°C. Jugular veins were cannulated for drug administration, and heart rate and blood pressure were measured via a carotid artery cannula to ensure adequate levels of anaesthesia. Animals were chemically sympathectomized with guanethedine (2 $mg \cdot kg^{-1}$, i.v., Bosche Scientific, New Brunswick, NJ, USA), paralysed with succinylcholine chloride (5 $\mu g \cdot min^{-1}$, i.v., Sigma-Aldrich), and mechanically ventilated via a tracheal cannula (tidal volume 2.5 mL, 100 $breaths \cdot min^{-1}$). Guinea-pigs were vagotomized by crushing both vagus nerves, and distal portions of both vagi were placed on platinum electrodes and submerged in mineral oil. Pulmonary inflation pressure was measured via a sidearm of the tracheal cannula.

Both vagus nerves were electrically stimulated simultaneously (1–25 Hz, 10 V, 0.2 ms pulse duration, for 5 s at 45–60 s intervals) to produce frequency-dependent bronchoconstriction, measured as an increase in pulmonary inflation pressure in mmH_20, and bradycardia. Atropine (1 $mg \cdot kg^{-1}$, i.v.) was given at the end of each experiment to confirm that cholinergic nerves mediated the vagally-induced bronchoconstriction.

Measurement of post-junctional muscarinic receptor function

Following measurement of vagal reactivity, ACh (1–10 $\mu g \cdot kg^{-1}$, i.v., Acros Organics, Ceel, Belgium) was administered to test the function of post-junctional M_2 receptors in cardiac muscle and post-junctional M_3 receptors in airway smooth muscle.

Inhibition of bronchoconstriction following i.v. administration of lactose and tiotropium in non-sensitized guinea-pigs

Vagus nerve-induced bronchoconstriction was elicited by electrically stimulating both vagus nerves every 2 min for 5 s (10 Hz, 10 V, 0.2 ms pulse duration) in anaesthetized animals. ACh (4 $\mu g \cdot kg^{-1}$, i.v.)-induced bronchoconstriction was elicited in the same animals following every third vagal stimulation. Gallamine (3 $mg \cdot kg^{-1}$, i.v.) was administered to block neuronal M_2 receptors. Once reproducible baseline bronchoconstrictions were achieved, tiotropium bromide (0.3–10 $\mu g \cdot kg^{-1}$) was given by administering Spiriva dissolved in PBS i.v. Each increasing dose of tiotropium was given after inhibition of bronchoconstriction reached a plateau (time determined in early experiments and maintained throughout). Time/vehicle control animals received equivalent amounts of i.v. lactose. Following each dose, the last two i.v. ACh-induced bronchoconstrictions were averaged and presented as a percentage of baseline. The second and third bronchoconstrictions following vagal stimulation were similarly averaged and presented as a percentage of baseline.

Bronchoalveolar lavage analysis

At the end of each experiment, lungs were lavaged with five 10 mL aliquots of PBS containing 10 $\mu g \cdot mL^{-1}$ isoprenaline (Sigma-Aldrich). Total cells were counted using a haemocytometer. Cells were also cytospun onto slides and stained with Hemacolor (EMD Chemicals Inc., Gibbstown, NJ, USA) to obtain differential cell counts.

Histopathological evaluation of eosinophils

Animals were then perfused with PBS and exsanguinated. Lungs were removed, inflated with zinc-buffered formalin (Anatech Ltd, Battle Creek, MI, USA), and fixed overnight at 4°C. Two transverse sections of the proximal region of each of two lobes were embedded in paraffin and processed for immunohistochemistry. Airway nerves were detected with a monoclonal antibody specific for PGP9.5 and eosinophils were stained with chromotrope 2R [modified from Evans *et al*. (2001)]. Antigen retrieval was carried out by microwaving slides in antigen unmasking solution (Vector Laboratories Inc., Burlingame, CA, USA). Endogenous peroxidase activity was quenched with 3% H_2O_2 diluted in cold methanol (10 min). Sections were permeabilized with 0.05% Tween 20 in PBS (2 × 3 min), blocked in 10% normal goat serum (30 min room temperature, Vector Laboratories Inc.), incubated with mouse anti-PGP9.5 (5 $\mu g \cdot mL^{-1}$, 1 h room temperature, AbD Serotec, Cat no. 7863-2004, Raleigh, NC, USA), and incubated with biotinylated goat anti-mouse IgG (7.5 $\mu g \cdot mL^{-1}$, 30 min room temperature, Vector Laboratories Inc., BA-9200). Antibody staining was detected with a streptavidin-linked horseradish peroxidase substrate (Vectastain ABC kit, Vector Laboratories Inc.) and Vector SG chromagen (Vector Laboratories Inc.). Normal mouse IgG or no primary antibody served as controls. Slides were then stained with 1% chromotrope 2R (Sigma-Aldrich) in diH_2O for 1 min, air dried overnight and mounted.

Eosinophils within airway walls (large bronchioles) were quantified by an investigator blinded to treatment. Overlapping 400× photographs of each airway were taken and overlaid to make a single image for each airway using a digital camera and NIH Image J Software [version 1.42q, NIH, Bethesda, MD, USA (Rasband, 1997–2009)] with Mosaic J plugin (Thevenaz and Unser, 2007). Total airway area between the epithelial basement membrane and surrounding alveoli was measured using MetaMorph imaging software (version 7.1.2.0, Molecular Devices, Sunnyvale, CA, USA). Total eosinophils and nerve-associated eosinophils (within

8 μm of a PGP9.5-positive nerve) were counted and expressed as eosinophils·mm^{-2} airway area. Values were averaged across three airways for each animal, and these numbers were used to calculate the mean for each group.

Statistical analysis

Values are means ± SEM. Baseline pulmonary and cardiovascular parameters were compared using one-way ANOVA with Bonferroni's correction for multiple comparisons. Frequency-response curves to nerve stimulation, dose-response curves to i.v. ACh, i.v. tiotropium or i.v. lactose were compared using two-way ANOVA for repeated measures with Bonferroni's correction. Bronchoalveoloar lavage leukocyte and tissue eosinophil data were log-transformed to equalize variances and then analysed using one-way ANOVA with Bonferroni's correction. A Kruskal–Wallis test was used to compare lymphoctye means in Figure 8 because variances could not be equalized. Significant *P*-values are reported as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Statistical data were analysed with GraphPad Prism (version 5, GraphPad Software, Inc., La Jolla, CA, USA).

Results

Effect of M_3 muscarinic receptor blockade at the time of antigen challenge on subsequent airway hyperreactivity

To test the effect of selectively blocking M_3 muscarinic receptors during antigen challenge on the development of airway hyperreactivity, tiotropium bromide was i.s. into the lungs of ovalbumin-sensitized guinea-pigs. Tiotropium (1 μg·kg^{-1}) was administered 24 h before antigen challenge to allow it to dissociate from M_1 and M_2 receptors, while still blocking M_3 muscarinic receptors at the time of antigen challenge. In separate experiments, atropine (1 mg·kg^{-1}, i.p.) was used to block all muscarinic receptor subtypes during antigen challenge and during the early response post-antigen challenge. Since atropine is short acting (elimination $t_{1/2}$ = 2.4 h (Kentala *et al.*, 1990)), it was administered 1 h before and 6 h after challenge. All physiological measurements were made 24 h after antigen challenge.

Antigen challenge did not change baseline pulmonary inflation pressure, heart rate or blood pressure measured in vagotomized guinea-pigs 24 h later relative to sensitized guinea-pigs (Table 1). Similarly, baseline pulmonary and cardiac parameters were not changed in those sensitized or sensitized and challenged animals pretreated with tiotropium, lactose vehicle or atropine (Table 1). Tiotropium, however, caused a small increase in baseline pulmonary inflation pressure in sensitized guinea-pigs.

Antigen challenge of sensitized guinea-pigs caused airway hyperreactivity that was mediated by the vagus nerves (Figure 1). Electrical stimulation of both vagus nerves induced frequency-dependent bronchoconstriction in vagotomized, sensitized guinea-pigs (Figure 1A) that was significantly potentiated 24 h after antigen challenge (Figure 1B). Pretreatment with lactose vehicle did not change this vagally induced bronchoconstriction in either sensitized (Figure 1A) or sensitized and challenged (Figure 1B) guinea-pigs. Pretreatment with tiotropium did not reduce vagally-induced bronchoconstriction in sensitized guinea-pigs (Figure 1A). However, tiotropium pretreatment completely prevented the development of airway hyperreactivity in sensitized and challenged guinea-pigs (Figure 1B). In contrast, atropine pretreatment only partially inhibited airway hyperreactivity following antigen challenge (Figure 1B). Thus, tiotropium pretreatment prevented subsequent vagally mediated airway hyperreactivity in antigen-challenged animals without reducing bronchoconstriction in sensitized control animals.

ACh released from parasympathetic nerves in the lungs stimulates M_3 muscarinic receptors on airway smooth muscle to induce bronchoconstriction. Smooth muscle M_3 receptor

Table 1

Baseline pulmonary and cardiovascular parameters for data in vagotomized guinea-pigs for Figures 1–2, 6 and 8–9

Pretreatment	*n*	Pulmonary inflation pressure (mmH_2O)	Heart rate (beats·min^{-1})	Blood pressure (mmHg) Systolic	Diastolic
Sensitized					
None	6	87 ± 4	291 ± 3	51 ± 4	29 ± 4
Lactose	6	103 ± 6	291 ± 10	49 ± 0.4	28 ± 2
Tiotropium	7	107 ± 5*	284 ± 8	44 ± 5	26 ± 3
Sensitized and challenged (24 h after challenge of sensitized guinea pigs)					
None	7	106 ± 2	278 ± 9	54 ± 2	29 ± 1
Lactose	6	98 ± 4	299 ± 13	50 ± 6	32 ± 5
Tiotropium	6	90 ± 3	293 ± 8	51 ± 2	30 ± 3
Atropine	7	97 ± 5	293 ± 10	49 ± 2	28 ± 2

Values are means ± SEM. *n*, number of guinea-pigs. All baseline parameter means were compared with the sensitized no treatment group; statistically significant changes are noted with *.

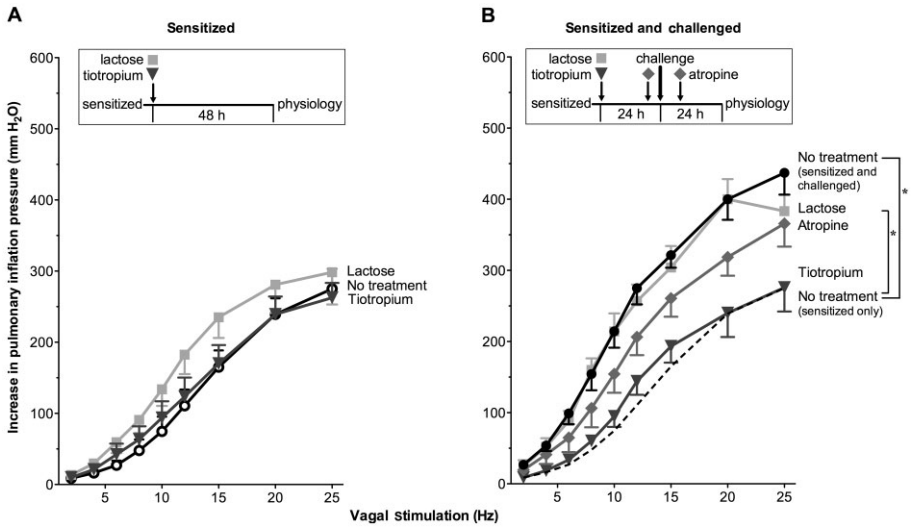

Figure 1

Selectively blocking M_3 receptors with tiotropium at the time of antigen challenge prevents development of subsequent airway hyperreactivity but does not inhibit bronchoconstriction. Electrical stimulation of both vagus nerves (2–25 Hz, 10 V, 0.2 ms, 5 s pulse train) produced frequency-dependent bronchoconstrictions (measured as an increase in pulmonary inflation pressure) in vagotomized, sensitized guinea-pigs (A, open circles; also shown in B as a dashed line) that were potentiated 24 h after antigen challenge (B, solid circles). Lactose vehicle did not change vagally induced bronchoconstriction 48 h after administration in either sensitized (A, squares) or sensitized and challenged guinea pigs (B, squares). Tiotropium (1 $\mu g \cdot kg^{-1}$, i.s.) did not change vagally-induced bronchoconstriction in sensitized guinea pigs (A) measured 48 h after administration but did prevent potentiation of vagally-induced bronchoconstriction in sensitized and challenged guinea-pigs (B). Unlike tiotropium, atropine (1 $mg \cdot kg^{-1}$, i.p.) administered 1 h before and 6 h after challenge only partially prevented the potentiation of vagally-induced bronchoconstriction in sensitized and challenged guinea-pigs (B). Data are expressed as means ± SEM, $n = 6–7$. All frequency response curves were compared with the sensitized, no treatment group (open circles) and statistically significant changes are noted with *.

function was measured with exogenous i.v. ACh. All of these animals were vagotomized to remove the confounding effects of reflex-induced bronchoconstriction. Antigen challenge did not increase i.v. ACh-induced bronchoconstriction (filled circles, Figure 2B) relative to sensitized controls (open circles, Figure 2A). Neither i.s. lactose nor atropine pretreatment had any affect on i.v. ACh-induced bronchoconstriction (Figure 2). Tiotropium inhibited i.v. ACh-induced bronchoconstriction in both sensitized (Figure 2A) and sensitized and challenged (Figure 2B) guinea-pigs 48 h after administration. Thus, some airway smooth muscle M_3 receptors are still blocked 48 h after tiotropium treatment, while no M_3 receptors are blocked 18 h after atropine treatment.

M_3 receptor blockade at the time of antigen challenge

Since vagally-induced bronchoconstriction was not blocked in sensitized guinea-pigs 48 h after tiotropium treatment (Figure 1A), we tested whether smooth muscle M_3 receptors were blocked at the time of antigen challenge. Either lactose (vehicle control) or one of two different doses of tiotropium (0.2 $\mu g \cdot kg^{-1}$ or 1 $\mu g \cdot kg^{-1}$) was i.s. into the lungs of ovalbumin-sensitized guinea-pigs, and physiological measurements were made 24 h later. Baseline pulmonary inflation pressure, heart rate and blood pressure were not significantly changed by either dose of tiotropium or by lactose vehicle relative to sensitized guinea-pigs that received no additional treatment (Table 2).

Bronchoconstriction induced by i.v. ACh in sensitized guinea-pigs was not changed by pretreatment with i.s. lactose vehicle (Figure 3A). However, i.v. ACh-induced bronchoconstriction was inhibited in a dose-dependent manner in sensitized animals pretreated with tiotropium (Figure 3A). The higher dose of tiotropium significantly decreased bronchoconstriction, by approximately 75%, demonstrating that 1 $\mu g \cdot kg^{-1}$ tiotropium still blocked M_3 muscarinic receptors on airway smooth muscle 24 h after administration, which was the time of antigen challenge in Figures 1 and 2.

Electrical stimulation of both vagus nerves caused frequency-dependent bronchoconstriction that was not inhibited by lactose vehicle in sensitized guinea-pigs (Figure 3B). Surprisingly, and in contrast to i.v. ACh, neither dose of tiotropium inhibited bronchoconstriction induced by electrical stimulation of both vagus nerves in sensitized guinea-pigs (Figure 3B). Since vagally-induced bronchoconstriction could be blocked with i.v. atropine (data not shown), it must have been mediated by ACh release onto muscarinic receptors. These data suggest that i.s. tiotropium did not block M_3 receptors at the neuromuscular junction with parasympathetic nerves.

Determining whether the route of tiotropium administration affects blockade of vagally induced bronchoconstriction

It is possible that i.s. tiotropium was unable to inhibit bronchoconstriction induced by electrical stimulation of the vagus nerves (Figure 3B) because the delivery method (insufflation) prevented tiotropium from distributing to M_3 muscarinic receptors at the junction with parasympathetic nerves.

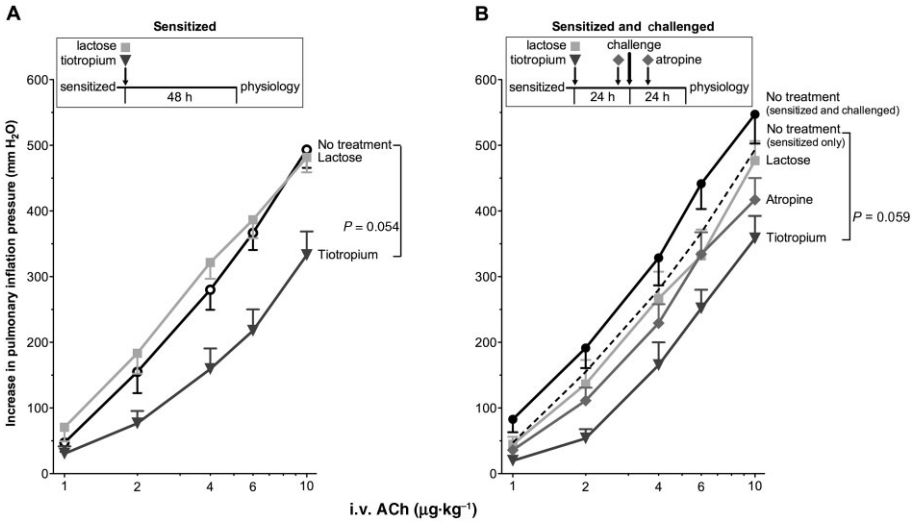

Figure 2

Tiotropium still blocks some M_3 muscarinic receptors on airway smooth muscle 48 h after administration in both sensitized (A) and sensitized and challenged (B) guinea-pigs. In vagotomized, sensitized guinea-pigs, i.v. ACh caused dose-dependent bronchoconstriction measured as an increase in pulmonary inflation pressure (A; also shown in B as a dashed line) that was not changed by antigen challenge (B). Bronchoconstriction was not changed by lactose vehicle 48 h after administration (A and B). Atropine (1 mg·kg^{-1}, i.p.) administered 1 h before and 6 h after antigen challenge also did not change i.v. ACh-induced bronchoconstriction 24 h after challenge (B). In contrast, tiotropium (1 μg·kg^{-1}, i.s.) inhibited i.v. ACh-induced bronchoconstriction in both sensitized (A) and sensitized and challenged (B) guinea-pigs when measured 48 h after administration. Data are expressed as means ± SEM, n = 6–7. All dose-response curves were compared with the sensitized, no treatment group (open circles). *P*-values approaching significance are reported following Bonferroni correction for six comparisons.

Table 2

Baseline pulmonary and cardiovascular parameters in vagotomized guinea-pigs for data in Figures 3, 5 and 7

Pretreatment	*n*	Pulmonary inflation pressure (mmH_2O)	Heart rate (beats·min^{-1})	Blood pressure (mmHg) Systolic	Diastolic
Sensitized					
None	3	107 ± 9	315 ± 10	41 ± 7	19 ± 2
Lactose	3	100 ± 0	317 ± 16	41 ± 4	26 ± 3
Tiotropium, 0.2 μg·kg^{-1}	4	108 ± 6	305 ± 9	43 ± 3	26 ± 5
Tiotropium, 1 μg·kg^{-1}	4	125 ± 16	293 ± 4	43 ± 2	24 ± 1

Values are means ± SEM. *n*, number of guinea pigs. None of the baseline parameter means were statistically different from the sensitized, no treatment group.

Therefore, we tested whether i.v. administration of tiotropium blocked equally M_3 muscarinic receptors stimulated by i.v. ACh and vagally released ACh. Reproducible baseline bronchoconstrictions to i.v. ACh (4 μg·kg^{-1}) and electrical stimulation of the vagus nerves (10 Hz) were measured in vagotomized, non-sensitized guinea-pigs treated with gallamine to block neuronal M_2 muscarinic receptors. Cumulative doses of i.v. tiotropium inhibited bronchoconstriction in a dose-dependent manner (Figure 4). The lowest dose of tiotropium (0.3 μg·kg^{-1}) significantly inhibited i.v. ACh-induced bronchoconstriction by more than 50%, whereas only the highest dose of tiotropium (10 μg·kg^{-1}) significantly inhibited vagally induced bronchoconstriction (relative to the appropriate i.v. lactose controls). At a minimum, i.v. tiotropium was 10-fold more potent at inhibiting i.v. ACh-induced bronchoconstriction than vagally-induced bronchoconstriction. Thus, lower doses of i.v. tiotropium were required to block M_3 muscarinic receptors involved in exogenous ACh-induced bronchoconstriction than were required to block endogenous ACh-induced bronchoconstriction.

For these experiments, lactose (filler in Spiriva) was used as a control and was given to guinea-pigs in separate experiments i.v. (dashed lines, Figure 4). We found no difference between the effect of lactose on i.v. ACh-induced bronchoconstriction and vagally induced bronchoconstriction. Yet we did find that overall bronchoconstriction appears to be inhibited in the lactose controls. Whether this inhibition is due to time, since the experiments lasted close to 2 h, or to lactose

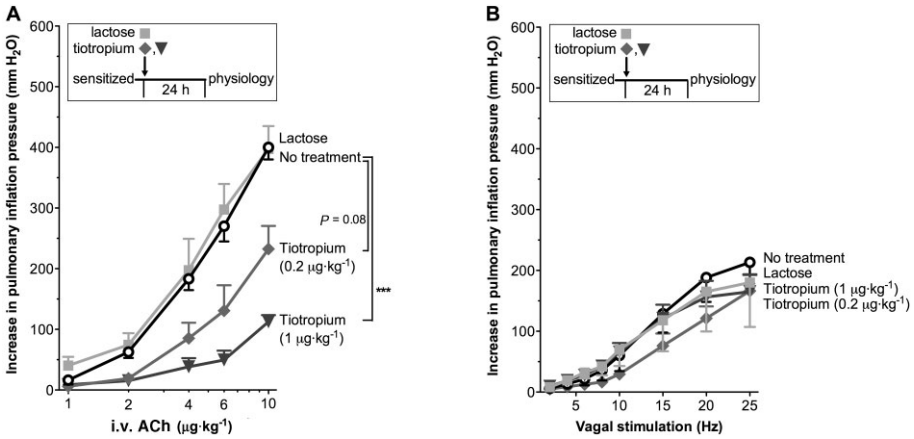

Figure 3

Tiotropium blocks smooth muscle M_3 receptors 24 h after administration when tested with i.v. ACh but not vagally released ACh. In vagotomized, sensitized guinea-pigs, i.v. ACh caused dose-dependent bronchoconstriction measured as an increase in pulmonary inflation pressure (A), and electrical stimulation of both vagus nerves caused frequency-dependent bronchoconstriction (B; 2–25 Hz, 10 V, 0.2 ms, 5 s pulse train). Bronchoconstriction was not changed by lactose vehicle 24 h after administration (A and B). Tiotropium, 0.2 $\mu g \cdot kg^{-1}$ i.s. and 1 $\mu g \cdot kg^{-1}$ i.s., inhibited bronchoconstriction induced by i.v. ACh (A) but not bronchoconstriction induced by vagal stimulation (B) 24 h after administration. Data are expressed as means ± SEM, n = 3–4. All dose- and frequency-response curves were compared with the no treatment group (open circles); statistically significant changes are noted with *.

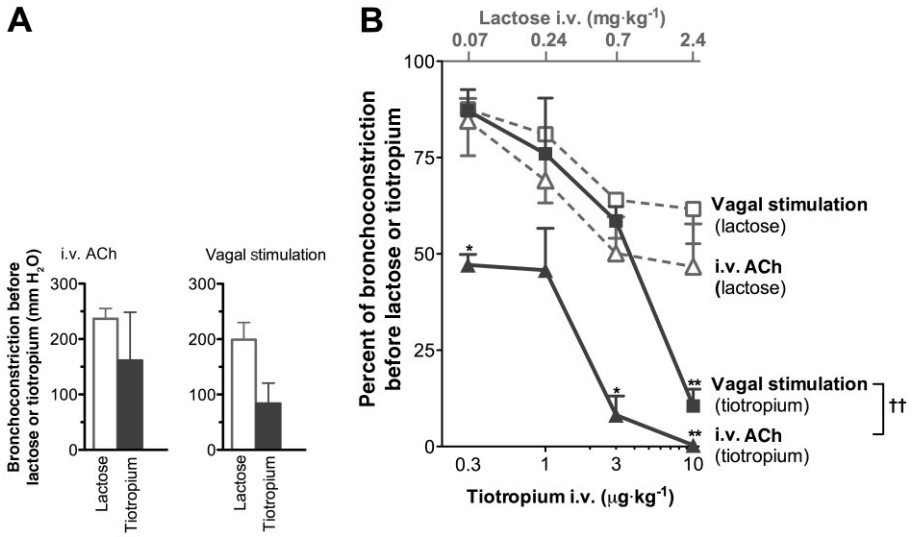

Figure 4

Intravenous administration of tiotropium blocks bronchoconstriction induced by i.v. ACh at lower doses than are required to block bronchoconstriction induced by electrically stimulating the vagus nerves. Bronchoconstriction, induced by either electrically stimulating both vagus nerves (10 Hz, 10 V, 0.2 ms, 5 s pulse train, at 2 min intervals) or by i.v. ACh (4 $\mu g \cdot kg^{-1}$, every 6 min between vagal stimulations), was measured in vagotomized guinea-pigs that were not sensitized or challenged. Bronchoconstriction before i.v. drug administration is shown on the left as an increase in pulmonary inflation pressure in mmH_2O (A), and on the right as a percentage of this bronchoconstriction (B). All animals received gallamine (3 $mg \cdot kg^{-1}$, i.v.) to block neuronal M_2 muscarinic receptors. Cumulative doses of tiotropium inhibited bronchoconstriction in a dose-dependent manner (solid lines, B), but it was significantly more effective against i.v. ACh-induced bronchoconstriction than it was against vagally induced bronchoconstriction. Lactose (dashed lines, B) was administered as a control and concentrations are shown on the upper axis. Bronchoconstriction was also inhibited with lactose, although the effect was not significantly different between i.v. ACh-induced bronchoconstriction and vagally-induced bronchoconstriction (B). Data are expressed as means ± SEM, n = 3. Inhibition of i.v. ACh-induced bronchoconstriction was compared with inhibition of vagally-induced bronchoconstriction for each treatment (lactose and tiotropium); significance is noted with †. Inhibition by tiotropium of bronchoconstriction induced by vagal stimulation or i.v. ACh was also compared at each dose to inhibition in the lactose control; significance is noted with *.

concentration was not tested. Regardless, the inhibitory effect of tiotropium was substantially greater than lactose.

Determining whether tiotropium blocks cardiac M_2 receptors

In the heart, ACh stimulates M_2 muscarinic receptors to cause bradycardia. Neither i.s. tiotropium nor i.s. lactose vehicle affected bradycardia induced by i.v. ACh or by electrical stimulation of the vagus nerves relative to untreated sensitized controls 24 h after insufflation into the lungs (Figure 5), which would be at the time of antigen challenge. These data indicate that cardiac M_2 receptors were not blocked by tiotropium at the time of antigen challenge.

Similarly, in the antigen challenge model, bradycardia induced by either i.v. ACh or electrical stimulation of the vagus nerves in sensitized (Figure 6, A and C) and sensitized and challenged (Figure 6, B and D) guinea-pigs was unchanged by antigen challenge, lactose pretreatment, tiotropium pretreatment or atropine pretreatment (Figure 6). Thus, cardiac M_2 muscarinic receptors were not blocked by tiotropium or atropine at the time physiological measurements were made (48 h after tiotropium and 18 h after atropine administration).

Effect of M_3 receptor blockade during antigen challenge on airway inflammation

We tested whether tiotropium pretreatment had any effect on airway inflammation. Insufflating powder into the lungs, regardless of whether it contained tiotropium, increased total bronchoalveolar cells (Figure 7). Both 24 h (Figure 7) and 48 h (Figure 8) after lactose or tiotropium powder administration, neutrophils were significantly increased relative to untreated sensitized controls. Antigen challenge did not further increase this neutrophilic inflammation (Figure 8).

Antigen challenge increased eosinophils in bronchoalveolar lavage of sensitized guinea-pigs regardless of whether they received no treatment, lactose, tiotropium or atropine pretreatment. However, this was only statistically significant following correction for multiple comparisons in animals treated with lactose 24 h prior to challenge (Figure 8).

In lung tissue, quantitative analysis of eosinophils in airway smooth muscle and adventitia, and around airway nerves demonstrated that antigen challenge of sensitized guinea-pigs increased total and nerve-associated eosinophils in lactose (vehicle)-pretreated animals (Figure 9). In tiotropium-pretreated guinea-pigs, antigen challenge did not significantly increase either total eosinophils or nerve-associated eosinophils (Figure 9). Thus, tiotropium inhibited eosinophilic inflammation following antigen challenge in airway tissues. In the absence of lactose, there was a similar increase in total airway eosinophils from 264 ± 33 eosinophils·mm^{-2} in sensitized guinea-pigs to 437 ± 73 eosinophils·mm^{-2} in sensitized and challenged guinea-pigs. Additionally, eosinophils specifically associated with airway nerves were also increased from 67 ± 13 eosinophils mm^{-2} in sensitized guinea-pigs to 134 ± 27 eosinophils·mm^{-2} in sensitized and challenged animals. In the presence of atropine, there were 379 ± 48 total airway eosinophils mm^{-2} and 126 ± 28 eosinophils·mm^{-2} associated with nerves in sensitized and challenged guinea-pigs.

Discussion

The data presented here demonstrate that selectively blocking M_3 muscarinic receptors during antigen challenge prevents subsequent development of airway hyperreactivity in guinea-pigs. Muscarinic receptor antagonists with 100-fold higher affinity for M_3 receptors over other muscarinic receptor subtypes are not readily available; therefore, we used tiotropium, a clinically used (GOLD, 2010; Peters *et al.*, 2010; Vogelmeier *et al.*, 2011), kinetically selective, M_3 receptor

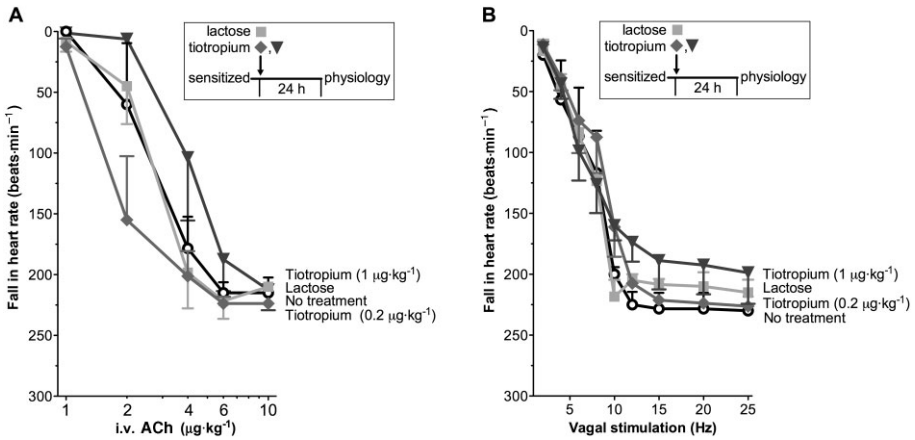

Figure 5

Tiotropium does not block M_2 muscarinic receptors in the heart 24 h after administration. In vagotomized, sensitized guinea-pigs, i.v. ACh caused dose-dependent bradycardia measured as a fall in heart rate in beats·min^{-1} (A). Electrical stimulation of both vagus nerves caused frequency-dependent bradycardia (B; 2–25 Hz, 10 V, 0.2 ms, 5 s pulse train). Neither lactose vehicle, 0.2 µg·kg^{-1} i.s. tiotropium, nor 1 µg·kg^{-1} i.s. tiotropium affected bradycardia induced by i.v. ACh (A) or vagal stimulation (B) 24 h after administration. Data are expressed as means ± SEM, n = 3–4. None of the dose- and frequency-response curves were statistically different from the no treatment group (open circles).

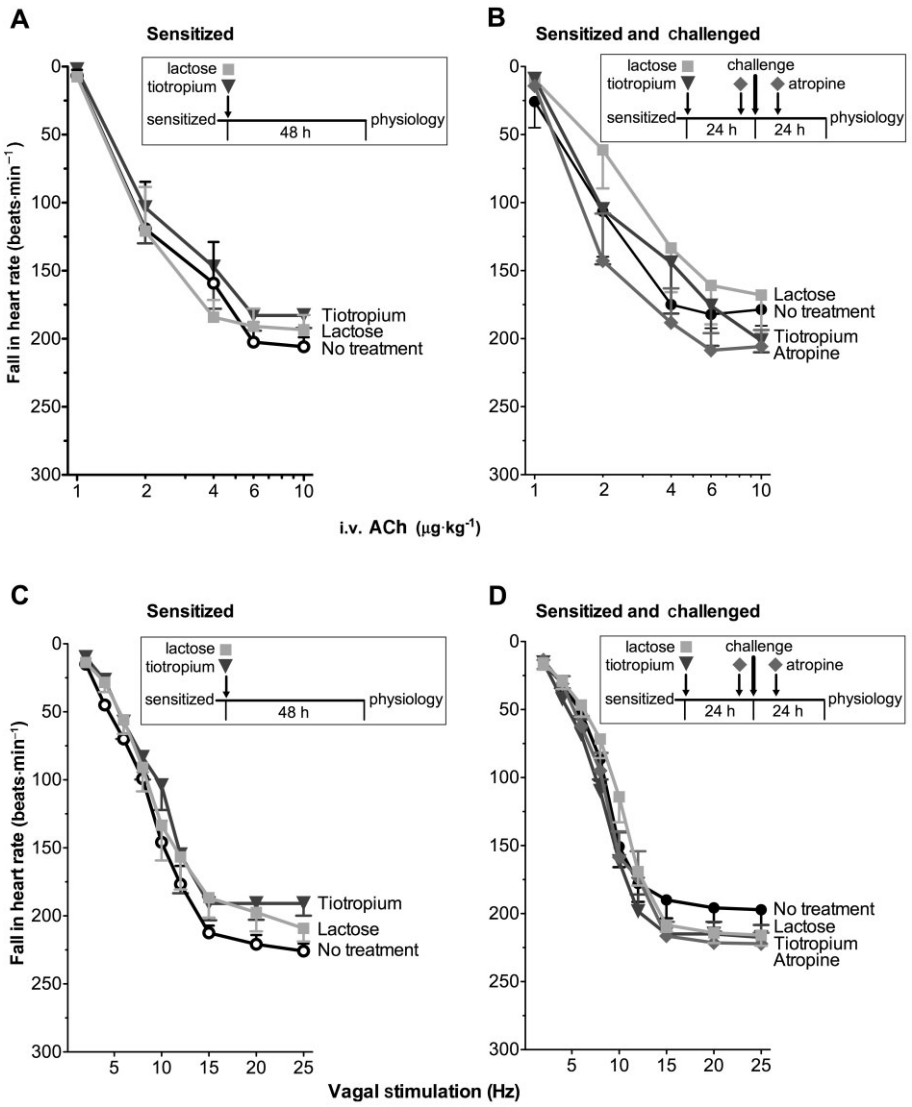

Figure 6

Lactose, tiotropium and atropine pretreatment each had no affect on bradycardia in sensitized or sensitized and challenged guinea-pigs. In vagotomized, sensitized guinea-pigs, i.v. ACh caused dose-dependent bradycardia (A) and electrical stimulation of both vagus nerves (2–25 Hz, 10 V, 0.2 ms, 5 s pulse train) caused frequency-dependent bradycardia (C), both measured as a fall in heart rate in beats·min^{-1}. Antigen challenge did not change bradycardia (B and D). Neither lactose nor tiotropium (1 μg·kg^{-1}, i.s.) changed bradycardia measured 48 h after administration in sensitized (A and C) or sensitized and challenged guinea-pigs (B and D). Similarly, atropine (1 mg·kg^{-1}, i.p.) administered 1 h before and 6 h after antigen challenge also did not change bradycardia in sensitized and challenged animals. Data are expressed as means ± SEM, n = 6–7. None of the dose- and frequency-response curves were statistically different from the sensitized, no treatment controls (open circles).

antagonist. Tiotropium was administered 24 h before antigen challenge to take advantage of its slow dissociation from M_3 receptors relative to M_2 and M_1 receptors (Disse *et al.*, 1993; Casarosa *et al.*, 2009). Functional studies in guinea-pig trachea show M_3 receptor function begins to return 9 h after tiotropium washout, while M_2 receptor function returns fully within 2 h (Takahashi *et al.*, 1994). In our study, we found no evidence for pharmacological blockade of M_2 muscarinic receptors 24 h after tiotropium administration since i.v. ACh-induced bradycardia was not reduced. However, as previously shown for guinea-pigs (Villetti *et al.*, 2006) tiotropium still blocked M_3 receptors on airway smooth muscle when tested with i.v. ACh.

Baseline heart rate was not changed with antigen challenge or pretreatments. However, there was a significant 20 mmH_2O increase in baseline pulmonary inflation pressure in sensitized guinea-pigs pretreated with tiotropium. This increase is unlikely to be physiologically significant, since greater increases (150 mmH_2O) following ozone do not suppress bronchoconstriction (Verhein *et al.*, 2008). We were unable to determine whether tiotropium pretreatment reduced vagal tone in our animals, since

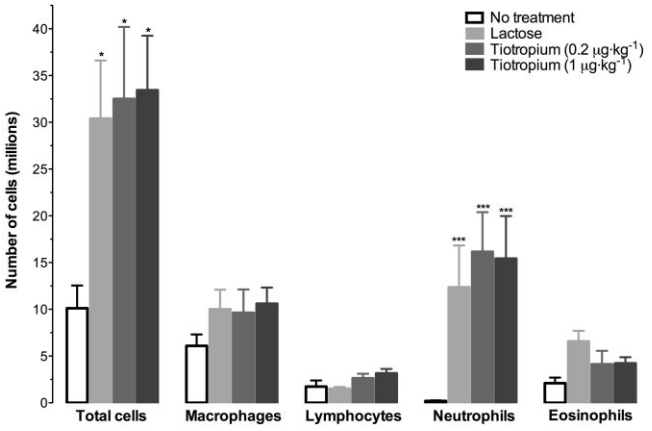

Figure 7

Neutrophils in bronchoalveolar lavage were increased 24 h after insufflation of lactose powder regardless of whether it also contained tiotropium. Inflammatory cells were counted in bronchoalveolar lavage of sensitized guinea-pigs. Lactose vehicle, 0.2 μg·kg^{-1} i.s. tiotropium, and 1 μg·kg^{-1} i.s. tiotropium all increased total and neutrophil cell numbers in sensitized guinea-pigs. Data are expressed as means ± SEM, $n = 3–4$. Mean leukocyte numbers were compared with the no treatment group; statistically significant changes are noted with *.

baseline pulmonary inflation pressure was measured after vagotomy.

We have demonstrated that airway hyperreactivity develops within 24 h of antigen challenge in sensitized guinea-pigs (Evans *et al.*, 1997, 2001; Fryer *et al.*, 2006; Verbout *et al.*, 2007). This was confirmed in these experiments where bronchoconstriction induced by electrical stimulation of both vagus nerves was significantly increased in sensitized and challenged guinea-pigs relative to sensitized controls. Airway hyperreactivity was mediated by the vagus nerves, since airway smooth muscle contraction to i.v. ACh, which bypasses the nerves in vagotomized animals, was not similarly increased. Tiotropium pretreatment completely prevented hyperreactivity in antigen-challenged guinea-pigs, but did not block smooth muscle M_3 muscarinic receptors innervated by the vagus nerves. This suggests that tiotropium prevents airway hyperreactivity in antigen-challenged guinea-pigs through a mechanism that is separate from inhibition of bronchoconstriction.

Eosinophilic inflammation correlates with asthma severity (Bousquet *et al.*, 1990). In patients with eosinophilic asthma, reducing lung eosinophils significantly decreases asthma exacerbations and allows decreased steroid use (Haldar *et al.*, 2009; Nair *et al.*, 2009). In antigen-challenged guinea-pigs, hyperreactivity is mediated by eosinophil recruitment to airway nerves and subsequent activation and release of eosinophil major basic protein (Costello *et al.*, 1997; Evans *et al.*, 1997, 2001; Verbout *et al.*, 2007). Tiotropium reduced eosinophil accumulation in airway tissue and around airway nerves following antigen challenge. This could be a mechanism for preventing airway hyperreactivity, since airway hyperreactivity is also prevented by treatments that inhibit eosinophil association with nerves or inhibit deposition of eosinophil major basic protein on nerves (Evans *et al.*, 1997, 2001; Fryer *et al.*, 2006; Nie *et al.*, 2009). It is noteworthy that bronchoalveolar neutrophils were increased by powder insufflation into the lungs. However, increased neutrophils were not associated with hyperreactivity, so the presence of neutrophils alone is not sufficient to cause airway hyperreactivity.

Tiotropium has already been shown to reduce airway remodelling, including smooth muscle thickening, smooth muscle hypercontractility and mucous gland hypertrophy. It also reduces Th_2 cytokines and lung eosinophils associated with chronic antigen challenge (Gosens *et al.*, 2005; Bos *et al.*, 2007; Ohta *et al.*, 2010). Furthermore, tiotropium's anti-inflammatory effects are not limited to antigen challenge since tiotropium also reduces cytokine production and neutrophil accumulation following cigarette smoke exposure in mice (Wollin and Pieper, 2010). Together, these studies support our data that tiotropium may prevent vagally mediated airway hyperreactivity by reducing inflammation.

Since smooth muscle M_3 receptors innervated by the nerves were not blocked at the time of antigen challenge, it is possible that tiotropium prevented airway hyperreactivity by blocking ACh released from non-neuronal sources such as epithelial cells or macrophages (Wessler and Kirkpatrick, 2008). Tiotropium could reduce eosinophil trafficking into tissue by blocking M_3 receptors on airway epithelial cells or alveolar macrophages that normally induce release of eosinophil chemoattractants (Koyama *et al.*, 1998; Sato *et al.*, 1998; Buhling *et al.*, 2007). Tiotropium could also reduce eosinophil accumulation by blocking M_3 muscarinic receptors on eosinophils (Verbout *et al.*, 2006). But, the role of M_3 muscarinic receptors on eosinophils is unknown. In addition, tiotropium could block M_3 receptors on smooth muscle cells not innervated by nerves to inhibit pro-inflammatory cytokine release (Gosens *et al.*, 2009) following antigen challenge.

In contrast to selectively blocking M_3 receptors, blockade of all muscarinic receptors with atropine during antigen challenge was less effective in preventing airway hyperreactivity, suggesting that blockade of all muscarinic receptors may counteract the benefit of blocking M_3 receptors. For example, M_1 muscarinic receptors on human airway mast cells inhibit evoked histamine release (Reinheimer *et al.*, 2000), and it may be important to maintain the activity of these inhibitory muscarinic receptors. Alternatively, differences in atropine and tiotropium kinetics might explain differences in prevention of airway hyperreactivity, since tiotropium, but not atropine, blocked some smooth muscle M_3 receptors when airway responsiveness was measured. However, this seems unlikely because neither drug blocked smooth muscle M_3 receptors innervated by vagal nerves (Figure 1A and Verbout *et al.*, 2007).

We have previously shown that atropine at the time of antigen challenge makes airway hyperreactivity significantly worse (Verbout *et al.*, 2007, 2009). Although, we were unable to see similar potentiation in this study, atropine was not completely protective either. Thus, tiotropium, but not the non-selective atropine, prevented development of hyperreactivity. Lack of atropine-induced hyperreactivity in our study may be explained by the additional use of antihistamines and

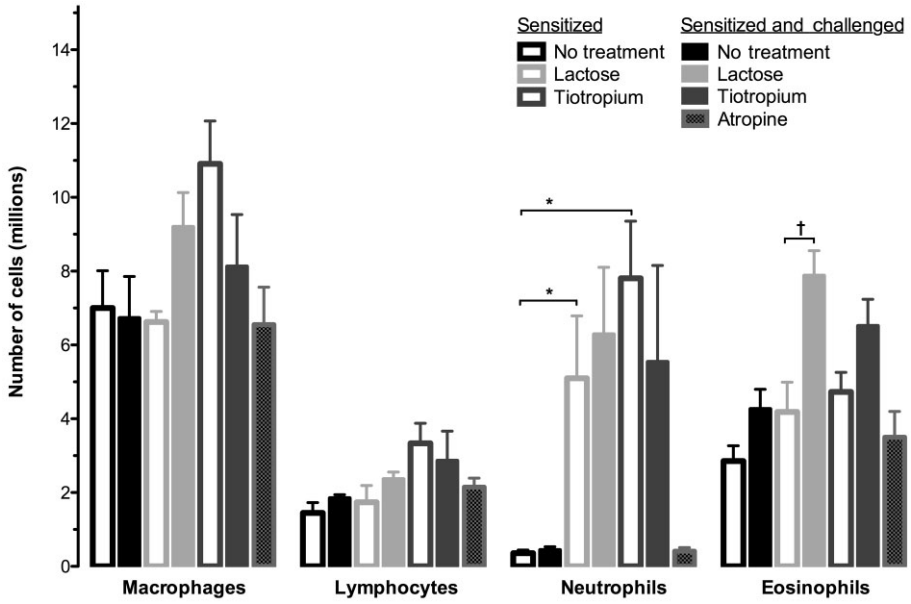

Figure 8

Neutrophils in bronchoalveolar lavage remain increased 48 h after powder insufflation. Inflammatory cells were counted in bronchoalveolar lavage of sensitized guinea-pigs and sensitized and challenged guinea-pigs. Neutrophils increased following pretreatment with lactose vehicle or 1 $\mu g \cdot kg^{-1}$ i.s. tiotropium relative to sensitized controls that received no treatment. Antigen challenge did not increase neutrophils further in animals that received no pretreatment, lactose, tiotropium, or atropine (1 $mg \cdot kg^{-1}$, i.p.) pretreatment. Antigen challenge did increase eosinophils, and this was significant in animals pretreated with lactose vehicle. Values are means ± SEM, n = 5–7. Mean leukocyte numbers in sensitized animals pretreated with lactose or tiotropium were compared with sensitized animals that received no treatment; significant changes are noted with *. Each antigen-challenged group was also compared with the appropriate unchallenged control group; significance is noted with †.

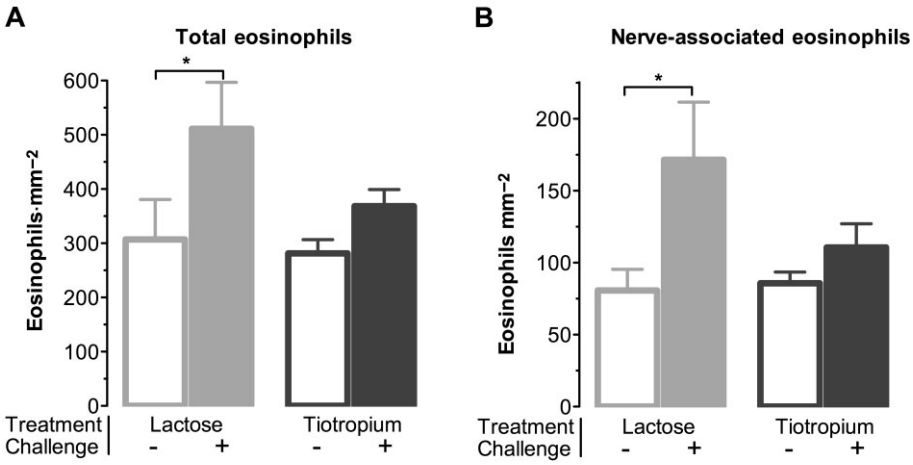

Figure 9

Tiotropium reduces total and nerve-associated eosinophils in the lungs of antigen-challenged guinea-pigs. Total (A) and nerve-associated (B, within 8 μm of a nerve) eosinophils were counted per square millimetre airway smooth muscle and adventitia in tissue sections. Nerves were labelled with antibody to protein gene product 9.5, and eosinophils were stained with chromotrope 2R. Eosinophils are found in the lungs and around nerves in sensitized guinea-pigs pretreated with lactose vehicle or tiotropium (1 $\mu g \cdot kg^{-1}$, i.s.). Antigen challenge increased total and nerve-associated eosinophils in sensitized guinea-pigs pretreated with lactose. In contrast, in sensitized guinea-pigs that received tiotropium 24 h prior to challenge, total and nerve-associated eosinophils were not significantly increased following antigen challenge. Data are expressed as means ± SEM, n = 6–7. Comparisons were made between all groups; statistically significant changes are noted with *.

isoprenaline (β-agonist) in the previous studies, while neither drug was used here.

During the course of these studies, we also observed that i.s. tiotropium blocked bronchoconstriction induced by i.v. ACh but did not block bronchoconstriction induced by vagally released ACh in sensitized guinea- pigs. These data are consistent with previous studies using atropine in humans with asthma and in anaesthetized dogs (Sheppard *et al.*, 1982, 1983; Holtzman *et al.*, 1983) that demonstrate there are several variables contributing to the efficacy of a competitive muscarinic antagonist. These include dose of antagonist, route of antagonist administration and regional differences in agonist concentration. In these previously published studies, low doses of inhaled atropine blocked bronchoconstriction induced by inhaled ACh (or methacholine) and also maximally decreased baseline vagally mediated cholinergic tone. However, much higher doses of atropine (8- to 16-fold) were required to block bronchoconstriction induced by either indirect (cold air) or direct (electrical) stimulation of the vagus nerves (Sheppard *et al.*, 1982; Holtzman *et al.*, 1983). Thus, similar to atropine, we show that doses of i.s. tiotropium that block exogenous ACh do not block vagally-induced bronchoconstriction. This is important because our data and these papers confirm that blockade of exogenous ACh-induced bronchoconstriction is not an adequate measure of blockade of ACh from its physiological source, the vagus nerves. Furthermore, these data demonstrate that inhibition of vagally-induced bronchoconstriction is not necessary to prevent subsequent development of airway hyperreactivity.

The difference in atropine's ability to inhibit bronchoconstriction induced by inhaled ACh and vagally-released ACh only occurs when atropine is administered by inhalation (Holtzman *et al.*, 1983; Sheppard *et al.*, 1983). Intravenous atropine distributes evenly to all airway smooth muscle muscarinic receptors and equally blocks bronchoconstriction induced by exogenous and endogenous ACh (Holtzman *et al.*, 1983). In contrast, in our study, i.v. tiotropium did not equally block exogenous ACh and vagally-induced bronchoconstriction; significantly higher doses were required to block vagally-induced bronchoconstriction. These data indicate that the dose of tiotropium is critical for bronchodilatation, independent of delivery route.

Tiotropium is a competitive antagonist (Casarosa *et al.*, 2009), but its slow dissociation kinetics make it functionally irreversible at M_3 receptors. Our data suggest a model where tonic neuronal stimulation maintains high local concentrations of ACh at smooth muscle M_3 receptors, while M_3 receptors further away from the nerves are exposed to lower endogenous ACh concentrations. Tiotropium would have to compete with ACh for receptor sites, and this would require more tiotropium around nerves where agonist concentrations are high. Thus, even though i.s. tiotropium blocked bronchoconstriction induced by exogenous agonists, it would require a higher dose to block vagally-induced bronchoconstriction.

In summary, the data presented here demonstrate that selectively blocking M_3 muscarinic receptors with tiotropium at the time of antigen challenge prevents subsequent vagally-mediated airway hyperreactivity. Importantly, airway hyperreactivity is prevented by a dose of tiotropium that is unable to inhibit vagally-induced bronchoconstriction. Furthermore, our data suggest that the mechanism may be anti-inflammatory, and that selective blockade of airway M_3 muscarinic receptors may be an effective treatment for asthma separate from bronchodilatation.

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Conflicts of interest

This research was partially funded by a grant from GlaxoSmithKline.

References

Bos IS, Gosens R, Zuidhof AB, Schaafsma D, Halayko AJ, Meurs H *et al.* (2007). Inhibition of allergen-induced airway remodelling by tiotropium and budesonide: a comparison. Eur Respir J 30: 653–661.

Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I *et al.* (1990). Eosinophilic inflammation in asthma. N Engl J Med 323: 1033–1039.

Brown JR, Kleimberg J, Marini M, Sun G, Bellini A, Mattoli S (1998). Kinetics of eotaxin expression and its relationship to eosinophil accumulation and activation in bronchial biopsies and bronchoalveolar lavage (BAL) of asthmatic patients after allergen inhalation. Clin Exp Immunol 114: 137–146.

Buhling F, Lieder N, Kuhlmann UC, Waldburg N, Welte T (2007). Tiotropium suppresses acetylcholine-induced release of chemotactic mediators *in vitro*. Respir Med 101: 2386–2394.

Canning BJ (2006). Reflex regulation of airway smooth muscle tone. J Appl Physiol 101: 971–985.

Casarosa P, Bouyssou T, Germeyer S, Schnapp A, Gantner F, Pieper M (2009). Preclinical evaluation of long-acting muscarinic antagonists: comparison of tiotropium and investigational drugs. J Pharmacol Exp Ther 330: 660–668.

Chung KF, Becker AB, Lazarus SC, Frick OL, Nadel JA, Gold WM (1985). Antigen-induced airway hyperresponsiveness and pulmonary inflammation in allergic dogs. J Appl Physiol 58: 1347–1353.

Cockcroft DW, Ruffin RE, Dolovich J, Hargreave FE (1977). Allergen-induced increase in non-allergic bronchial reactivity. Clin Allergy 7: 503–513.

Costello RW, Schofield BH, Kephart GM, Gleich GJ, Jacoby DB, Fryer AD (1997). Localization of eosinophils to airway nerves and effect on neuronal M2 muscarinic receptor function. Am J Physiol 273 (1 Pt 1): L93–103.

Disse B, Reichl R, Speck G, Traunecker W, Ludwig Rominger KL, Hammer R (1993). Ba 679 BR, a novel long-acting anticholinergic bronchodilator. Life Sci 52: 537–544.

EPR-3 (2007). Expert Panel Report 3 (EPR-3): guidelines for the Diagnosis and Management of Asthma-Summary Report 2007. J Allergy Clin Immunol 120 (Suppl.): S94–S138.

Evans CM, Fryer AD, Jacoby DB, Gleich GJ, Costello RW (1997). Pretreatment with antibody to eosinophil major basic protein prevents hyperresponsiveness by protecting neuronal M2 muscarinic receptors in antigen-challenged guinea pigs. J Clin Invest 100: 2254–2262.

Evans CM, Jacoby DB, Fryer AD (2001). Effects of dexamethasone on antigen-induced airway eosinophilia and M(2) receptor dysfunction. Am J Respir Crit Care Med 163: 1484–1492.

Fryer AD, Jacoby DB (2008). Cholinergic mechanisms and anticholinergic therapy in respiratory diseases. In: Adkinson NF Jr, Busse WW, Bochner BS, Holgate ST, Simons FER, Lemanske RF (eds). *Middleton's Allergy: Principles and Practice*, 7th edn. Elsevier: Philadelphia, PA, pp. 1603–1618.

Fryer AD, Maclagan J (1987). Ipratropium bromide potentiates bronchoconstriction induced by vagal nerve stimulation in the guinea-pig. Eur J Pharmacol 139: 187–191.

Fryer AD, Stein LH, Nie Z, Curtis DE, Evans CM, Hodgson ST *et al*. (2006). Neuronal eotaxin and the effects of CCR3 antagonist on airway hyperreactivity and M2 receptor dysfunction. J Clin Invest 116: 228–236.

GOLD (2010). *Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease*. Global Initiative for Chronic Obstructive Lung Disease. Available at: http//www.goldcopd.org (accessed 11 November 2011).

Gold WM, Kessler GF, Yu DY (1972). Role of vagus nerves in experimental asthma in allergic dogs. J Appl Physiol 33: 719–725.

Gosens R, Bos IS, Zaagsma J, Meurs H (2005). Protective effects of tiotropium bromide in the progression of airway smooth muscle remodeling. Am J Respir Crit Care Med 171: 1096–1102.

Gosens R, Rieks D, Meurs H, Ninaber DK, Rabe KF, Nanninga J *et al*. (2009). Muscarinic M3 receptor stimulation increases cigarette smoke-induced IL-8 secretion by human airway smooth muscle cells. Eur Respir J 34: 1436–1443.

Haldar P, Brightling CE, Hargadon B, Gupta S, Monteiro W, Sousa A *et al*. (2009). Mepolizumab and exacerbations of refractory eosinophilic asthma. N Engl J Med 360: 973–984.

Holtzman MJ, McNamara MP, Sheppard D, Fabbri LM, Hahn HL, Graf PD *et al*. (1983). Intravenous versus inhaled atropine for inhibiting bronchoconstrictor responses in dogs. J Appl Physiol 54: 134–139.

Kentala E, Kaila T, Iisalo E, Kanto J (1990). Intramuscular atropine in healthy volunteers: a pharmacokinetic and pharmacodynamic study. Int J Clin Pharmacol Ther Toxicol 28: 399–404.

Koyama S, Sato E, Nomura H, Kubo K, Nagai S, Izumi T (1998). Acetylcholine and substance P stimulate bronchial epithelial cells to release eosinophil chemotactic activity. J Appl Physiol 84: 1528–1534.

Nair P, Pizzichini MM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E *et al*. (2009). Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. N Engl J Med 360: 985–993.

Nie Z, Jacoby DB, Fryer AD (2009). Etanercept prevents airway hyperresponsiveness by protecting neuronal M2 muscarinic receptors in antigen-challenged guinea pigs. Br J Pharmacol 156: 201–210.

Ohta S, Oda N, Yokoe T, Tanaka A, Yamamoto Y, Watanabe Y *et al*. (2010). Effect of tiotropium bromide on airway inflammation and remodelling in a mouse model of asthma. Clin Exp Allergy 40: 1266–1275.

Peters SP, Kunselman SJ, Icitovic N, Moore WC, Pascual R, Ameredes BT *et al*. (2010). Tiotropium bromide step-up therapy for adults with uncontrolled asthma. N Engl J Med 363: 1715–1726.

Rasband WS (1997–2009). ImageJ. U. S. National Institutes of Health: Bethesda, MD. Available at: http://imagej.nih.gov/ij/ (accessed 11 November 2011).

Reinheimer T, Mohlig T, Zimmermann S, Hohle KD, Wessler I (2000). Muscarinic control of histamine release from airways. Inhibitory M1-receptors in human bronchi but absence in rat trachea. Am J Respir Crit Care Med 162 (2 Pt 1): 534–538.

Sato E, Koyama S, Okubo Y, Kubo K, Sekiguchi M (1998). Acetylcholine stimulates alveolar macrophages to release inflammatory cell chemotactic activity. Am J Physiol 274 (6 Pt 1): L970–L979.

Sheppard D, Epstein J, Holtzman M, Nadel J, Boushey H (1982). Dose-dependent inhibition of cold air-induced bronchoconstriction by atropine. Respir Environ Exerc Physiol 53: 169–174.

Sheppard D, Epstein J, Holtzman MJ, Nadel JA, Boushey HA (1983). Effect of route of atropine delivery on bronchospasm from cold air and methacholine. J Appl Physiol 54: 130–133.

Takahashi T, Belvisi MG, Patel H, Ward JK, Tadjkarimi S, Yacoub MH *et al*. (1994). Effect of Ba 679 BR, a novel long-acting anticholinergic agent, on cholinergic neurotransmission in guinea pig and human airways. Am J Respir Crit Care Med 150 (6 Pt 1): 1640–1645.

Thevenaz P, Unser M (2007). User-friendly semiautomated assembly of accurate image mosaics in microscopy. Microsc Res Tech 70: 135–146.

Verbout NG, Lorton JK, Jacoby DB, Fryer AD (2006). A functional role for muscarinic receptors on eosinophils in the airways. Proc Am Thorac Soc 3: A587.

Verbout NG, Lorton JK, Jacoby DB, Fryer AD (2007). Atropine pretreatment enhances airway hyperreactivity in antigen-challenged guinea pigs through an eosinophil-dependent mechanism. Am J Physiol Lung Cell Mol Physiol 292: L1126–L1135.

Verbout NG, Jacoby DB, Gleich GJ, Fryer AD (2009). Atropine-enhanced, antigen challenge-induced airway hyperreactivity in guinea pigs is mediated by eosinophils and nerve growth factor. Am J Physiol Lung Cell Mol Physiol 297: L228–L237.

Verhein KC, Jacoby DB, Fryer AD (2008). IL-1 receptors mediate persistent, but not acute, airway hyperreactivity to ozone in guinea pigs. Am J Respir Cell Mol Biol 39: 730–738.

Villetti G, Bergamaschi M, Bassani F, Bolzoni PT, Harrison S, Gigli PM *et al*. (2006). Pharmacological assessment of the duration of action of glycopyrrolate vs tiotropium and ipratropium in guinea-pig and human airways. Br J Pharmacol 148: 291–298.

Vogelmeier C, Hederer B, Glaab T, Schmidt H, Rutten-van Molken MP, Beeh KM *et al*. (2011). Tiotropium versus salmeterol for the prevention of exacerbations of COPD. N Engl J Med 364: 1093–1103.

Ward MJ, Fentem PH, Smith WH, Davies D (1981). Ipratropium bromide in acute asthma. Br Med J (Clin Res Ed) 282: 598–600.

Wessler I, Kirkpatrick CJ (2008). Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. Br J Pharmacol 154: 1558–1571.

Wollin L, Pieper MP (2010). Tiotropium bromide exerts anti-inflammatory activity in a cigarette smoke mouse model of COPD. Pulm Pharmacol Ther 23: 345–354.