

Combination Therapy with Budesonide and Salmeterol in Experimental Allergic Inflammation

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Abstract

The aim of this study was to determinate bronchodilator, antitussive, and ciliomodulatory activity of inhaled combination therapy with budesonide and salmeterol, and to correlate the results with the anti-inflammatory effect. The experiments were performed using two models of allergic inflammation (21 and 28 days long sensitization with ovalbumine) in guinea pigs. The animals were treated daily by aerosols of budesonide (1 mM), salmeterol (0.17 mM), and a half-dose combination of the two drugs. Antitussive and bronchodilator activities were evaluated *in vivo*. The ciliary beat frequency (CBF) was assessed *in vitro* in tracheal brushed samples, and inflammatory cytokines (IL-4, IL-5, IL-13, GM-CSF, and TNF- α) were determined in bronchoalveolar lavage fluid (BALF). We found that the combination therapy significantly decreased the number of cough efforts, airway reactivity, and the level of inflammatory cytokines in both models of allergic asthma. Three weeks long sensitization led to an increase in CBF and all three therapeutic approaches have shown a ciliostimulatory effect in order: salmeterol < budesonid < combination therapy. Four weeks long ovalbumine sensitization, on the other hand, decreased the CBF, increased IL-5, and decreased IL-13. In this case, only the combination therapy was able to stimulate the CBF. We conclude that a half-dose combination therapy of budesonide and salmeterol shows comparable antitussive, bronchodilator, and the anti-inflammatory effect to a full dose therapy with budesonide alone, but had a more pronounced stimulatory effect on the CBF.

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Airway reactivity • Ciliary beat frequency • Cough • Inhaled corticosteroids • Th2 cytokines

1 Introduction

Allergic asthma is a chronic inflammatory disease of conducting airways in which many inflammatory cells (mast cells, macrophages, T-lymphocytes, eosinophils, and neutrophils) and epithelial cells play a role. These cells release a multitude of mediators such as cytokines, chemokines, and growth factors resulting in chronic sustained inflammation that affects the airway function. In allergic asthma, bronchial smooth muscles become more responsive and contract in response to usually harmless particles (Halwani et al. 2011). Many inflammatory mediators have a deleterious effect on the airway epithelium. They stimulate the production of pathological mucus, induce goblet cell hyperplasia, cause epithelial shedding, and alter the ciliary movement. All these changes disrupt the normal function of mucociliary clearance and lead to mucus retention (Erle and Sheppard 2014; Rogers 2004). Resulting mucus airway obstruction significantly contributes to airway responsiveness, clinically manifested by cough, wheezing, and chest tightness (Barnes 2011a).

Since there is no cure for asthma, attenuation of ongoing inflammation is the main target in asthma treatment, achieved mainly by regular administration of inhaled corticosteroids (ICS). Although several new anti-inflammatory drugs, designated against specific inflammatory molecules, are currently in clinical studies, taking into the consideration the complexity of the disease, it is unlikely that blocking a single mediator could provide a better anti-inflammatory spectrum than currently used ICS (Durham et al. 2015). Their regular administration is associated with improvements in symptoms and lung function, and reduces disease exacerbations. The efficacy of ICS demonstrates the dose-

response relationship, but their higher doses, more absorbed through the lungs, increase risk of side effects. Thus, one of the goals of asthma treatment is to optimize the use of ICS to increase treatment potency while maintaining side effects at bay (Chung et al. 2009). In this regard, long acting β_2 -agonists (LABA), used in asthma due to their bronchodilator activity, have been shown to possess steroid-sparing effect. Adding LABA to ICS therapy seems more effective than simply doubling the dose of ICS. In addition to have a positive effect on asthma symptoms control, ICS may have an additive effect on airway inflammation (Tamm et al. 2012). In the present study, we set out to gain insight into the interaction between LABA and ICS, by examining their effects on several defense mechanisms in animal models of experimentally induced allergic inflammation. We focused attention on a half-dose combination therapy with inhaled budesonide and salmeterol, monitoring the anti-tussive, bronchodilator, and ciliomodulatory effects of such a combination. We further correlated these effects with the level of inflammatory mediators, interleukins (IL) IL-4, IL-5, IL-13, tumor necrosis factor (TNF- α), and Granulocyte-macrophage colony-stimulating factor (GM-CSF) in the bronchoalveolar lavage fluid (BALF).

2 Methods

The study was approved by the Institutional Ethics Committee of Jessenius Faculty of Medicine in Martin, Slovakia (permit IRB 00005636) and all experimental procedures were carried out according the Slovakian and European Community regulations for the use of laboratory animals and guidelines on animal welfare (EU decision

No. 1249/2013). Healthy, adult male TRIK-strain guinea pigs were purchased from the Department of Experimental Pharmacology of the Slovak Academy of Sciences in Dobrá Voda, Slovakia, an accredited breeding facility. The animals were housed under the controlled conditions with access to food and water *ad libitum*.

Drugs and other substances used in the experiment were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Cytokine concentration was determined with a multiplex kit, Bio-Plex Pro Human cytokine Th1/Th2 immunoassay, purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.1 Experimental Groups

The experiments were performed using two models of experimentally induced allergic inflammation, where animals were submitted to 3- or 4-week-long ovalbumine (OVA) sensitization. The animals were randomly divided into several experimental groups, each consisting of eight guinea pigs. In the control group, animals received saline instead of OVA and drug treatments. In the negative control groups, animals were exposed to OVA during 21 and 28 days according to sensitization scheme. There were three therapeutic groups, in which the animals were treated during the ongoing sensitization with aerosols of budesonide (1 mM) or salmeterol (0.17 mM), or their half-dose combination, applied on a daily basis.

Drug solutions were aerosolized by a PARI jet nebulizer (output $5 \text{ l} \cdot \text{s}^{-1}$, particles mass median diameter $1.2 \text{ } \mu\text{m}$; Paul Ritzau, Pati-Werk GmbH, Starnberg, Germany) and delivered to the head chamber of a double body plethysmograph (HSE type 855; Hugo Sachs Electronic, March, Germany), where the animals were placed.

Antitussive and bronchodilator activities of inhaled drugs were assessed 24 h after the last exposure, under *in vivo* laboratory conditions. After sacrificing the animals, ciliary beat

frequency (CBF) was assessed in tracheal brushed-out ciliary samples and the level of inflammatory mediators (IL-4, IL-5, IL-13, TNF- α , and GM-CSF) were determined in BALF.

2.2 Experimentally Induced Allergic Airway Inflammation

The animals were sensitized to ovalbumin (OVA), applied as a 1 % solution, intraperitoneally, subcutaneously, or by inhalation. For intraperitoneal and subcutaneous administration, OVA was injected together with aluminium hydroxide [$\text{Al}(\text{OH})_3$] (0.5 ml saline solution of 5 mg of OVA in conjunction with 100 g of $\text{Al}(\text{OH})_3$ per animal). Three weeks long sensitization was conducted according to the method described by Franova et al. (2013). During the 4 weeks long sensitization, animals were injected with OVA intraperitoneally on Days 1 and 4 and subcutaneously on Days 1, 12, and 20. On Days 15, 18, 20, 22, 24, and 27 of sensitization, animals were exposed to OVA aerosol for 1–3 min.

2.3 Evaluation of Airway Smooth Muscle Reactivity *in Vivo*

Airway smooth muscle reactivity was evaluated in a double chamber plethysmograph (HSE type 855, Hugo Sachs Electronic, March, Germany), consisting of nasal and thoracic chamber. Airway reactivity was assessed from changes in thoracic and nasal airflow induced by 30 s inhalation of the bronchoconstrictor mediator histamine (10^{-6} M). From the resulting phase shift between nasal and thoracic respiratory flows, specific airway resistance (sRaw) was calculated according to Pennock et al's (1979) method using the HSE Pulmodyn Pennock respiratory software. sRaw was used as a measure of bronchodilator effects of inhaled drugs.

2.4 Evaluation of Cough Reflex *in Vivo*

Conscious guinea pigs were individually placed in a double chamber plethysmograph. The cough reflex was provoked by inhalation of aerosol of citric acid (0.3 M) for 3 min. A sudden enhancement of expiratory flow during coughing was detected by a pneumotachograph head connected to the nasal chamber of the plethysmograph. PC-recorded changes in expiratory airflow, which were simultaneously accompanied by characteristic cough sound and movement, were regarded as cough efforts. The sound and movement typical for cough reflex were evaluated by two trained observers and verified with video recordings.

2.5 Evaluation of Ciliary Beat Frequency (CBF) *in Vitro*

After sacrificing the animal, a small window was dissected in a precisely cleaned area of the upper part of trachea to expose the epithelium for a brushing collection of ciliated cells. The brushing method is little invasive, relatively simple, and reliable technique to obtain ciliated epithelium and is an accepted method for studying ciliary function. The material acquired was suspended in a drop of warm saline solution of $36.5 \pm 0.5^\circ$ placed on a microscope slide and was covered. Undamaged strips of ciliated epithelium, with the presence of beating cilia, were selected using an inverted phase contrast microscope (Zeiss Aixio vert. A1; Carl Zeiss AG; Göttingen, Germany) and the beating was recorded by a high speed video camera (Basler A504kc; Adept Turnkey Pty Ltd, Brookvale, Australia) with the frame rate of 256–512 frames per sec. The recorded video sequences of beating regions, approximately 10 sequences per sample, were analyzed with Labview™ software to identify ciliary regions of interest (ROI). For every ROI, a median of CBF was calculated and used

as an evaluation parameter. A final value of CBF, expressed in Hz, was an average of ten median values obtained from each specimen.

2.6 Evaluation of Cytokines in Bronchoalveolar Lavage Fluid (BALF)

BALF was collected by washing out the right bronchus with warm saline (37°C). The volume of saline introduced to right bronchus was calculated according to animal's body weight of animal (10 ml/kg). The level of IL-4, IL-5, IL-13, TNF- α , and GM-CSF cytokines was determined in the supernatant that was centrifuged for 2 min at $377 \times g$. For the determination and quantification of cytokines we used a commercial Th1/Th2 panel Human Cytokine kit (Bio-Rad; Hercules, California, US) containing all required reagents and antibodies. A simultaneous detection of different molecules is based on fluorescently dyed magnetic beads, having a distinct color code, which are covalently coupled to antibodies directed against the desired biomarkers. Magnetic beads were first incubated with standards, samples, or controls, followed by incubation with capture antibody, and finally with the fluorescent reporter streptavidin-phycoerythrin conjugate. Between each incubation, a series of washes, using Bio-Plex Pro wash station (Bio-Rad, Hercules, California, USA), was performed to remove unbound proteins. The reaction data were acquired with the Bio-Plex® 200 array system (Bio-Rad, Hercules, California, USA) in which the beads flow in single file through a region illuminated by two lasers. One laser (635 nm excitation) illuminates the fluorescent dye within each bead to provide the analyte identification and the second one excites streptavidin-phycoerythrin conjugate generating a signal used for the analyte quantification. A high-speed digital processor manages data output and Bio-Plex Manager™ 6.0 software presents results as a concentration in pg/ml.

2.7 Statistical Analysis

Data are represented as means $\pm$ SE. Statistical analysis was performed using one-way analysis of variance ANOVA. A p -value < 0.05 was taken as a threshold defining the statistical significance.

3 Results

3.1 Cough Reflex

Changes in the cough reflex were evaluated as a change in the number of cough efforts induced by citric acid inhalation. Three weeks long OVA sensitization led to a significant increase of cough. The increase was attenuated after the extension of OVA sensitization up to 4 weeks. In both models of sensitization, all three therapeutic approaches suppressed cough, with the greatest suppression observed in the group receiving a half-dose combination of budesonide and salmeterol (Fig. 1).

3.2 Airway Reactivity – Specific Airway Resistance (sRaw)

Comparing the effects on sRaw of histamine inhalation in two different models of experimental allergic inflammation, the shorter OVA

sensitization caused a greater increase in the reactivity of bronchial smooth muscle. However, a significant increase in sRaw was observed in both sensitization models. Also, in both models, chronic treatment with budesonide, salmeterol, or their half-dose combination showed a significant protective effect against bronchoconstriction provoked by histamine inhalation (Fig. 2).

3.3 Ciliary Beat Frequency (CBF)

Three weeks long OVA sensitization caused a slight increase in CBF. In this experimental model, chronic administration of drugs increased CBF in the following increasing order: salmeterol $<$ budesonide $<$ combination of both drugs. By contrast, 4 weeks long sensitization caused a significant suppression of CBF and only a half-dose combination treatment was capable of increasing CBF (Fig. 3).

3.4 Inflammatory Cytokines in Bronchoalveolar Fluid (BALF)

In both models of OVA-induced allergic inflammation, the cytokines IL-4, IL-5, IL-13, TNF- α , and GM-CSF were increased in BALF. The longer 4-weeks-sensitization was characterized by a marked increase in the level of IL-5 and a drop of

Fig. 1 Changes in number of cough efforts after chronic administration of inhaled budesonide, salmeterol, or their half-dose combination (bud/sal) in experimental allergic inflammation induced by 21 or 28 days long ovalbumin (OVA) sensitization in guinea pigs; +++ $p < 0.001$ vs. control; *** $p < 0.001$ and * $p < 0.5$ vs. OVA

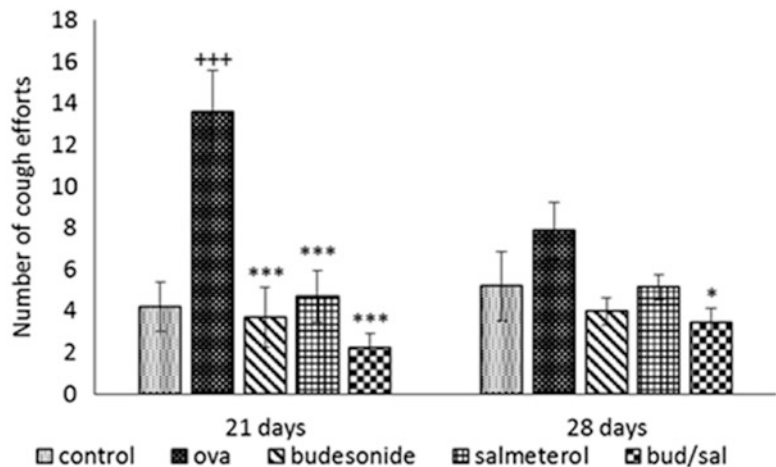


Fig. 2 Changes in specific airway resistance (sRaw) after chronic administration of inhaled budesonide, salmeterol, or their half-dose combination (bud/sal) in experimental allergic inflammation induced by 21 or 28 days long ovalbumin (OVA) sensitization in guinea pigs; +++p < 0.01 and +p < 0.001 vs. control; ***p < 0.5 and *p < 0.001 vs. OVA

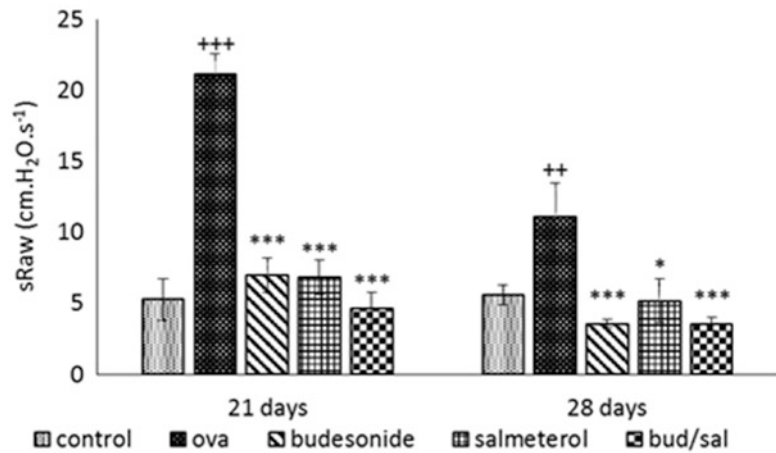
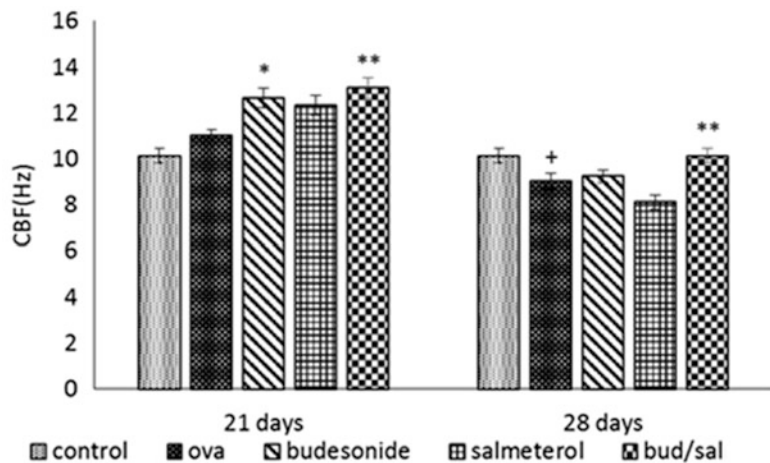


Fig. 3 Changes in ciliary beat frequency (CBF) after chronic administration of inhaled budesonide, salmeterol, or their half-dose combination (bud/sal) in experimental allergic inflammation induced by 21 or 28 days long ovalbumin (OVA) sensitization in guinea pigs; *p < 0.5 and **p < 0.01 vs. OVA; +p < 0.05 vs. control



IL-13 compared with the shorter 3-week-sensitization. Salmeterol had no effect on the expression of inflammatory markers. Budesonide and a half dose budesonide/salmeterol treatment had a comparable suppressive effect on all cytokines assessed (Fig. 4).

4 Discussion

Chronic combination therapy with ICS and LABA is the recommended approach in the treatment of moderate and severe asthma (GINA 2015). Many studies demonstrated a clinically beneficial interaction of the two drugs, leading to a better control of asthma symptoms, fewer

exacerbations, and improved lung function (Koopmans et al. 2006). The goal of the present study was to evaluate the bronchodilator, antitussive, and ciliomodulatory effect of a half-dose combination of budesonide and salmeterol in the context of their anti-inflammatory efficacy. These effects were assessed in two experimental models of allergic inflammation, both characterized by a significant increase in the Th2 cytokines IL-4, IL-5, and IL-13, which are operational in shaping the inflammatory process (Holgate 2011). The major differences in the expression of these cytokines in the two inflammatory models was an augmentation of IL-5 and a reduction of IL-13 in the longer 4 weeks compared with the shorter 3 weeks model.

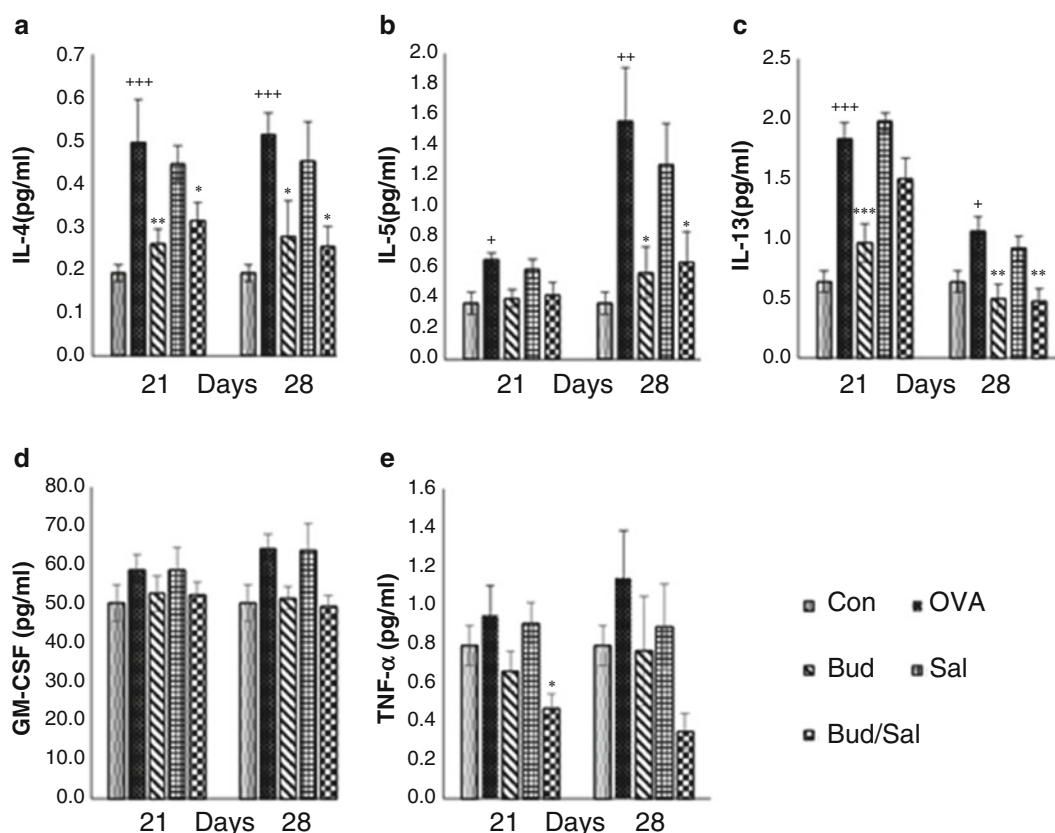


Fig. 4 Changes in the cytokines IL-4 (a), IL-5 (b), IL-13 (c), GM-CSF (d), and TNF- α (e) after chronic administration of inhaled budesonide (Bud), salmeterol (Sal), or their half-dose combination (Bud/Sal) in experimental allergic inflammation induced by 21 or 28 days

long ovalbumin (OVA) sensitization in guinea pigs; + $p < 0.05$, ++ $p < 0.01$, and +++ $p < 0.001$ vs. control (Con); * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. OVA

Since IL-13 has been identified as a potent bronchoconstrictor and main inducer of airway hyperreactivity (Mattes et al. 2001), its reduction may be associated with the observed decrease in specific airway resistance after 4 weeks long sensitization. Mechanical changes of the airways induced by bronchoconstriction stimulate mechanoreceptors involved in mediation of cough reflex (Mazzone 2005). Thus, a decrease in airway reactivity in the 4 weeks model of allergic asthma may explain the attenuation of the cough reflex. Another possible explanation for less cough may also be the destruction of C-fibers due to persistent inflammation and subsequent depletion of tachykinins, potent cough triggers. Some studies have confirmed that inflammatory changes in airways may

actually decrease cough reflex sensitivity (Franova et al. 2013).

IL-13 in addition to inducing bronchoconstriction may also reduce ciliary movement (Gomperts et al. 2007; Laoukili et al. 2001). In the present study we failed to observe a cilioinhibitory effect when IL-13 concentration was the most. On the contrary, decreased CBF was recorded after 4 weeks OVA sensitization, when IL-13 concentration was not as high. However, in this condition we noted a substantial increase in IL-5 that, along with GM-CSF, acts as a promoter and activator of eosinophils. These cells release toxic substances that have marked cilioinhibitory effect (Thomas et al. 2010). IL-5, rather than IL-13, may plausibly inhibit CBF through the activation of eosinophils. Moreover,

according to the study of Svartengren et al. (1989), mucociliary clearance may be either increased or decreased in asthma. These authors submit that there may be a principal irritation caused by inflammation, which actually leads to an increase in mucociliary clearance, at least in the early stages of asthma. Taking into consideration that CBF is one of the key factors regulating the rate of mucociliary clearance (Braiman and Priel 2008), the present findings of increased CBF in the 3 weeks model and decreased CBF in the four weeks model of allergic asthma are in line with the reasoning above outlined.

Since inflammation plays a key role in the asthma pathogenesis, its suppression is one of the main goals in asthma therapy (Chung et al. 2009). In this regard, ICS represent the drugs of choice whose effects are mediated through the activation of anti-inflammatory (transactivation) genes and transrepression of pro-inflammatory genes (Strehl and Buttgereit 2013). In the present study, budesonide, a representative member of ICS, in monotherapy, significantly reduced the bronchoconstriction of histamine challenge, suppressed the cough reflex in both models of allergic asthma, and led to a slight increase in CBF.

Some anti-inflammatory activity has also been demonstrated in several *in vitro* studies with β_2 -agonists. Airway β_2 -adrenoreceptors, beside of being widely expressed in airway smooth muscles, have been identified in epithelial cells and in many pro-inflammatory and immune cells, including mast cells, lymphocytes, macrophages, eosinophils, and neutrophils. β_2 -agonists suppress the pro-inflammatory activity of the cells above mentioned, mainly by activation of protein kinase A (PKA). Activated PKA phosphorylates and inactivates several proteins involved in the abrupt transient increase in cytosolic calcium, which is necessary for immune cell activation (Theron et al. 2013). A clear anti-inflammatory effect of β_2 -agonists observed *in vitro* has not been confirmed *in vivo* due likely to rapid receptor desensitization. As the inflammatory and immune cells express β_2 -receptors at a lower

density than airway smooth muscles do, these cells are more susceptible to the development of tolerance (Barnes 1999). Although salmeterol did not suppress inflammatory markers in the present study, its protective effect against inhaled histamine-induced bronchoconstriction persisted. It is also well known that β_2 -agonists possess ciliostimulatory activity (Wohlsen et al. 2010). In the three weeks model of allergic inflammation salmeterol stimulated CBF, but the stimulation was smaller than that after budesonide alone. In the 4 weeks model, characterized by a marked increase in IL-5, salmeterol's ability to stimulate CBF was abolished.

To avoid the development of receptor tolerance, LABA should be administrated in combination with ICS in chronic use. Glucocorticoids, in the process of transactivation, not only increase the transcription of β_2 -receptors, but also improve the receptor coupling leading to better receptor responsiveness after stimulation (Sin and Man 2006). That may be important for the non-bronchodilator effects of β_2 -agonists, such as down-regulation of calcium in inflammatory cells. (Theron et al. 2013; Barnes 2011a). On the other hand, β_2 -agonists can enhance the anti-inflammatory activity of ICS *via* interaction with the glucocorticoid signal transduction. LABA activate glucocorticoid receptors and enhance transcription of anti-inflammatory mediators (Tamm et al. 2012). In the present study, half-dose combination therapy with budesonide and salmeterol demonstrated a comparable suppressive effect on IL-4, IL-5, IL-13, and GM-CSF as monotherapy with budesonide in a full dose. However, regarding TNF- α , the suppressive effect of half-dose combination therapy was greater than that observed after budesonide alone. This cytokine is released from a variety of cells, including macrophages and epithelial cells, and may be important in amplifying the allergic inflammatory response in severe asthma (Barnes 2011b). Glucocorticoids and LABA alter the expression of TNF- α by differential mechanisms. The former increase the expression of proteins that degrade TNF- α messenger RNA.

The latter directly suppress the synthesis of TNF- α via cAMP-dependent activation of guanine nucleotide exchange protein (Epac1). A combination of these two effects, along with increased density of β_2 -receptors on target cells and enhancement of transcription activity of budesonide may be responsible for a substantial decrease in TNF- α .

In the present study, combination therapy also demonstrated a beneficial effect on defense mechanisms investigated. It remarkably suppressed the bronchoconstriction of histamine challenge and the number of cough efforts induced by citric acid inhalation. In comparison with monotherapy, half-dose combination therapy stronger enhanced ciliary movement. The enhancement was present only with the combination therapy in the presence of high levels of IL-5 in the 4 weeks model of allergic inflammation. That could be a result of mutual potentiation of anti-inflammatory effect of either drug, along with increased expression of β_2 -receptors on the surface of epithelial cells. In addition, enhanced mucociliary clearance accelerates the expulsion of noxious particles (Braiman and Priel 2008) and protects epithelial cells from activation and consequent production of inflammatory mediators (Holgate 2011), which contributes to decreased airway reactivity.

In conclusion, half-dose combination therapy consisting of budesonide and salmeterol demonstrates comparable antitussive, bronchodilator, and anti-inflammatory effects as monotherapy with budesonide. The major advantage of combination therapy seems its enhancing the ciliary movement.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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