

Bronchoprotection by Olodaterol Is Synergistically Enhanced by Tiotropium in a Guinea Pig Model of Allergic Asthma

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ABSTRACT

The novel once-daily β_2 -agonist bronchodilator drug olodaterol has recently been shown to be effective in patients with allergic asthma for >24 hours. An increased cholinergic tone common to these patients may decrease the effectiveness of β_2 -agonists. This could provide a rationale for combination therapy with olodaterol and the long-acting anticholinergic tiotropium to aim for a once-daily treatment regimen. In guinea pigs, we evaluated the protective effects of olodaterol, alone and in combination with tiotropium, on airway responsiveness to histamine, which is partially mediated by a cholinergic reflex mechanism. In addition, using a guinea pig model of acute allergic asthma, we examined the cooperative effects of these bronchodilators on allergen-induced early (EAR) and late (LAR) asthmatic reactions, airway hyper-responsiveness (AHR) to histamine, and airway

inflammation. It was demonstrated that the protective effect of olodaterol against histamine-induced bronchoconstriction was synergistically enhanced and prolonged in the presence of tiotropium. In addition, tiotropium synergistically augmented both the reversal of and the protection against the allergen-induced AHR after the EAR by olodaterol. Olodaterol and tiotropium were highly effective in inhibiting the magnitude of the allergen-induced EAR and LAR, and both reactions were fully inhibited by the combination of these drugs. It is remarkable that these effects were not associated with an effect on inflammatory cell infiltration in the airways. In conclusion, the results indicate that combination therapy with olodaterol and tiotropium may be highly effective in the treatment of allergen-induced asthmatic reactions and AHR.

Introduction

Allergic asthma is a chronic inflammatory disorder of the airways. Characteristic features of allergic asthma are allergen-induced early (EAR) and late (LAR) asthmatic reactions, transient airway hyper-responsiveness (AHR) after these reactions, and airway inflammation. In addition, structural changes in the airway wall may develop, associated with a progressive decline in lung function and chronic AHR (Bousquet et al., 2000; Cockcroft and Davis, 2006; Meurs et al., 2008).

Inhaled β_2 -adrenoceptor agonists are presently the mainstay bronchodilator therapy for asthma (www.ginasthma.org). Short-acting β_2 -agonists, such as salbutamol and terbutaline, are used as reliever therapy for episodes of dyspnea, whereas long-acting β_2 -agonists, such as formoterol and salmeterol, are used as controller therapy for persistent asthma in combination with inhaled corticosteroids. According to current guidelines, short-acting anticholinergics, such as ipratropium and oxitropium, may be used as alternative bronchodilators to relieve symptoms; however, these drugs are generally less effective than β_2 -agonists (Gross, 2006).

The bronchodilating effectiveness of β_2 -agonists is dependent on functional antagonism by bronchoconstricting agents. Thus, studies in human (Raffestin et al., 1985; Van Amsterdam et al., 1990) and animal (Torphy et al., 1985; Van Amsterdam et al., 1989b) airway smooth muscle preparations have demonstrated that the potency and efficacy of β_2 -agonists are gradually reduced in the presence of increasing concentrations of contractile stimuli, including muscarinic receptor agonists and histamine. The reduced β -adrenergic responsiveness may importantly be attributed to cross-talk between G_q -coupled muscarinic M_3 - or histamine H_1 -receptors and G_s -coupled β_2 -adrenoceptors (Van Amsterdam et al., 1989b, 1990; Grandordy et al., 1994; Meurs et al., 2001), presumably via protein kinase C-induced phosphorylation of the β_2 -adrenoceptor and/or G_s (Sibley and Lefkowitz, 1985; Pyne et al., 1992a,b; Boterman et al., 2005). In addition, it has been demonstrated that protein kinase C activation enhances β -agonist-induced β_2 -adrenoceptor desensitization in airway smooth muscle (Boterman et al., 2006), which could involve phosphorylation and activation of G-protein-coupled receptor kinase 2 (Chuang et al., 1995, 1996). Evidence for reduced β -adrenergic responsiveness in the presence of increasing concentrations of contractile agonists has also been observed in vivo (Jenne et al., 1987) and could well explain why

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ABBREVIATIONS: AHR, airway hyper-responsiveness; BAL, bronchoalveolar lavage; EAR, early asthmatic reaction; LAR, late asthmatic reaction; OVA, ovalbumin; PBS, phosphate-buffered saline; PC₁₀₀, provocative concentration causing 100% increase in pleural pressure; P_{pi} , pleural pressure.

β_2 -agonists become less effective during severe asthmatic episodes.

Because cholinergic tone is increased in asthma (Gosens et al., 2006), it may be expected that anticholinergics both attenuate bronchoconstriction and potentiate β_2 -agonist-induced bronchodilation by relieving the cholinergic restraint on β_2 -adrenoceptor function. This provides a strong rationale for combination treatment with β_2 -agonists and anticholinergics in this disease. In support, it has previously been demonstrated that treatment with a combination of short-acting anticholinergics and β_2 -agonists may be of benefit in acute severe asthma by improving pulmonary function and reducing the risk of exacerbations and hospital admissions (Lanes et al., 1998; Rodrigo et al., 1999). Moreover, more recent studies demonstrated that the long-acting once-daily anticholinergic tiotropium improved lung function and reduced exacerbations in patients with severe asthma not controlled by standard maintenance therapy (Peters et al., 2010; Kerstjens et al., 2011, 2012).

Combination therapy with long-acting once-daily β_2 -agonists and anticholinergics may be advantageous in improving airflow over a 24-hour period, thereby simplifying treatment regimens as much as possible. The effect of such a combination therapy in asthma is currently unknown. In the present study, we evaluated the effects of the novel once-daily β_2 -agonist olodaterol (O'Byrne et al., 2009; Bouyssou et al., 2010a,b; Casarosa et al., 2011; Van Noord et al., 2011), alone and in combination with tiotropium, on allergen-induced EAR, LAR, AHR, and airway inflammation, using a guinea pig model of acute allergic asthma.

Materials and Methods

Animals

Outbred, male, specified pathogen-free Dunkin Hartley guinea pigs (Harlan, Heathfield, UK), weighing approximately 250 g, were used in this study. Animals used for the allergic asthma model were actively IgE-sensitized to ovalbumin (OVA) as described previously (Van Amsterdam et al., 1989a). In short, 0.5 ml of an allergen solution containing 100 $\mu\text{g/ml}$ OVA and 100 mg/ml $\text{Al}(\text{OH})_3$ in saline was injected intraperitoneally, whereas another 0.5 ml was divided over seven subcutaneous injection sites in the proximity of lymph nodes in the paws, lumbar region, and neck. Two weeks after sensitization, the animals were surgically provided with a balloon catheter in the thoracic cavity, as outlined below. The animals entered the experimental protocol 4–8 weeks after sensitization. All protocols described were approved by the University of Groningen Committee for Animal Experimentation.

Measurement of Airway Function

Airway function was assessed by online measurement of pleural pressure (P_{pl}) under conscious and unrestrained conditions as described previously (Santing et al., 1992; Meurs et al., 2006). In short, a small fluid-filled latex balloon catheter was surgically implanted inside the thoracic cavity. The free end of the cannula was driven subcutaneously to the neck of the animal where it was exposed and attached permanently. Via an external saline-filled cannula, the intrapleural balloon catheter was connected to a pressure transducer (TXX-R; Viggo-Spectramed, Bithoven, The Netherlands), and P_{pl} was continuously measured using an online computer system. By use of a combination of flow measurement with a pneumotachograph implanted inside the trachea and pressure measurement with the intrapleural balloon catheter, it was previously shown that

changes in P_{pl} are linearly related to changes in airway resistance and hence can be used as a sensitive index for allergen- and histamine-induced bronchoconstriction (Santing et al., 1992). In this way, airway function can be monitored continuously and repeatedly for prolonged periods of time, whereas the animals are unaware of the measurements being taken.

Provocations

Histamine and OVA provocations were performed by inhalation of aerosolized solutions. These provocations were carried out in a specially designed perspex cage of 9 liters in which the guinea pigs could move freely, as previously described (Santing et al., 1992; Meurs et al., 2006). A DeVilbiss nebulizer (type 646) driven by an airflow of 8 l/min provided the aerosol with an output of 0.33 ml/min. The animals were habituated to the experimental conditions and the provocation procedure at least 1 week after surgery when preoperative weight was restored, as described previously (Meurs et al., 2006). On the experimental days following the habituation procedure, histamine and allergen provocations were performed as described below. All provocations were preceded by an adaptation period of at least 30 minutes, followed by a control provocation with saline lasting 3 minutes. A baseline P_{pl} value was calculated by averaging the P_{pl} values from the last 20 minutes of the adaptation period.

To assess the airway reactivity to histamine, subsequent provocations with increasing concentration steps (3.13, 6.25, 12.5, 25, 50, 75, 100, 125, 150, 175, and 200 $\mu\text{g/ml}$, and up to 800 $\mu\text{g/ml}$ using 50 $\mu\text{g/ml}$ steps) in saline were performed. Histamine provocations lasted maximally 3 minutes and were separated by 7-minute intervals. Animals were challenged until P_{pl} was increased by >100% above baseline for at least 3 consecutive minutes. The provocation concentration of histamine causing a 100% increase of P_{pl} (PC_{100}) was derived by linear interpolation of the concentration- P_{pl} curve and was used as an index for airway reactivity toward histamine. P_{pl} returned to baseline within 15 minutes after the last histamine provocation.

Allergen provocations were performed by inhalation of 0.05% OVA in saline. The OVA inhalation was discontinued when the first signs of respiratory distress were observed and an increase in P_{pl} of >100% was reached. When this did not occur within 3 minutes, a 0.1% OVA solution in saline was subsequently used.

Bronchoalveolar Lavage

Animals were anesthetized with pentobarbital (Euthasol 20%), administered intraperitoneally. The trachea was exposed and cannulated, and the lungs were lavaged gently using 5 ml of sterile saline at 37°C, followed by three subsequent aliquots of 8 ml of saline. The recovered bronchoalveolar lavage (BAL) samples were kept on ice and centrifuged at 200g for 10 minutes at 4°C. The pellets were combined and resuspended to a final volume of 1.0 ml in phosphate-buffered saline (PBS), and total cell numbers were counted using a Casy cell counter (Model TT; Innovatis, Reutlingen, Germany). For cytologic examination, cytospin preparations were stained with May-Grünwald and Giemsa stain. Cell differentiation was performed by counting at least 400 cells in duplicate.

Protective Effects of Olodaterol, Tiotropium, and Their Combination on Basal Airway Responsiveness to Histamine

PBS- and drug-induced effects on basal histamine responsiveness were established. Thirty minutes after the assessment of basal histamine PC_{100} , an aerosol of PBS (control) or 10, 100, or 1000 μM or 10 mM (nebulizer concentrations) olodaterol in PBS was inhaled for 3 minutes. After these inhalations, histamine PC_{100} values were reassessed 0.5, 2, 6, 12, and 24 hours later to establish the dose- and time-dependent effects of olodaterol on histamine responsiveness. Likewise, the effects of 100 μM tiotropium (3 minutes) and the combination of 1000 μM olodaterol plus 100 μM tiotropium

(3 minutes) were established at 0.5, 6, and 24 hours after inhalation of the drugs to investigate the potentiation of the olodaterol effect by tiotropium. A dose of 100 μM tiotropium (3 minutes) was chosen, because previous studies had shown that this dose is effective in attenuating allergen-induced airway inflammation and remodeling in a guinea pig model of chronic allergic asthma (Gosens et al., 2005; Bos et al., 2007), whereas 1000 μM olodaterol (3 minutes) showed a submaximal effect in the protection study with the β_2 -agonist (see *Results*).

Reversal of Allergen-Induced AHR by Olodaterol, Tiotropium, and Their Combination

Histamine PC_{100} values were assessed 24 hours before OVA challenge (basal) and 5 hours after the allergen challenge to assess AHR after the EAR. PBS, 1000 μM olodaterol, and/or 100 μM tiotropium (nebulizer concentrations, 3 minutes) were inhaled 5.5 hours after allergen challenge, and airway responsiveness was reassessed at 6.5 hours to measure reversibility of allergen-induced AHR. In addition, histamine PC_{100} values were determined 24 hours after allergen challenge to establish treatment effects on the AHR after the LAR.

Prevention of Allergen-Induced AHR, EAR, LAR, and Airway Inflammation by Olodaterol, Tiotropium, and Their Combination

Allergen challenges were performed twice, with a 1-week interval. On the first occasion, histamine sensitivity (PC_{100}) was assessed 24 hours before OVA challenge. One hour before allergen challenge, the animals were treated with inhaled PBS (3 minutes). Subsequently, the animals were challenged with OVA until the first signs of obstruction were visible and P_{pl} increased by $>100\%$ to establish the sensitivity of each animal to the allergen. This protocol was repeated 1 week later, but now the animals were treated with inhaled PBS (3 minutes), tiotropium (100 μM), olodaterol (1000 μM), or the combination of olodaterol (1000 μM) and tiotropium (100 μM) (nebulizer concentrations, 3 minutes) 1 hour before allergen challenge. For the PBS-treated control animals, a 2-fold-higher OVA concentration compared with the first challenge was needed on average to obtain airway obstruction because of minor desensitization to the allergen. Based on this control, 2-fold-higher OVA concentrations compared with the first week were also used for the tiotropium-, olodaterol-, and tiotropium/olodaterol-treated animals. This approach was chosen to minimize the influence of interindividual variation in sensitivity toward the allergen. For quantitative assessment of the EAR and LAR after the various treatments, airway function was continuously measured during the whole procedure. The magnitudes of the EAR and LAR were calculated as areas under the P_{pl} time-response curves between 0 and 5 hours and between 8 and 24 hours, respectively, using trapezoid integration of percent P_{pl} changes over discrete 5-minute intervals. BAL was performed 25 hours after the last allergen challenge. Animals challenged with saline and treated with PBS on both occasions served as controls.

Chemicals. Histamine dihydrochloride, OVA (grade III), and May-Grünwald and Giemsa were obtained from Sigma-Aldrich (St. Louis, MO). Aluminum hydroxide was obtained from Wako Pure Chemical Industries (Osaka, Japan). Euthasol was purchased from Produlab Pharma (Raamsdonksveer, The Netherlands). (1*R*,2*R*,4*S*,5*S*,7*S*)-7-[2-Hydroxy(di-2-thienyl)acetoxy]-9,9-dimethyl-3-oxa-9-azoniatricyclo[3.3.1.0^{2,4}]nonane (tiotropium) and 6-hydroxy-8-[(1*R*)-1-hydroxy-2-[[2-(4-methoxyphenyl)-1,1-dimethylethyl]amino]ethyl]-2*H*-1,4-benzoxazin-3(4*H*)-one monohydrochloride (olodaterol) were provided by Boehringer Ingelheim GmbH (Ingelheim, Germany).

Data Analysis. All data are expressed as means $\pm$ S.E.M. Statistical evaluation of differences was performed using a (repeated-measure) analysis of variance with a Newman-Keuls post hoc

test as appropriate. Synergism between tiotropium and olodaterol was calculated by comparing the calculated sum of the effects of the individual drugs to the measured effect of the combination therapy, using a Student's *t* test. Differences were considered to be statistically significant when $P < 0.05$.

Results

The Protective Effect of Olodaterol on Basal Histamine Responsiveness Is Synergistically Enhanced and Prolonged by Tiotropium. The protective effects of olodaterol, tiotropium, and their combination on basal histamine responsiveness are demonstrated in Figs. 1 and 2. Olodaterol protected against histamine-induced airway obstruction in a dose- and time-dependent manner (Fig. 1). Maximal protection was obtained 0.5 hour after treatment and ranged from 2.7 ± 0.2 -fold for 10 μM up to 7.0 ± 0.2 -fold for 10 mM olodaterol (nebulizer concentrations, 3-minute inhalation). The duration of bronchoprotection was up to 2 hours for 10 μM (2.0 ± 0.2 -fold) to >24 hours for 10 mM olodaterol (2.0 ± 0.3 -fold at 24 hours). PBS inhalations (3 minutes) had no effect on histamine PC_{100} values at the various time points (Fig. 1).

Cotreatment with tiotropium (100 μM , 3 minutes), which had only a minor effect on histamine PC_{100} by itself, markedly increased the protective effect and duration of action of a submaximal dose of olodaterol (1000 μM , 3 minutes) in a synergistic manner (Fig. 2), indicating that endogenous acetylcholine release considerably attenuates β_2 -agonist bronchodilator activity.

The Reversal of Allergen-Induced AHR after the EAR by Olodaterol Is Synergistically Enhanced by Tiotropium. In Figs. 3 and 4, the reversal of established AHR after the EAR by olodaterol, tiotropium, and their combination is shown. Allergen challenge induced a mean AHR to histamine of 8.4 ± 1.2 -fold decrease in PC_{100} 5 hours after challenge (i.e., after the EAR). The allergen-induced

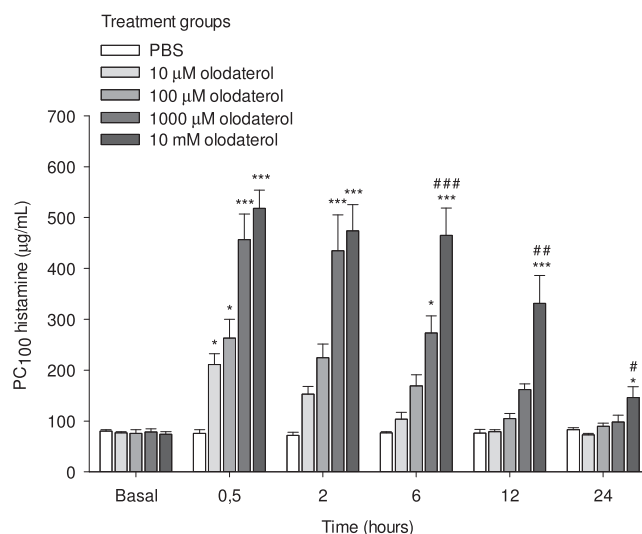


Fig. 1. Protective effects of olodaterol against basal histamine-induced bronchoconstriction. Airway responsiveness to histamine (PC_{100}) was determined 0.5 hour before (basal) and 0.5, 2, 6, 12, and 24 hours after inhalation of PBS or 10, 100, or 1000 μM or 10 mM olodaterol (nebulizer concentrations, 3 minutes) at $t = 0$ hour. At the highest dose, olodaterol protects against histamine-induced airway obstruction for at least 24 hours. Data represent means $\pm$ S.E.M. of 3–5 animals. * $P < 0.05$; *** $P < 0.001$ vs. PBS. # $P < 0.05$ vs. 1000 μM olodaterol; ## $P < 0.01$; ### $P < 0.001$ vs. 1000 μM olodaterol.

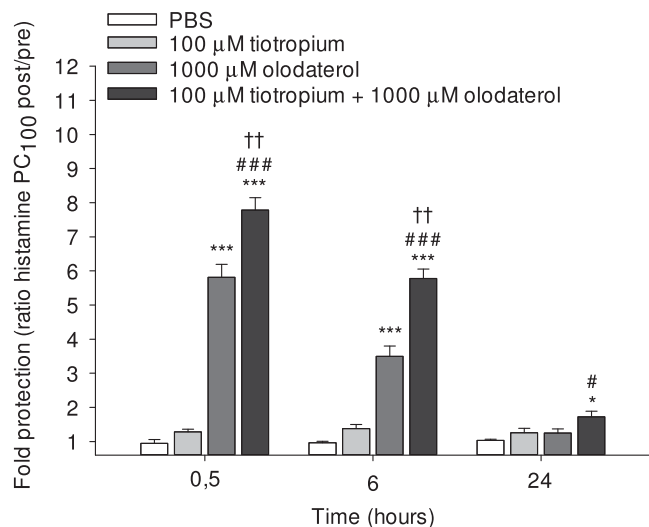


Fig. 2. The protective effect of olodaterol against histamine-induced bronchoconstriction is synergistically enhanced by tiotropium. Histamine PC_{100} values were determined 0.5 hour before and 0.5, 6, and 24 hours after inhalation of 100 μ M tiotropium, 1000 μ M olodaterol, or 100 μ M tiotropium + 1000 μ M olodaterol (nebulizer concentrations, 3 minutes) at $t = 0$ hour. Synergism between olodaterol and tiotropium is observed for all time points after administration, while cotreatment with tiotropium prolongs the protective effect of olodaterol. Data are expressed as the ratio of PC_{100} post-treatment to pretreatment and represent means $\pm$ S.E.M. of 3–5 animals. * $P < 0.05$; *** $P < 0.001$ vs. PBS. # $P < 0.05$; ### $P < 0.001$ vs. olodaterol. †† $P < 0.01$ vs. calculated sum of tiotropium and olodaterol.

AHR was unaffected by inhalation of PBS at 5.5 hours after the challenge (Figs. 3A and 4). By contrast, treatment with olodaterol (1000 μ M, 3 minutes) 5.5 hours after allergen challenge strongly reversed the AHR after the EAR (11.4 ± 3.1 -fold increase in PC_{100} ; Figs. 3C and 4). Tiotropium (100 μ M, 3 minutes), which slightly reversed the AHR by itself

(2.4 ± 0.5 -fold; Figs. 3B and 4), synergistically enhanced the effect of olodaterol to 21.0 ± 2.3 -fold (Figs. 3D and 4). After the LAR, the PBS-treated animals were still hyper-responsive (2.3 ± 0.7 -fold decrease in PC_{100} ; Fig. 3A). Tiotropium (given 5.5 hours after allergen challenge) did not affect this AHR (Fig. 3B), whereas olodaterol, with and without tiotropium, was still fully protective (Fig. 3, C and D).

The Protection against Allergen-Induced AHR after the EAR by Olodaterol Is Synergistically Enhanced by Tiotropium. The protection against allergen-induced AHR after the EAR and LAR by pretreatment with the bronchodilators before allergen challenge is demonstrated in Figs. 5 and 6. To determine the protective effects of olodaterol (1000 μ M, 3 minutes), tiotropium (100 μ M, 3 minutes), and their combination against the development of allergen-induced AHR, drugs were inhaled 1 hour before allergen challenge. In PBS-treated animals (controls), allergen challenge induced AHR to histamine after both the EAR (4.9 ± 1.2 -fold decrease in PC_{100}) and LAR (2.8 ± 1.3 -fold decrease; Figs. 5A and 6). The AHR after the EAR was absent in the animals treated with either olodaterol (2.3 ± 0.2 -fold increase in PC_{100} compared with basal; Figs. 5C and 6) or tiotropium (1.3 ± 0.2 -fold increase; Figs. 5B and 6). When combined, a synergistic 4.8 ± 0.5 -fold increase in PC_{100} was observed (Figs. 5D and 6). After the LAR, AHR was still fully protected by olodaterol, tiotropium, and their combination (1.5 ± 0.2 -, 1.3 ± 0.1 -, and 1.6 ± 0.2 -fold increase in PC_{100} , respectively; Figs. 5, B–D, and 6); however, synergism was no longer observed. No changes in airway responsiveness were observed in the PBS-treated, saline-challenged animals (Fig. 6).

Protection against Allergen-Induced EAR and LAR by Olodaterol, Tiotropium, and Their Combination. Figure 7 demonstrates the effects of olodaterol, tiotropium, and their combination on allergen-induced EAR and LAR, defined as the AUCs of the P_{pl} time-response curve between 0 and 5 hours and

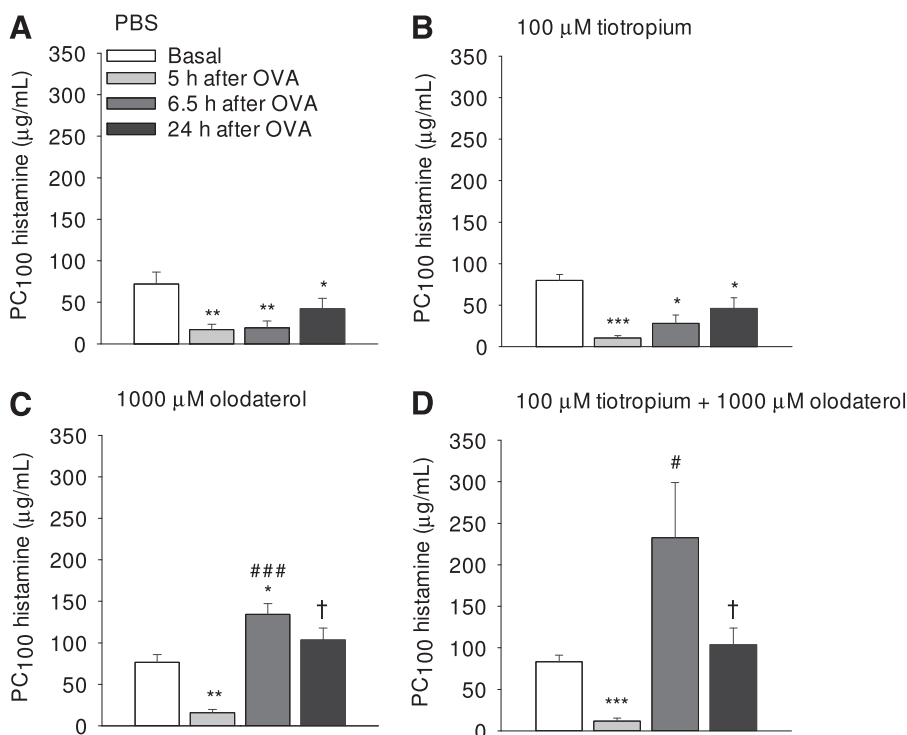


Fig. 3. Effects of olodaterol, tiotropium, and their combination on allergen-induced AHR. Airway responsiveness to histamine (PC_{100}) was determined 24 hours before (basal) and 5 hours after OVA challenge to assess allergen-induced AHR after the EAR. At $t = 5.5$ hours after OVA challenge, the animals inhaled PBS (A), 100 μ M tiotropium (B), 1000 μ M olodaterol (C), or the combination of 1000 μ M olodaterol and 100 μ M tiotropium (D) (nebulizer concentrations, 3 minutes). Drug-induced reversibility of allergen-induced AHR was measured 6.5 hours after OVA challenge. In addition, histamine PC_{100} values were determined 24 hours after allergen challenge to establish treatment effects on the AHR after the LAR. Data represent means $\pm$ S.E.M. of 5–6 animals. * $P \leq 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. basal. # $P < 0.05$; ### $P < 0.001$ vs. PC_{100} at 5 hours. † $P < 0.05$ vs. PBS-treated animals at $t = 24$ hours.

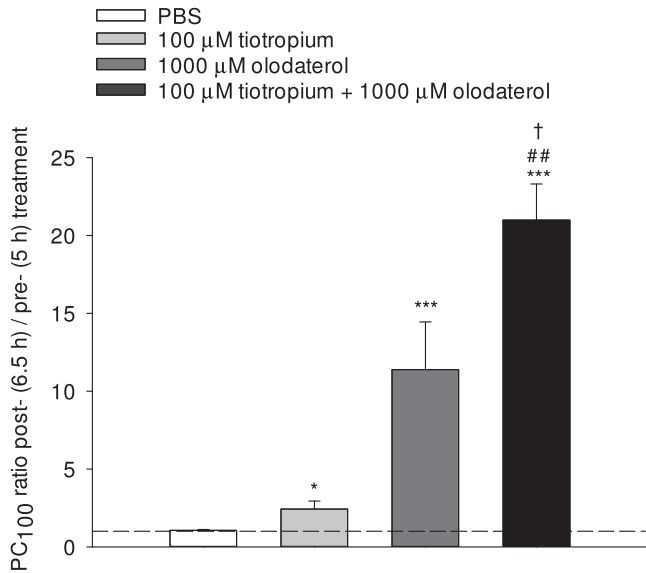


Fig. 4. The reversal of allergen-induced AHR after the EAR by olodaterol is synergistically enhanced by tiotropium. Effects of inhaled PBS, 100 μ M tiotropium, 1000 μ M olodaterol, or the combination of 1000 μ M olodaterol and 100 μ M tiotropium (nebulizer concentrations, 3 minutes) on allergen-induced AHR after the EAR. Data are expressed as the ratio of PC₁₀₀ post-treatment (measured 6.5 hours after challenge) to pretreatment (measured at 5 hours after challenge). Values represent means $\pm$ S.E.M. of 5–6 animals. * P < 0.05; *** P < 0.001 vs. PBS-treated animals. † P < 0.01 vs. olodaterol-treated animals. ‡ P < 0.05 vs. calculated sum of tiotropium and olodaterol.

between 8 and 24 hours after allergen challenge, respectively. As demonstrated, OVA challenge of the PBS-treated animals caused significant EAR and LAR. Both the EAR and LAR were largely inhibited by tiotropium and olodaterol. Remarkably, the combination caused full inhibition of these reactions (Fig. 7).

No Significant Effects of Olodaterol, Tiotropium, and Their Combination on Inflammatory Cell Infiltration in the Airways.

Allergen challenge induced a significant infiltration of total inflammatory cells in the BAL after the LAR (Fig. 8A). Increased numbers of particularly eosinophils and macrophages were observed (Fig. 8B), whereas the numbers of neutrophils (Fig. 8B) as well as lymphocytes and epithelial cells (not shown) were not significantly changed. Total and differentiated cell numbers in the BAL were not significantly affected by any of the treatments (Fig. 8, A and B).

Discussion

In this study, we demonstrate that the long-acting anticholinergic tiotropium synergistically enhances the protective effect of the novel long-acting once-daily β_2 -agonist olodaterol on basal histamine responsiveness in guinea pigs. This finding indicates that endogenous, histamine-induced, acetylcholine release considerably attenuates β_2 -agonist responsiveness and that cross-talk between muscarinic receptors and β_2 -adrenoceptors may be involved. Accordingly, in a guinea pig model of acute allergic asthma, tiotropium synergistically enhanced both the reversal of and protection against the allergen-induced AHR to histamine after the EAR by olodaterol. Moreover, olodaterol, tiotropium, and their combination were highly effective in inhibiting the allergen-induced EAR and LAR without affecting infiltration of inflammatory cells in the airways.

A clear dose- and time-dependent protection against histamine-induced airway constriction by olodaterol was found. At a single dose of 10 mM (nebulizer concentration, 3 minutes), ~7-fold protection was observed after 30 minutes, while the duration of action was >24 hours. Our data correspond well with previous reports of dose-dependent protection for >24 hours against methacholine in guinea pigs, dogs, and man (O'Byrne et al., 2009; Bouyssou et al., 2010a). In addition, olodaterol caused a dose- and time-dependent bronchodilation for up to 24 hours in patients with chronic obstructive pulmonary disease (Van Noord et al., 2011).

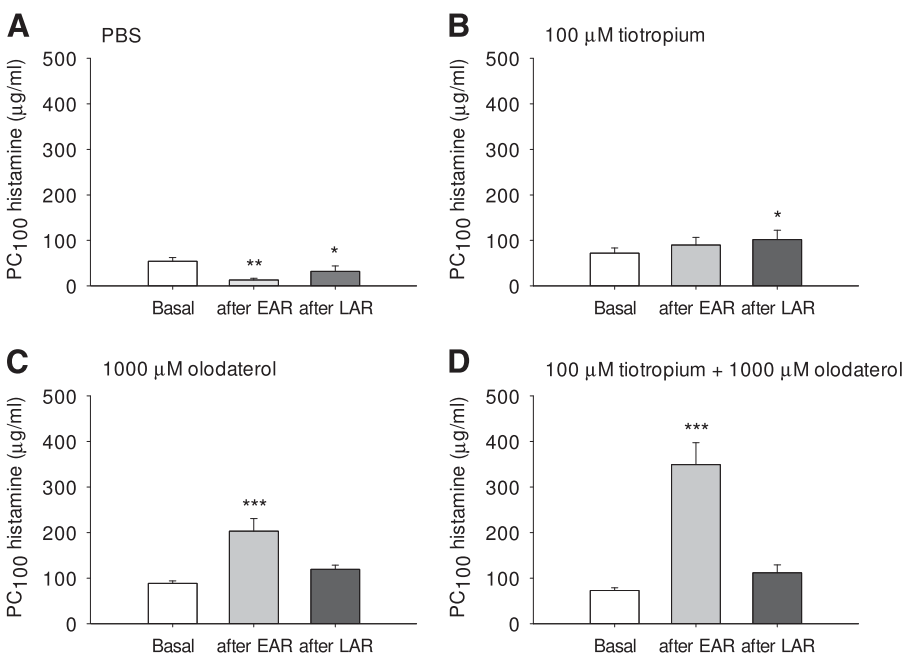


Fig. 5. Effects of pretreatment with olodaterol, tiotropium, and their combination on allergen-induced AHR. Airway responsiveness to histamine (PC₁₀₀) was determined 24 hours before (basal) and at 6 and 24 hours after OVA challenge to assess allergen-induced AHR after the EAR and LAR. One hour before OVA challenge, the animals inhaled PBS (A), 100 μ M tiotropium (B), 1000 μ M olodaterol (C), or the combination of 1000 μ M olodaterol and 100 μ M tiotropium (D) (nebulizer concentrations, 3 minutes). Data represent means $\pm$ S.E.M. of 5–6 animals. * P < 0.05; ** P < 0.01; *** P < 0.001 vs. basal.

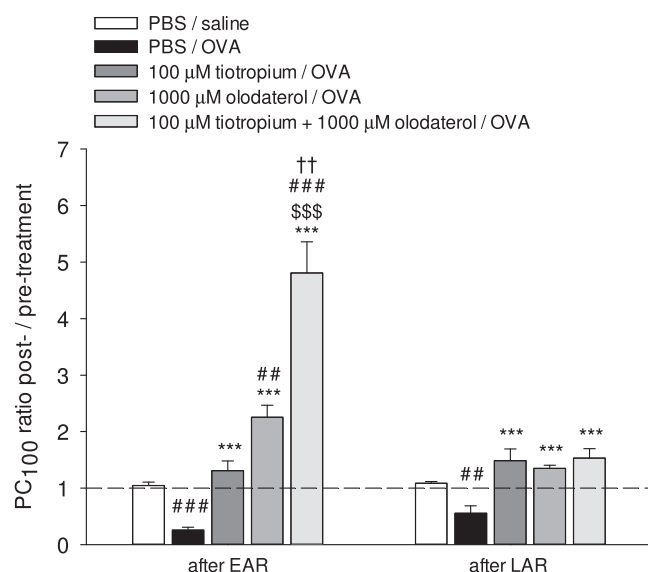


Fig. 6. The protection of allergen-induced AHR after the EAR by olodaterol is synergistically enhanced by tiotropium. Effects of inhaled PBS, 100 μM tiotropium, 1000 μM olodaterol, or the combination of 1000 μM olodaterol and 100 μM tiotropium (nebulizer concentrations, 3 minutes) on allergen (OVA)-induced AHR after the EAR and LAR. A control group of PBS-treated, saline-challenged guinea pigs is also shown. Data are expressed as the ratio of PC₁₀₀ post-treatment (measured 6 and 24 hours after allergen challenge) to pretreatment (measured 24 hours before challenge). Values represent means $\pm$ S.E.M. of 5–6 animals. ****P* < 0.001 vs. PBS/OVA. ##*P* < 0.01; ###*P* < 0.001 vs. PBS/saline. \$\$\$*P* < 0.001 vs. olodaterol/OVA. ††*P* < 0.01 vs. calculated sum of tiotropium and olodaterol.

Olodaterol (1000 μM) also proved to be highly effective in reversing the allergen-induced AHR to histamine after the EAR (~11-fold increase in PC₁₀₀ at 6.5 hours after allergen

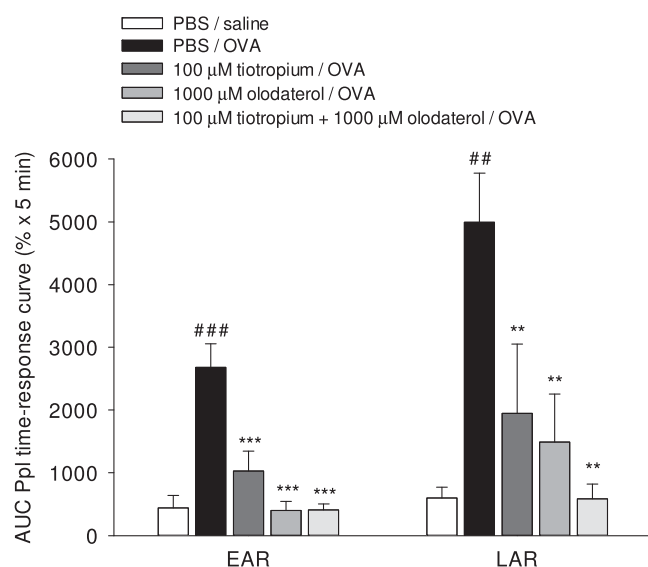


Fig. 7. Effects of pretreatment with olodaterol, tiotropium, and their combination on allergen-induced EAR and LAR. Effects of inhaled PBS, 100 μM tiotropium, 1000 μM olodaterol, or the combination of 1000 μM olodaterol and 100 μM tiotropium (nebulizer concentrations, 3 minutes) on allergen (OVA)-induced changes in airway function (*P*_{pl}) were measured. A control group of PBS-treated, saline-challenged guinea pigs is also shown. The magnitudes of the EAR and the LAR are presented as area under the *P*_{pl} time-response curve between 0 and 5 hours and between 8 and 24 hours, respectively. Data represent means $\pm$ S.E.M. of 4–6 animals. ***P* < 0.01; ****P* < 0.001 vs. PBS/OVA-treated animals. ##*P* < 0.01; ###*P* < 0.001 vs. PBS/saline.

challenge). In a similar setup, we previously demonstrated that a clinically relevant dose of 2.5 mM salbutamol (nebulizer concentration, 3 minutes) also reversed allergen-induced AHR to histamine in guinea pigs, though with a smaller effect (~3-fold increase in PC₁₀₀) (Westerhof et al., 2005).

Because cholinergic activity is increased in allergic asthma (Gosens et al., 2006), it is not surprising that a more pronounced synergism between olodaterol and tiotropium in the reversal of allergen-induced AHR to histamine was found than in the inhibition of histamine responsiveness at basal conditions. Thus, tiotropium at a concentration (100 μM) that by itself only slightly reversed the AHR after the EAR synergistically enhanced the effect of olodaterol (1000 μM) by almost 2-fold to a 21-fold increase in PC₁₀₀. Of note, olodaterol—with and without tiotropium—administered 5.5 hours after allergen provocation to reverse the AHR after the EAR was still effective in protecting against the AHR after the LAR (at 24 hours after challenge), confirming its long duration of action.

When inhaled prior to the allergen challenge, olodaterol was also highly effective, causing considerable protection against the allergen-induced EAR and LAR, as well as the AHR after both reactions. However, cell infiltration in the BAL after the LAR was not affected. These findings correspond well with previous findings with salbutamol inhalations in our model (Westerhof et al., 2005), although, as might be expected, the effects of the long-acting olodaterol on the allergen-induced asthmatic reactions and AHR in the present study were more pronounced.

It is remarkable that, in contrast to the minor reversal of allergen-induced AHR after the EAR by tiotropium, pretreatment with the same dose of the anticholinergic (100 μM, 3 minutes) fully prevented the development of AHR after the EAR, which suggests that nonbronchodilatory, anti-inflammatory effects of tiotropium may be involved. This holds similarly for the AHR after the LAR. Although inflammatory cell infiltration in the BAL (measured at 25 hours after allergen challenge) appeared not to be inhibited by tiotropium, it cannot be excluded that inhibition of mediator release by inflammatory or structural cells in the airways may be involved (Kistemaker et al., 2012). Interestingly, evidence for a non-bronchodilating effect of tiotropium on AHR, as assessed by electrical field stimulation-induced vagal stimulation, was also recently found by Buels et al. (2012). Similarly, this was not associated with a change in inflammatory cell numbers in the BAL. Remarkably, however, there appeared to be a reduction of vagal nerve-associated eosinophils in the airway tissue, which might be involved in the reduced vagal hyper-responsiveness by tiotropium (Buels et al., 2012).

It is also of interest that tiotropium caused marked inhibition of the LAR without affecting inflammatory cell infiltration in the BAL. The inhibition of the LAR corresponds with recent observations in rats (Raemdonck et al., 2012), indicating that allergen challenge induces activation of TRPA1 channels on sensory nerves during the LAR, leading to enhanced cholinergic reflex bronchoconstriction. Remarkably, inhibition of the LAR by tiotropium or any other anticholinergic in asthmatic patients has thus far not been reported.

As with the reversal of allergen-induced AHR, we found a highly synergistic effect of olodaterol and tiotropium in

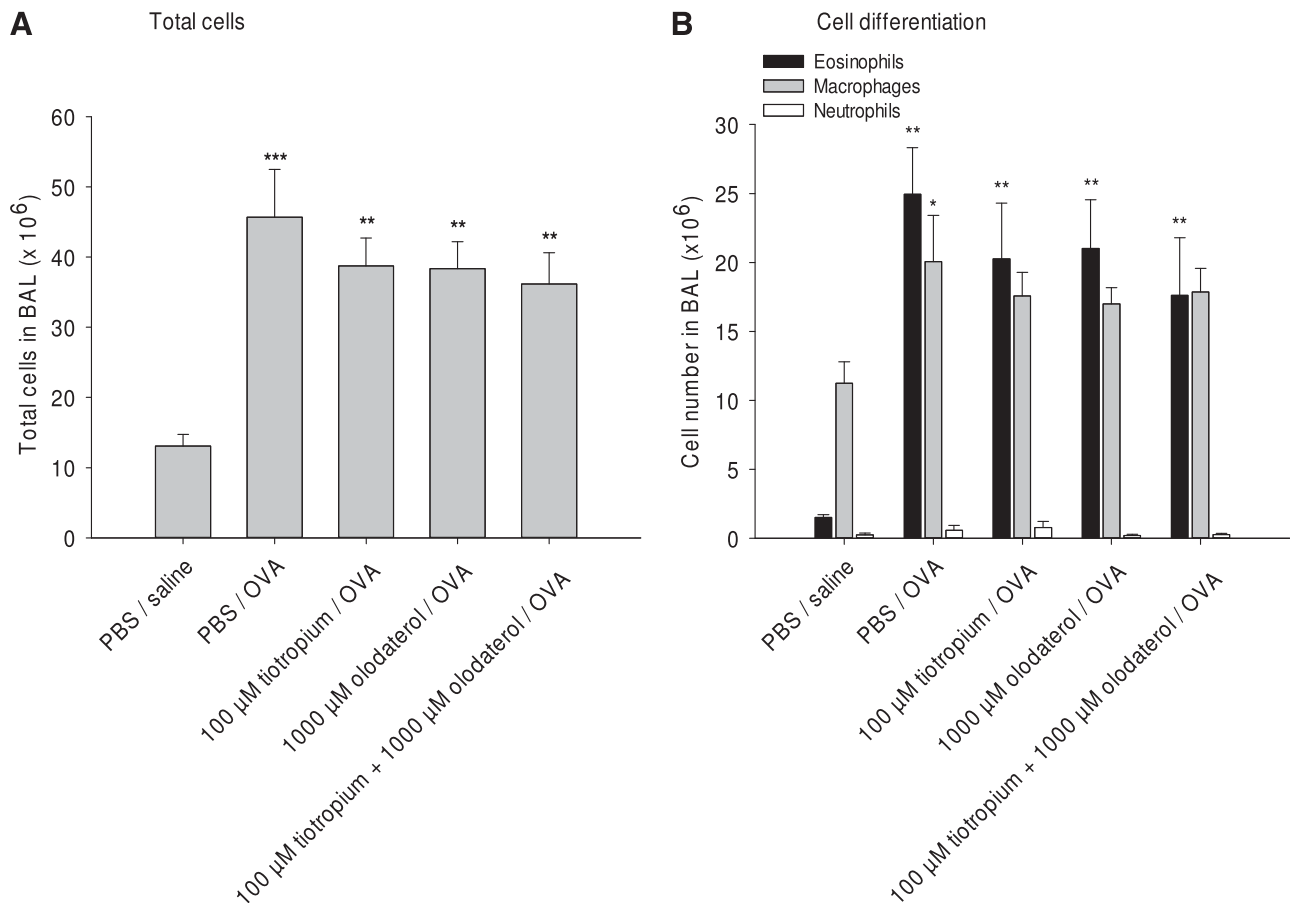


Fig. 8. Effects of pretreatment with olodaterol, tiotropium, and their combination on allergen-induced inflammatory cell infiltration in the BAL. Total cell number (A) and cell differentiation (B) of BAL obtained 25 hours after saline or allergen (OVA) challenge (after the LAR) were analyzed. Allergen-challenged animals were treated by 3-minute inhalations of PBS, tiotropium (100 μ M), olodaterol (1000 μ M), or the combination of tiotropium (100 μ M) and olodaterol (1000 μ M) 1 hour before challenge, whereas saline-challenged animals were treated by a 3-minute inhalation of PBS. Data represent means $\pm$ S.E.M. of 5–6 animals. * P < 0.05 vs. PBS/saline-treated animals; ** P < 0.01; *** P < 0.001 vs. PBS/saline-treated animals.

protecting against the allergen-induced AHR after the EAR, when administered before the allergen challenge. In addition, although not synergistic, it was found that olodaterol, tiotropium, and their combination fully inhibited the AHR after the LAR. The cause of the lack of synergism in protecting against the AHR after the LAR is unknown, but could well be related to a relatively lower cholinergic tone after the LAR than after the EAR (Ten Berge et al., 1995), thereby not exposing a potential synergism. Nevertheless, from a clinical perspective, the protection against the AHR after the LAR by both olodaterol and tiotropium, as well as their combination, is of importance. Inhibition of inflammatory cell infiltration does not seem to contribute to the protective effects of the combination therapy, although, as discussed above, inhibition of mediator release by these or structural cells in the airways could potentially be involved. Remarkably, in contrast to the present observation after single inhalation in acute asthma, inhibition of allergen-induced inflammatory cell infiltration has been observed after repeated inhalations of a similar dose of tiotropium in guinea pig and mouse models of chronic asthma (Bos et al., 2007; Ohta et al., 2010). This could obviously be of relevance for prolonged treatment with the combination of tiotropium and olodaterol, and requires further examination.

Both olodaterol and tiotropium inhibited the EAR and LAR to a considerable extent. Remarkably, even full inhibition of

both reactions was observed with the combination of these drugs. No synergism was observed, however, due to the high efficacy of the individual drugs to inhibit these reactions; lower doses of olodaterol and tiotropium would be required to demonstrate such an effect.

Fully in line with our observations, it has been shown that intratracheally applied tiotropium potentiated the inhibitory effects of the once-daily β_2 -agonist carmoterol on acute allergen-, acetylcholine-, and histamine-induced bronchoconstriction in anesthetized, actively sensitized guinea pigs (Rossoni et al., 2007).

In conclusion, olodaterol has a pronounced long-acting (up to 24-hour) protective effect against histamine-induced airway obstruction and allergen-induced AHR to histamine in a guinea pig model of acute allergic asthma. These responses are synergistically enhanced by tiotropium, indicating that acetylcholine reduces the effectiveness of the β_2 -agonist under these conditions. In addition, the combination of olodaterol and tiotropium fully inhibited both the EAR and LAR in the allergic asthma model. These findings indicate that combination therapy with olodaterol and tiotropium may be highly effective in the treatment of allergic asthma.

Authorship Contributions

Participated in research design: Maarsingh, Gosens, Zaagsma, Meurs.

Conducted experiments: Smit, Zuidhof, Bos, Maarsingh.

Performed data analysis: Smit, Zuidhof, Bos, Maarsingh, Gosens, Zaagsma, Meurs.

Wrote or contributed to the writing of the manuscript: Smit, Maarsingh, Gosens, Zaagsma, Meurs.

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