

Comparison of the effects of two long-acting beta₂-agonists on cytokine secretion by human airway epithelial cells

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Background and Purpose: Long-acting beta₂-agonists (LABAs) have proved to be useful in the management of asthma and prevention of exacerbations. LABAs can modulate inflammatory and repair processes in the airways of individuals affected by many respiratory disorders. This study assessed the effects of LABAs on the release of inflammatory mediators by bronchial epithelia.

Methods: The effects of the LABAs salmeterol and formoterol on the synthesis of soluble interleukin-8 (IL-8), granulocyte-macrophage colony-stimulating factor (GM-CSF), and vascular endothelial growth factor (VEGF) in the human airway epithelial cell line A549 was investigated in vitro. Cells cultured for 8 h in the presence of an LABA were stimulated with tumor necrosis factor- α for 16 h and then enzyme-linked immunosorbent assays for IL-8, GM-CSF, and VEGF were performed on the supernatants.

Results: Both salmeterol and formoterol significantly suppressed IL-8, GM-CSF, and VEGF secretion from tumor necrosis factor- α -stimulated A549 cells. Results indicated that formoterol was more potent than salmeterol in suppressing IL-8 and VEGF production. In contrast, salmeterol appeared to be more potent than formoterol in suppressing GM-CSF production.

Conclusion: LABAs have some anti-inflammatory effects on bronchial epithelia. The differences between salmeterol and formoterol and mechanisms for the observed effects need further evaluation.

Key words: Adrenergic beta-agonists; Formoterol; Granulocyte-macrophage colony-stimulating factor; Interleukin-8; Salmeterol; Vascular endothelial growth factor A

Introduction

Recent clinical studies have demonstrated that a combination of inhaled short- or long-acting beta (β)₂-agonists (LABAs) with inhaled glucocorticoids results in better asthma control than higher doses of glucocorticoids alone [1-3]. These clinical findings have been supported by the results of in vitro mechanistic studies, which demonstrated the additive effects of β ₂-agonists or other cyclic adenosine monophosphate (cAMP) elevating agents and glucocorticoids on the inhibition of cytokine release and adhesion molecule expression in human blood mononuclear cells [4,5]. Moreover, some in vivo studies revealed that LABAs

have positive effects on some indicators of inflammation in inflammatory cells (e.g., eosinophils) and tissues (e.g., bronchial mucosa), but the clinical importance of these findings has not been established [6-9].

The effect of LABAs is based on their interactions with cells that contain β -adrenergic receptors. Although the main target of β -agonist action is the airway smooth muscle cell, there is now evidence that β -receptors are widely distributed in cells that both reside in and infiltrate the lung [10,11]. Moreover, LABAs can inhibit the histamine release from mast cells and neutrophils [12,13]. This means that LABAs may exert their anti-inflammatory effects via some other as yet unclear β -adrenoceptor-independent mechanisms.

Asthma is characterized by airway inflammation, bronchial hyper-responsiveness, and reversible airflow obstruction. The asthmatic inflammatory response consists of infiltration into the airway of a variety of

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activated inflammatory cells and the release of a variety of mediators. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-8 (IL-8) are the most potent cytokines involved in airway inflammation; vascular endothelial growth factor (VEGF) is involved in the airway remodeling process [14-16]. This *in vitro* study compared the effect of the LABAs salmeterol and formoterol on cytokine secretion in airway epithelial cells, in order to assess their anti-inflammatory effects on bronchial epithelia.

Methods

Cells and culture medium

The human airway epithelial cell line, A549 (American Type Culture Collection [ATCC], Rockville, MD, USA), was derived from a human pulmonary adenocarcinoma and is regarded as a model of the type II pneumocyte [17]. Cells were grown to confluence in RPMI-1640 medium (Cambrex Corp., East Rutherford, NJ, USA) with 10% fetal bovine serum (ATCC), 100 U/mL penicillin (Invitrogen, Carlsbad, CA, USA), and 100 U/mL streptomycin (Invitrogen).

Cell culture

Cells were grown to $\geq 60\%$ confluence in uncoated 24-well plates (BD Biosciences, San Jose, CA, USA). For subsequent experiments, the cell suspension was diluted to a final concentration of 2.5×10^5 cells/mL. One mL of this suspension was transferred to each well of 24-well tissue culture plates (BD Biosciences) and incubated overnight to allow for cell adherence.

Experiments were started by culturing the cells in RPMI-1640 medium, pre-incubating with formoterol (GlaxoSmithKline, Taipei, Taiwan) or salmeterol (AstraZeneca, Taipei, Taiwan) at different concentrations (0, 25, 50, 75, or 100 μM) for 8 h, and then adding 10 ng/mL human recombinant tumor necrosis factor- α (TNF- α ; BD Biosciences). Cells were then cultured for an additional 16 h along with appropriate vehicle controls. Cell-free supernatants were collected by centrifugation and stored at -70°C until an enzyme-linked immunosorbent assay (ELISA) was performed. At all stages of culture, cells were maintained at 37°C in a moist 5% carbon dioxide atmosphere. The experiments were performed independently at least 3 times.

Drug sensitivity assay

Drug sensitivities of A549 to either salmeterol or formoterol were assayed with a 3-(4,5-dimethyl thiazol)-

2,5-diphenyltetrazolium bromide (MTT) colorimetric cell survival assay [18]. In brief, cells were incubated with various concentrations of either salmeterol or formoterol for 24 h. MTT solution was then added to each well up to a final concentration of 5 mg/mL, and then the plates were incubated for another 4 h at 37°C . Cells were dissolved in dimethyl sulfoxide. The absorbance at 450 nm was recorded immediately using an ELISA reader (Thermo Clinical LabSystems, Vantaa, Finland).

Measurement of GM-CSF, IL-8, and VEGF

GM-CSF, IL-8, and VEGF concentrations in cell supernatants were measured using ELISA kits (BD Biosciences) according to the manufacturer's specifications. Absorbance was read at 450 nm with the ELISA reader.

Statistical analysis

Results of experiments were analyzed by the independent *t* test. Differences with a probability value < 0.05 were considered significant.

Results

Drug sensitivity assay

An MTT colorimetric assay [18] was used to determine the survival of A549 cells exposed to either salmeterol or formoterol (0, 25, 50, 75, or 100 μM). Their survival (Fig. 1) was 98% that of control (untreated cells).

Salmeterol and formoterol inhibition of IL-8 secretion

Both salmeterol and formoterol dose-dependently inhibited IL-8 secretion from TNF- α -induced A549 cells (Fig. 2). However, the salmeterol-associated inhibition was only significant at a concentration of 100 μM ($p < 0.01$ compared with control; Fig. 2). On the other hand, formoterol-associated inhibition was dose-dependent and significant from 25 μM to 100 μM ($p < 0.05$ at 25 μM , 75 μM , and 100 μM , and $p < 0.01$ at 50 μM , compared with control; Fig. 2). Formoterol was the more potent inhibitor of IL-8 production at a concentration of 50 μM ($p = 0.001$, Table 1).

Salmeterol and formoterol inhibition of GM-CSF secretion

Both salmeterol and formoterol dose-dependently inhibited GM-CSF secretion from TNF- α -stimulated A549 cells ($p < 0.01$, Fig. 3). Salmeterol was the more

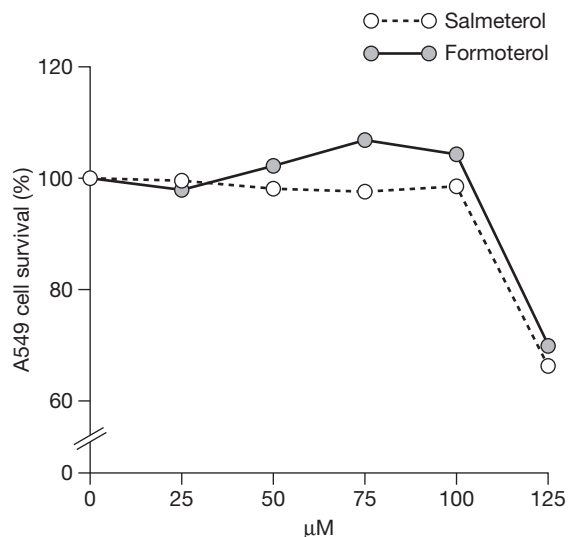


Fig. 1. Twenty four-hour survival curves of A549 cells. Drug sensitivities of A549 to either salmeterol or formoterol were assayed by 3-(4,5-dimethyl thiazol)-2,5-diphenyltetrazolium bromide colorimetric cell survival assay.

potent inhibitor of GM-CSF production at concentrations of 25 µM and 50 µM ($p=0.001$ at 25 µM and $p<0.001$ at 50 µM, Table 1).

Salmeterol and formoterol inhibition of VEGF secretion

Salmeterol and formoterol dose-dependently inhibited VEGF secretion from TNF- α -stimulated A549 cells

($p<0.01$, Fig. 4). Formoterol was the more potent inhibitor of VEGF secretion at a concentration of 75 µM ($p=0.003$, Table 1).

Discussion

Contemporary treatment strategies for moderate to severe persistent asthma represent a move away from inhaled corticosteroid (ICS) monotherapy towards a combination of ICS and LABAs [19]. Comparison of the effectiveness of these combinations with monotherapy has provided consistent clinical evidence that the former better improved pulmonary function, symptomatology, and exacerbation rates [2,20]. Although LABAs and ICS are known to work by different mechanisms, the mechanism of the combination remains unclear and underlying mechanisms are under investigation. Distinguishing the anti-inflammatory activity of LABAs will help define optimal long-term treatment regimens for asthma that not only improve pulmonary function, symptoms and exacerbation rates, but also protect against airway remodeling.

As observed in in vitro studies [5,21], β_2 -receptor agonists reduce TNF- α -stimulated GM-CSF production in T lymphocytes and human blood mononuclear cells. The high potency of formoterol depends on its strong lipophilicity, resulting in plasma membrane accumulation of the drug during a 24-h experiment [22]. The cell membrane is the target of β_2 -agonist activity,

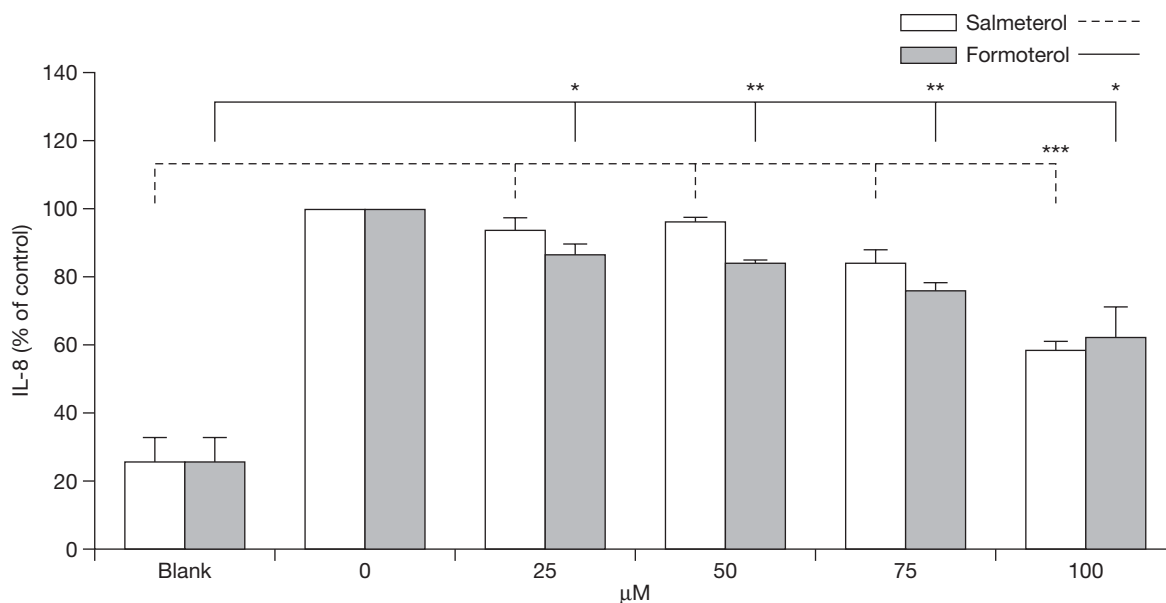


Fig. 2. Interleukin (IL)-8 levels produced by A549 cells exposed to 0, 25, 50, 75, and 100 µM of salmeterol and formoterol for 24 h and then stimulated with tumor necrosis factor-alpha 10 ng/mL. Columns and bars represent the mean \pm standard error of the mean. * $p<0.05$, ** $p<0.01$, *** $p<0.01$ vs control.

Table 1. Comparison of effects of salmeterol (SAL) and formoterol (FOR) on cytokine secretion in tumor necrosis factor- α -stimulated A549 cells

Concentration (μM)	% of control (mean \pm SEM)		p^a
	SAL	FOR	
IL-8 % of control			
25	93.50 \pm 3.94	86.46 \pm 3.27	0.241
50	96.40 \pm 0.99	84.13 \pm 0.67	0.001
75	84.25 \pm 3.74	75.75 \pm 2.51	0.132
100	58.52 \pm 2.61	62.06 \pm 9.26	0.732
GM-CSF			
25	49.32 \pm 1.49	73.04 \pm 2.20	0.001
50	44.53 \pm 0.97	67.18 \pm 1.54	<0.001
75	39.77 \pm 1.88	49.30 \pm 3.50	0.074
100	28.59 \pm 3.96	35.40 \pm 3.56	0.270
VEGF			
25	78.29 \pm 3.49	85.51 \pm 0.27	0.108
50	79.33 \pm 2.06	74.18 \pm 0.28	0.069
75	75.66 \pm 1.39	64.07 \pm 1.26	0.003
100	47.76 \pm 0.90	46.74 \pm 1.13	0.520

Abbreviations: SEM = standard error of the mean; IL-8 = interleukin-8; GM-CSF = granulocyte-macrophage colony-stimulating factor; VEGF = vascular endothelial growth factor

^aIndependent t test.

as the β_2 -receptors are located there, but few studies have monitored change in the membrane concentration of drug over time. Our study focusing on the effect of increasing concentrations of LABAs was based on a previous in vitro study of the effect of salmeterol on immunoglobulin E-mediated histamine release from human basophils by Kleine-Tebbe et al [12] and the

results of our drug sensitivity assay. We found that 100 μM salmeterol and a much lower level of formoterol (25 μM) significantly suppressed IL-8 secretion from TNF- α -stimulated A549 cells (58.52% \pm 2.61, $p=0.004$ and 86.46% \pm 3.27 of control value, $p=0.014$). At a dose of 50 μM , formoterol was more potent than salmeterol in suppressing IL-8 secretion (84.13% vs 96.40% of

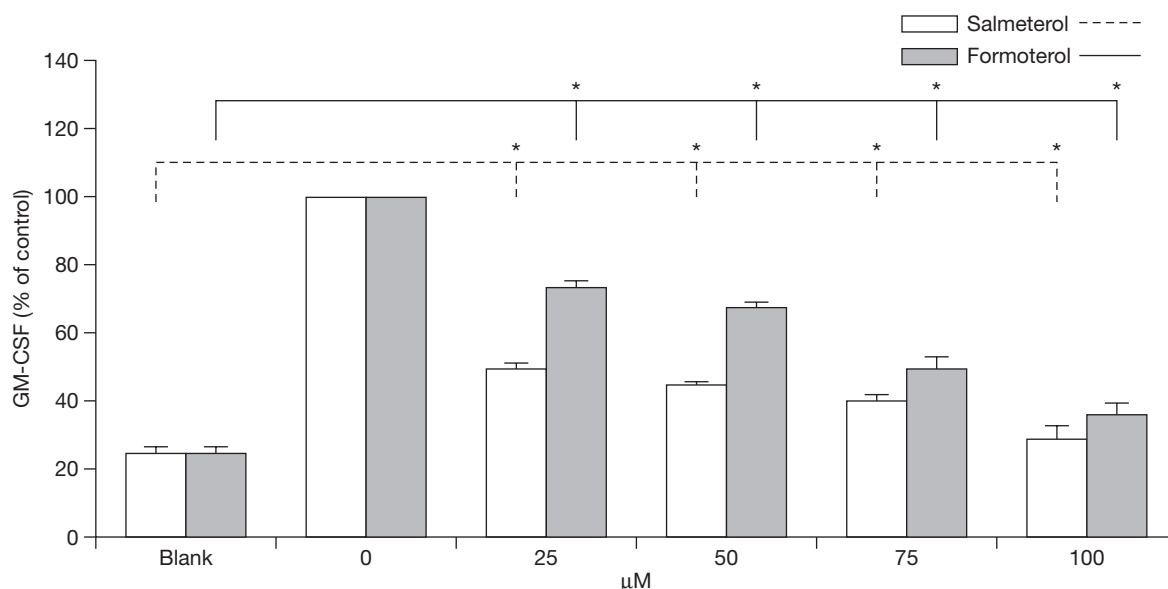


Fig. 3. Granulocyte macrophage-colony stimulation factor (GM-CSF) levels produced by A549 cells exposed to 0, 25, 50, 75, and 100 μM of salmeterol and formoterol for 24 h and then stimulated with tumor necrosis factor- α 10 ng/mL. Columns and bars represent mean \pm standard error of the mean. * $p < 0.01$ vs control.

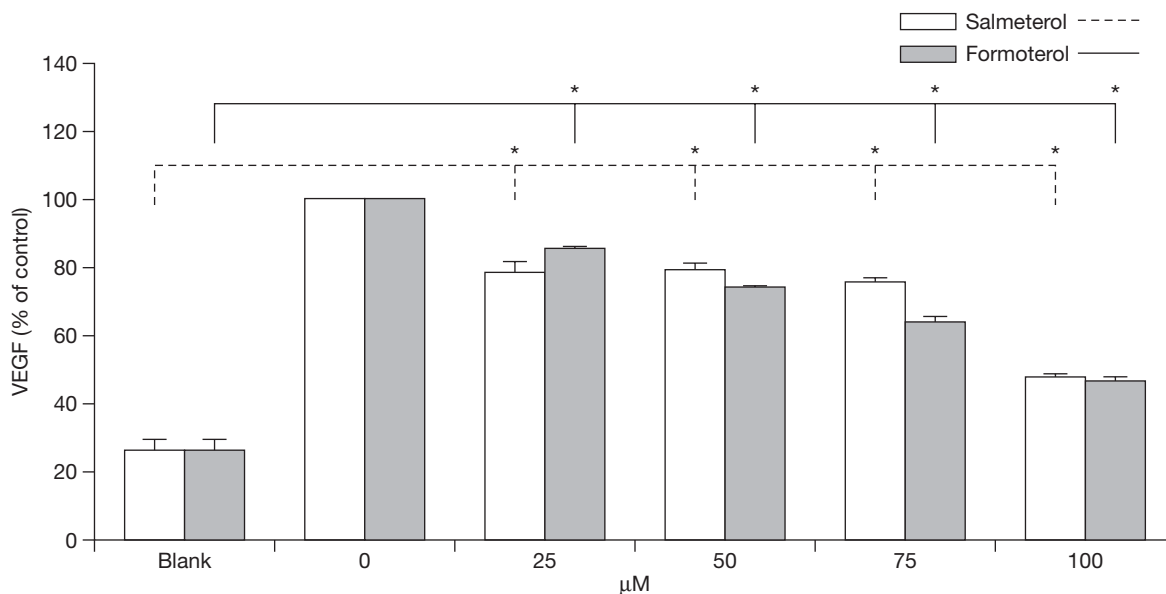


Fig. 4. Vascular endothelial growth factor (VEGF) levels produced by A549 cells exposed to 0, 25, 50, 75, and 100 μM of salmeterol and formoterol for 24 h and then stimulated with tumor necrosis factor- α 10 ng/mL. Columns and bars represent mean \pm standard error of the mean. * $p < 0.01$ vs control.

control value, $p = 0.001$). Both salmeterol and formoterol significantly and dose-dependently suppressed GM-CSF and VEGF secretion from TNF- α -stimulated A549 ($p < 0.01$). Salmeterol was more active than formoterol in suppressing GM-CSF production (25 μM : 49.32% vs 73.04% of control value, $p < 0.001$; 50 μM : 44.53% vs 67.18% of control value, $p < 0.001$) whereas formoterol was more active than salmeterol in suppressing VEGF production (75 μM : 64.07% vs 75.66% of control value, $p = 0.003$). Thus, LABAs can suppress proinflammatory cytokine secretion from airway epithelium. The underlying mechanisms remain unclear and the differences in the activities of salmeterol and formoterol action needs further evaluation.

It has recently been suggested that the anti-inflammatory properties of β_2 -agonists might be partially mediated through activation of the glucocorticoid receptor (GR). Eickelberg et al [23] demonstrated in primary pulmonary fibroblasts and vascular smooth muscle cells that β_2 -agonists could activate the GR, resulting in nuclear translocation, DNA binding, and initiation of transcription of a glucocorticoid responsive element-regulated reporter gene. Protein kinase A, a cAMP-dependent kinase activated by β_2 -agonists, apparently activates GR [24], as its inhibition blocks GR activation. An in vitro study on the anti-inflammatory effects of salmeterol and fluticasone on lung myofibroblasts revealed that the 2 drugs block the TNF- α -induced nuclear translocation of the

proinflammatory transcription factor, nuclear factor-kappaB (NF- κB). Finally, salmeterol decreases TNF- α -induced production of the inflammatory cytokine, IL-6 [25]. These findings suggest that blocking the TNF- α -induced nuclear translocation of NF- κB may contribute to the anti-inflammatory effect of LABAs. However, another in vitro study on human bronchial epithelial cells showed that the GR antagonist, RU486, did not influence the effect of formoterol, suggesting no involvement of the GR in the mechanism of action. In contrast, formoterol rapidly induced an elevation in intracellular cAMP, an effect which was reduced in the presence of propranolol. In addition, formoterol-induced cytokine secretion was fully blocked by propranolol, demonstrating that this effect is β_2 -receptor mediated [26]. The above findings suggest that the effect of formoterol is not fully mediated through the GR and may involve interactions between glucocorticosteroids and β_2 -agonists at other levels.

One recently published paper pointed out that β_2 -adrenergic receptor agonists can reduce the release of GM-CSF by human airway smooth muscle cells. These effects are considered anti-inflammatory and are ascribed to the activity of the (R)-enantiomer of the agonist. However, the effect of the (S)-enantiomer, once thought to be inert, on GM-CSF release has not been extensively explored. The study concluded that GM-CSF release by human airway smooth muscle cells is downregulated by (R)-enantiomers and enhanced by

(S)-enantiomers. The reversal of (R)-enantiomer and dexamethasone effects suggests suppression of their anti-inflammatory effects by the (S)-enantiomer, perhaps through an antagonistic mechanism similar to that of propranolol [27].

A subsequent study was designed on the hypothesis that the acute β_2 -adrenergic stimulation of airway epithelial cells with albuterol could suppress the production and release of inflammatory mediators, specifically GM-CSF, via a pathway involving inducible nitric oxide synthase. The results showed that inducible nitric oxide synthase was significantly upregulated in a concentration-dependent manner by the active (R)-enantiomer of albuterol. (R)-albuterol also attenuated cytokine-induced increases in GM-CSF steady-state mRNA expression and protein release. The (S)-enantiomer of albuterol had no effect on these parameters. Overall, this study identified a novel pathway by which β_2 -adrenergic agonists may exhibit anti-inflammatory effects in the airway epithelium and surrounding milieu [28]. However, the final mechanisms of the anti-inflammatory effects of LABAs have yet to be fully elucidated and will remain the main objective of our studies.

In the present study, we assessed the effects of 2 LABAs, salmeterol or formoterol, on IL-8, GM-CSF, and VEGF secretion by a human bronchial epithelial cell line and concluded that both had anti-inflammatory effects, as evidenced by inhibition of these inflammatory mediators.

Acknowledgments

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References

1. Woolcock A, Lundback B, Ringdal N, Jacques LA. Comparison of addition of salmeterol to inhaled steroids with doubling of the dose of inhaled steroids. *Am J Respir Crit Care Med.* 1996; 153:1481-8.
2. Pauwels RA, Löfdahl CG, Postma DS, Tattersfield AE, O'Byrne P, Barnes PJ, et al. Effect of inhaled formoterol and budesonide on exacerbations of asthma. Formoterol and Corticosteroids Establishing Therapy (FACET) International Study Group. *N Engl J Med.* 1997;337:1405-11.
3. Hancox RJ, Cowan JO, Flannery EM, Herbison GP, McLachlan CR, Wong CS, et al. Randomised trial of an inhaled beta2 agonist, inhaled corticosteroid and their combination in the treatment of asthma. *Thorax.* 1999;54:482-7.
4. Seldon PM, Stevens DA, Adcock IM, O'Connor BJ, Barnes PJ, Giembycz MA. Albuterol does not antagonize the inhibitory effect of dexamethasone on monocyte cytokine release. *Am J Respir Crit Care Med.* 1998;157:803-9.
5. Oddera S, Silvestri M, Testi R, Rossi GA. Salmeterol enhances the inhibitory activity of dexamethasone on allergen-induced blood mononuclear cell activation. *Respiration.* 1998;65: 199-204.
6. Stelmach I, Gorski P, Jerzynska J, Stelmach W, Majak P, Kuna P. A randomized, double-blind trial of the effect of treatment with formoterol on clinical and inflammatory parameters of asthma in children. *Ann Allergy Asthma Immunol.* 2002;89: 67-73.
7. Jeffery PK, Venge P, Gizycki MJ, Egerod I, Dahl R, Faurschou P. Effects of salmeterol on mucosal inflammation in asthma: a placebo-controlled study. *Eur Respir J.* 2002;20:1378-85.
8. Pinto Pereira LM, Clement Y, Rouse J, Matthew J, Asgarali Z, Ramoutar D. Salmeterol decreases eosinophilic cationic protein and rescue medication in patients inhaling beclomethasone dipropionate: preliminary study in mild and moderate asthma in Trinidad, West Indies. *Int J Clin Pharmacol Res.* 2003;23: 69-74.
9. Reid DW, Ward C, Wang N, Zheng L, Bish R, Orsida B, et al. Possible anti-inflammatory effect of salmeterol against interleukin-8 and neutrophil activation in asthma in vivo. *Eur Respir J.* 2003;21:994-9.
10. Abraham G, Kneuer C, Ehrhardt C, Honscha W, Ungemach FR. Expression of functional beta2-adrenergic receptors in the lung epithelial cell lines 16HBE14o(-), Calu-3 and A549. *Biochim Biophys Acta.* 2004;1691:169-79.
11. Johnson M. Effects of beta2-agonists on resident and infiltrating inflammatory cells. *J Allergy Clin Immunol.* 2002;110(Suppl 6):S282-90.
12. Kleine-Tebbe J, Frank G, Josties C, Kunkel G. Influence of salmeterol, a long-acting beta 2-adrenoceptor agonist, on IgE-mediated histamine release from human basophils. *J Investig Allergol Clin Immunol.* 1994;4:12-7.
13. Chong LK, Cooper E, Vardey CJ, Peachell PT. Salmeterol inhibition of mediator release from human lung mast cells by beta-adrenoceptor-dependent and independent mechanisms. *Br J Pharmacol.* 1998;123:1009-15.
14. McDonald DM. Angiogenesis and remodeling of airway vasculature in chronic inflammation. *Am J Respir Crit Care Med.* 2001;164:S39-45.
15. Norzila MZ, Fakes K, Henry RL, Simpson J, Gibson PG. Interleukin-8 secretion and neutrophil recruitment accompanies

- induced sputum eosinophil activation in children with acute asthma. *Am J Respir Crit Care Med.* 2000;161:769-74.
16. Ackerman V, Marini M, Vittori E, Bellini A, Vassali G, Mattoli S. Detection of cytokines and their cell sources in bronchial biopsy specimens from asthmatic patients. Relationship to atopic status, symptoms, and level of airway hyperresponsiveness. *Chest.* 1994;105:687-96.
 17. Lieber M, Smith B, Szakal A, Nelson-Rees W, Todaro G. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int J Cancer.* 1976;17:62-70.
 18. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983;65:55-63.
 19. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention, NHLBI/WHO Workshop Report, Updated 2003. London: Global Initiative for Asthma. Available from: https://ginasthma.com/wr_clean.pdf
 20. Shrewsbury S, Pyke S, Britton M. Meta-analysis of increased dose of inhaled steroid or addition of salmeterol in symptomatic asthma (MIASMA). *BMJ.* 2000;320:1368-73.
 21. Borger P, Hoekstra Y, Esselink MT, Postma DS, Zaagsma J, Vellenga E, et al. Beta-adrenoceptor-mediated inhibition of IFN-gamma, IL-3, and GM-CSF mRNA accumulation in activated human T lymphocytes is solely mediated by the beta2- adrenoceptor subtype. *Am J Respir Cell Mol Biol.* 1998; 19:400-7.
 22. Anderson GP. Formoterol: pharmacology, molecular basis of agonism, and mechanism of long duration of a highly potent and selective beta 2-adrenoceptor agonist bronchodilator. *Life Sci.* 1993;52:2145-60.
 23. Eickelberg O, Roth M, Lörx R, Bruce V, Rüdiger J, Johnson M, et al. Ligand-independent activation of the glucocorticoid receptor by beta2-adrenergic receptor agonists in primary human lung fibroblasts and vascular smooth muscle cells. *J Biol Chem.* 1999;274:1005-10.
 24. Cadepond F, Ulmann A, Baulieu EE. RU486 (mifepristone): mechanisms of action and clinical uses. *Annu Rev Med.* 1997; 48:129-56.
 25. Baouz S, Giron-Michel J, Azzarone B, Giuliani M, Cagnoni F, Olsson S, et al. Lung myofibroblasts as targets of salmeterol and fluticasone propionate: inhibition of alpha-SMA and NF-kappaB. *Int Immunol.* 2005;17:1473-81.
 26. Korn SH, Jerre A, Brattsand R. Effects of formoterol and budesonide on GM-CSF and IL-8 secretion by triggered human bronchial epithelial cells. *Eur Respir J.* 2001;17:1070-7.
 27. Ameredes BT, Calhoun WJ. Modulation of GM-CSF release by enantiomers of beta-agonists in human airway smooth muscle. *J Allergy Clin Immunol.* 2005;116:65-72.
 28. Chorley BN, Li Y, Fang S, Park JA, Adler KB. (R)-albuterol elicits antiinflammatory effects in human airway epithelial cells via iNOS. *Am J Respir Cell Mol Biol.* 2006;34:119-27.