



Original article

Effect of beta2-adrenergic agonists on eosinophil adhesion, superoxide anion generation, and degranulation



Toru Noguchi^{a, b, c}, Kazuyuki Nakagome^{a, b, *}, Takehito Kobayashi^{a, b, c}, Yutaka Ueda^{a, d}, Tomoyuki Soma^{a, b}, Hidetomo Nakamoto^c, Makoto Nagata^{a, b}

^a Allergy Center, Saitama Medical University, Saitama, Japan

^b Department of Respiratory Medicine, Saitama Medical University, Saitama, Japan

^c Department of General Internal Medicine, Saitama Medical University, Saitama, Japan

^d Department of Pediatrics, Saitama Medical University, Saitama, Japan

ARTICLE INFO

Article history:

Received 18 January 2015

Received in revised form

17 May 2015

Accepted 20 May 2015

Available online 5 July 2015

Keywords:

Adhesion molecule

Beta2-adrenergic agonist

Chemokine

Cysteinyl leukotrienes

Eosinophils

Abbreviations:

ANOVA, analysis of variance; cAMP, cyclic adenosine 3',5'-monophosphate; CXCR, CXC chemokine receptor; cysLT, cysteinyl leukotriene; EDN, eosinophil-derived neurotoxin; EPO, eosinophil peroxidase; FOR, formoterol; HBSS, Hank's balanced salt solution; ICAM, intercellular cell adhesion molecule; IL, interleukin; IP-10, IFN- γ -inducible protein of 10 kDa; O₂⁻, superoxide anion; PAF, platelet activating factor; rh, recombinant human; RV, rhinovirus; SAL, salbutamol; SOD, superoxide dismutase; Th, T helper

ABSTRACT

Background: Eosinophils play important roles in the development of asthma exacerbation. Viral infection is a major cause of asthma exacerbation, and the expression of IFN- γ -inducible protein of 10 kDa (IP-10) and cysteinyl leukotrienes (cysLTs) is up-regulated in virus-induced asthma. As β 2-adrenergic agonists, such as formoterol or salbutamol, are used to treat asthma exacerbation, we examined whether formoterol or salbutamol could modify eosinophil functions such as adhesiveness, particularly those activated by cysLTs or IP-10.

Methods: Eosinophils were isolated from the blood of healthy subjects and were pre-incubated with either formoterol or salbutamol, and subsequently stimulated with IL-5, LTD₄, or IP-10. Adhesion of eosinophils to intercellular cell adhesion molecule (ICAM)-1 was measured using eosinophil peroxidase assays. The generation of eosinophil superoxide anion (O₂⁻) was examined based on the superoxide dismutase-inhibitable reduction of cytochrome C. Eosinophil-derived neurotoxin (EDN) release was evaluated by ELISA as a marker of degranulation.

Results: Neither formoterol nor salbutamol suppressed the spontaneous adhesion of eosinophils to ICAM-1. However, when eosinophils were activated by IL-5, LTD₄, or IP-10, formoterol, but not salbutamol, suppressed the adhesion to ICAM-1. Formoterol also suppressed IL-5, LTD₄, or IP-10 induced eosinophil O₂⁻ generation or EDN release.

Conclusions: These findings suggest that formoterol, but not salbutamol, suppresses eosinophil functions enhanced by IL-5, LTD₄, or IP-10. As these factors are involved in the development of asthma exacerbation, our results strongly support the hypothesis that administration of formoterol is a novel strategy for treating asthma exacerbation.

Copyright © 2015, Japanese Society of Allergy. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Eosinophils play important roles in the pathophysiology of asthma through the release of a variety of inflammatory mediators including major basic protein, cysteinyl leukotrienes (cysLTs),

reactive oxygen species, and cytokines.^{1,2} Eosinophils are also involved in the development of asthma exacerbation. For example, Green *et al.* reported that a treatment strategy directed at normalization of the ratio of eosinophils in induced sputum reduces asthma exacerbation.³ Among T helper (Th) 2 cytokines, interleukin (IL)-5 is a well-recognized mediator in eosinophilic inflammation,⁴ and the *in vitro* effects on eosinophils include prolongation of eosinophil survival and modification of their functions.^{5,6} Recent studies suggested that in asthmatics with persistent sputum eosinophilia, treatment with anti-IL-5 mAb reduced asthma exacerbations and the requirement of systemic

* Corresponding author. Department of Respiratory Medicine, Allergy Center, Saitama Medical University, 38 Morohongo, Moroyama-cho, Iruma-gun, Saitama 350-0495, Japan.

E-mail address: nakagomek-ky@umin.ac.jp (K. Nakagome).

Peer review under responsibility of Japanese Society of Allergy.

corticosteroids,^{7–9} implicating the role of eosinophils or IL-5 in the development of asthma exacerbation.

CysLTs, such as LTC₄, LTD₄, and LTE₄, also contribute to the accumulation of eosinophils in the tissues of asthmatic airways. We have reported that LTD₄ up-regulates the expression of β 2 integrins on human eosinophils *in vitro* and augments eosinophil adhesion.¹⁰ We also reported that LTD₄ directly induces transendothelial migration, superoxide anion (O₂⁻) generation, and degranulation.¹¹ In fact, administration of cysLT1 receptor antagonists reduces the number of eosinophils in sputum or blood in asthma patients^{12,13} and the frequency of asthma exacerbation in children.¹⁴ Therefore, suppression of IL-5 (or Th2)-mediated or cysLT-mediated eosinophilic inflammation could be a useful strategy for inhibiting asthma exacerbation.

Acute respiratory infections are a major cause of asthma exacerbation.^{15,16} The role of IFN- γ -inducible protein of 10 kDa (IP-10) in virus-induced asthma exacerbation has been highlighted.^{17–19} We reported that IP-10 up-regulates eosinophil functions such as adhesion and O₂⁻ generation through CXCR3 and β 2 integrin.²⁰ We also reported that IP-10 induces degranulation and produces a number of cytokines/chemokines.²⁰ Therefore, during virus-induced asthma exacerbation IP-10 could directly activate the function of eosinophils in the airway.

β 2-adrenoreceptor agonists, such as formoterol and salbutamol, are used to treat asthma exacerbation. Among these drugs, a combination of formoterol and budesonide, especially when used as reliever therapy, suppressed the rate of moderate or severe exacerbation,^{21–23} suggesting that formoterol may have other functions in addition to its bronchodilating effect. To date, whether β 2-adrenoreceptor agonists are capable of suppressing eosinophilic inflammation remains unknown. Some reports suggest that β 2-adrenoreceptor agonists suppress the function of mast cells,^{24,25} neutrophils,^{26–30} eosinophils,^{31–33} and T cells,³⁴ although they are still controversial.^{35–38} However, the effect of formoterol or salbutamol on the function of eosinophils, particularly eosinophils activated by IL-5, cysLTs, or IP-10, has not been fully established.

The initial steps of eosinophil accumulation in the asthmatic airway are adhesion to and subsequent transmigration across endothelial cells. Therefore, interaction between eosinophils and adhesion molecules would contribute to the development of airway inflammation in bronchial asthma. In this study, we examined whether formoterol or salbutamol could modify eosinophil functions such as adhesiveness, activated by IL-5, cysLTs, or IP-10. We found that formoterol, but not salbutamol, suppressed eosinophil adhesion to intercellular cell adhesion molecule (ICAM)-1 when eosinophils were stimulated with IL-5, cysLTs, or IP-10. We also found that formoterol suppressed the IL-5, LTD₄, or IP-10-induced eosinophil O₂⁻ generation or degranulation.

Methods

Preparation of eosinophils

Eosinophils were isolated from peripheral blood collected from non-atopic healthy donors with a peripheral blood differential eosinophil count of <5%. The numbers of males and females, ranging in age from 21 to 59 years, were comparable among the donors. We received approval from the Ethical Committee of Saitama Medical University, and informed consent was obtained before collection of each blood sample. Eosinophils were isolated by negative selection using immunomagnetic beads, as described.^{10,11,20,39,40} Over 98% of the cells were eosinophils, as determined by morphologic criteria using May-Grünwald-Giemsa staining. Eosinophil viability was >99%, as determined by Trypan

blue dye exclusion. Eosinophils were resuspended in Hank's balanced salt solution (HBSS) supplemented with gelatin to a final concentration of 0.1% (HBSS/gel).

Eosinophil adhesion assay

Eosinophil adhesion to recombinant human (rh)-ICAM-1-coated plates was assessed based on the residual eosinophil peroxidase (EPO) activity of adherent eosinophils, as described.^{10,11,20,39,40} Eosinophils were pre-incubated with formoterol (10 nM/100 nM/1000 nM) or salbutamol (10 nM/100 nM/1000 nM) at 37 °C for 15 min. The eosinophils (100 μ l of 1×10^5 cells/ml in HBSS/gel) were then incubated with or without IL-5 (100 pM), LTD₄ (100 pM), or IP-10 (100 nM) in rh-ICAM-1-coated plates at 37 °C for 20 min. The plates were washed with HBSS and 100 μ l of HBSS/gel was then added to the wells. Standards comprised of 100 μ l of serially diluted cell suspensions (1×10^3 , 3×10^3 , 1×10^4 , 3×10^4 , and 1×10^5 cells/ml) were added to the empty wells. The EPO substrate (1 mM o-phenylenediamine, 1 mM H₂O₂, and 0.1% Triton X-100 in Tris buffer, pH 8.0) was then added to all wells and the plates were incubated for 30 min at room temperature. The reaction was stopped by adding 20 μ l of 4 M H₂SO₄ and absorbance was measured at 490 nm. Each experiment was performed in quadruplicate using eosinophils from a single donor, and the percentage eosinophil adhesion was determined from mean values that were calculated from log dose response curves. Eosinophil viability after incubation was >98%, as determined by Trypan blue dye exclusion.

Eosinophil O₂⁻ generation

Eosinophil O₂⁻ generation was measured in 96-well plates (Corning, NY, USA) as described based on the superoxide dismutase (SOD)-inhibitable reduction of cytochrome C.^{11,20} We initially added SOD (0.2 mg/ml in HBSS/gel; 20 μ l) to SOD control wells and then HBSS/gel to all wells to bring the final volume to 100 μ l. The eosinophil density was adjusted to 1.25×10^6 cells/ml of HBSS/gel mixed 4:1 with cytochrome C (12 mg/ml of HBSS/gel), and 100 μ l of eosinophil suspension was then added to all wells. After treatment with formoterol and stimulation with IL-5, LTD₄, or IP-10, the absorbance of the cell suspensions in the wells was measured at 550 nm in an Immuno-Mini (NJ-2300; Japan Intermed, Tokyo, Japan), followed by repeated measurements over the next 240 min. The plates were incubated in a 5% CO₂ incubator at 37 °C between measurements. Each reaction was evaluated in duplicate against the control reaction in wells containing 20 μ g/ml of SOD. The results were adjusted for a 1-ml reaction volume, and O₂⁻ generation was calculated at an extinction coefficient of 21.1 mM⁻¹ cm⁻¹, as nanomoles of cytochrome C reduced per 1.0×10^6 cells/ml minus the SOD control. The maximum value during the incubation time was examined to evaluate the effects of various factors on eosinophil O₂⁻ generation. Cell viability, determined by Trypan blue exclusion at the end of each experiment, remained at 95% after a 240-min incubation with the activator.

Eosinophil degranulation

Eosinophils (1×10^6 cells/ml) in 96-well plates were incubated for the 240 min that were required for measurement of O₂⁻ generation, and were then immediately centrifuged (700 g) at 4 °C for 15 min. Recovered cell-free supernatants were subjected to eosinophil-derived neurotoxin (EDN) analysis, as described previously.^{11,20} Levels of EDN were quantified using ELISA kits (Medical and Biological Laboratory, Nagoya, Japan).

Statistical analysis

Values are expressed as means $\pm$ SEM. Results were compared using a one-way factorial analysis of variance (ANOVA) with Tukey's test for multiple comparisons. Values of $P < 0.05$ were considered statistically significant.

Results

Effect of formoterol or salbutamol on spontaneous adhesion of eosinophils to ICAM-1

The effect of formoterol or salbutamol on spontaneous eosinophil adhesion to ICAM-1 was studied. Eosinophils were incubated with formoterol (10 nM/100 nM/1000 nM) or salbutamol (10 nM/100 nM/1000 nM) and eosinophil adhesion to rh-ICAM-1 was measured using residual EPO assays. In the absence of eosinophil activators, it was found that formoterol or salbutamol did not modify the adhesiveness of eosinophils to ICAM-1 (Fig. 1A, B). The viability of eosinophils after treatment of formoterol or salbutamol was more than 98%.

Effect of formoterol or salbutamol on eosinophil adhesion to ICAM-1 enhanced by IL-5

The effects of formoterol or salbutamol on IL-5-stimulated eosinophil adhesion to ICAM-1 were assessed because IL-5 plays important roles in the development of Th2-mediated eosinophilic inflammation.^{4–9} Eosinophils were pre-incubated with formoterol (10 nM/100 nM/1000 nM) or salbutamol (10 nM/100 nM/1000 nM) for 15 min, stimulated with IL-5 (100 pM), and adhesion to rh-ICAM-1 was evaluated. At 100 pM, IL-5 enhanced eosinophil adhesiveness to ICAM-1 (Fig. 2A, B). Formoterol (1000 nM) significantly suppressed IL-5-stimulated eosinophil adhesion to ICAM-1 (Fig. 2A; medium [without formoterol (FOR)] $21.0 \pm 1.1\%$, FOR (1000 nM) $15.4 \pm 0.6\%$; $P < 0.05$), although 10 nM or 100 nM

formoterol did not suppress adhesion [FOR (10 nM) $19.1 \pm 1.2\%$, FOR (100 nM) $18.0 \pm 1.1\%$]. In contrast, salbutamol (10–1000 nM) did not suppress eosinophil adhesion [Fig. 2B; medium (without salbutamol (SAL)] $22.9 \pm 2.2\%$, SAL (10 nM) $20.4 \pm 1.7\%$, SAL (100 nM) $21.3 \pm 1.3\%$, SAL (1000 nM) $20.6 \pm 1.5\%$; not significant]. As for pre-incubation time, 15 or 30 min treatment of eosinophils with formoterol suppressed IL-5-induced adhesion although no pre-incubation (simultaneous administration of formoterol) did not suppress it (Fig. 2C). So we pre-incubated eosinophils with β_2 -adrenoreceptor agonists for 15 min before addition of activator in the following experiments.

Effect of formoterol or salbutamol on eosinophil adhesion to ICAM-1 enhanced by LTD₄

LTD₄ plays an important role in the development of eosinophilic inflammation,^{10–14} and the effects of formoterol or salbutamol on LTD₄-stimulated eosinophil adhesion to ICAM-1 were assessed. As we previously reported,¹⁰ 100 pM LTD₄ enhanced the adhesiveness of eosinophils to ICAM-1 (Fig. 3A, B). Formoterol (1000 nM) significantly suppressed the eosinophil adhesion enhanced by LTD₄ [Fig. 3A; medium (without FOR) $16.7 \pm 1.3\%$, FOR (1000 nM) $9.4 \pm 1.0\%$; $P < 0.05$]. In contrast, salbutamol did not suppress eosinophil adhesion [Fig. 3B; medium (without SAL) $15.1 \pm 1.7\%$, SAL (1000 nM) $13.3 \pm 1.3\%$; not significant].

Effect of formoterol or salbutamol on eosinophil adhesion to ICAM-1 enhanced by IP-10

The effects of formoterol or salbutamol on IP-10-stimulated eosinophil adhesion to ICAM-1 were studied because IP-10 plays an important role in the development virus-induced asthma.^{17–19} As we previously reported,²⁰ IP-10 (100 nM) enhanced the adhesiveness of eosinophils to ICAM-1 (Fig. 4A, B). Formoterol (100–1000 nM) significantly suppressed IP-10-stimulated eosinophil adhesion to ICAM-1 [Fig. 4A; medium (without FOR)

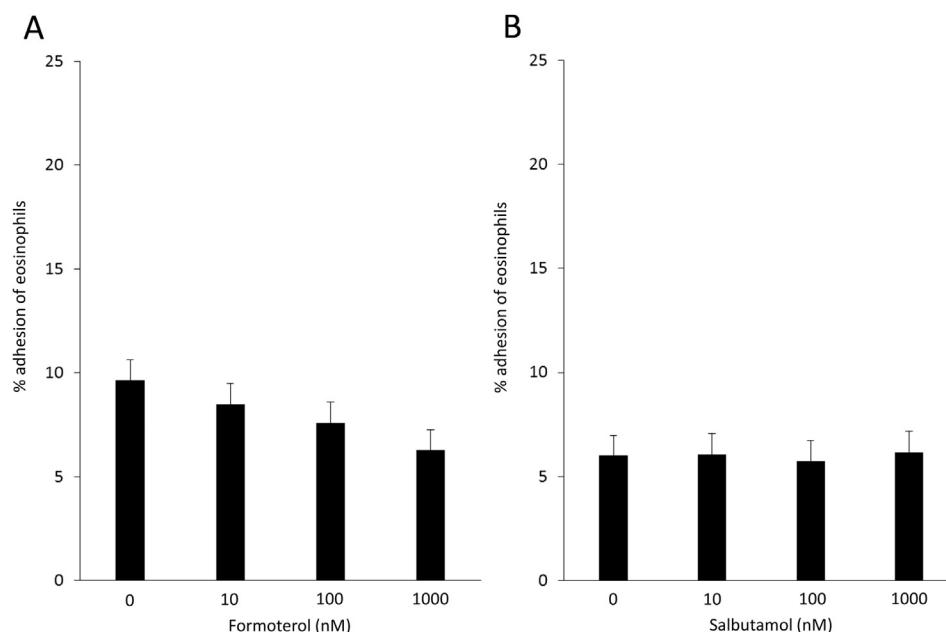


Fig. 1. Effect of formoterol or salbutamol on spontaneous eosinophil adhesiveness to ICAM-1. Eosinophils ($100 \mu\text{l}$ of 1×10^5 cell/ml in HBSS/gel) were incubated with or without formoterol (10 nM/100 nM/1000 nM) (A) or salbutamol (10 nM/100 nM/1000 nM) (B) at 37°C for 15 min. The adhesiveness of eosinophils to rh-ICAM-1 was assessed using residual EPO assays. Data are shown as means $\pm$ SEM of 6 experiments using cells from different donors.

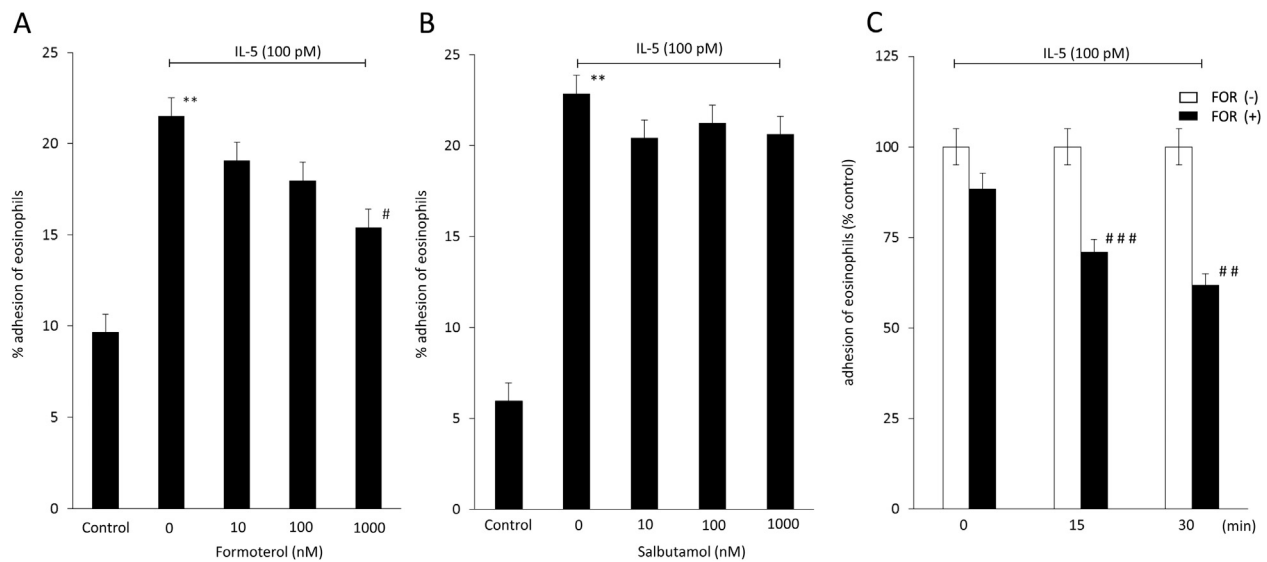


Fig. 2. Effect of formoterol or salbutamol on eosinophil adhesiveness to ICAM-1 enhanced by IL-5. Eosinophils were pre-incubated with formoterol (10 nM/100 nM/1000 nM) (A) or salbutamol (10 nM/100 nM/1000 nM) (B) for 15 min, and then stimulated with IL-5 (100 pM). The adhesiveness of eosinophils to rh-ICAM-1 was assessed using residual EPO assays. Data are shown as means $\pm$ SEM of 6 experiments using cells from different donors. ** $P < 0.01$ vs. eosinophil adhesion without IL-5 (control). # $P < 0.05$ vs. eosinophil adhesion by IL-5 without formoterol. (C) Effect of pre-incubation time of formoterol on eosinophil adhesion. Eosinophils were pre-incubated with formoterol (1000 nM) for 0 (no pre-incubation), 15, or 30 min, and then stimulated with IL-5 (100 pM). The adhesiveness of eosinophils to rh-ICAM-1 was assessed ($n = 4$). The adhesion of eosinophils observed in response to IL-5 without formoterol treatment was used as a control (100%). ## $P < 0.01$ or ### $P < 0.001$ vs. eosinophil adhesion by IL-5 without formoterol.

13.9 $\pm$ 0.6%, FOR (100 nM) 10.8% $\pm$ 1.0%; FOR (1000 nM) 8.5 $\pm$ 0.7%; $P < 0.01$, respectively.]. Conversely, salbutamol did not suppress IP-10-stimulated eosinophil adhesion [Fig. 4B; medium (without SAL) 11.0 $\pm$ 1.8%, SAL (1000 nM) 9.6 $\pm$ 1.6%; not significant]. We also examined the effect of pre-incubation time of formoterol on IP-10-induced eosinophil adhesion. Pre-incubation of formoterol for more than 15 min was needed to suppress IP-10-induced eosinophil adhesion (Fig. 4C).

Effect of formoterol on eosinophil O_2^- generation and degranulation

Finally, we examined whether formoterol could suppress other functions of eosinophils such as O_2^- generation and degranulation. Eosinophils were incubated with formoterol (1000 nM), stimulated with IL-5 (100 pM), LTD₄ (100 pM), or IP-10 (100 nM), and then eosinophil O_2^- generation and EDN release were measured. Formoterol (1000 nM) significantly suppressed IL-5, LTD₄, or IP-10-

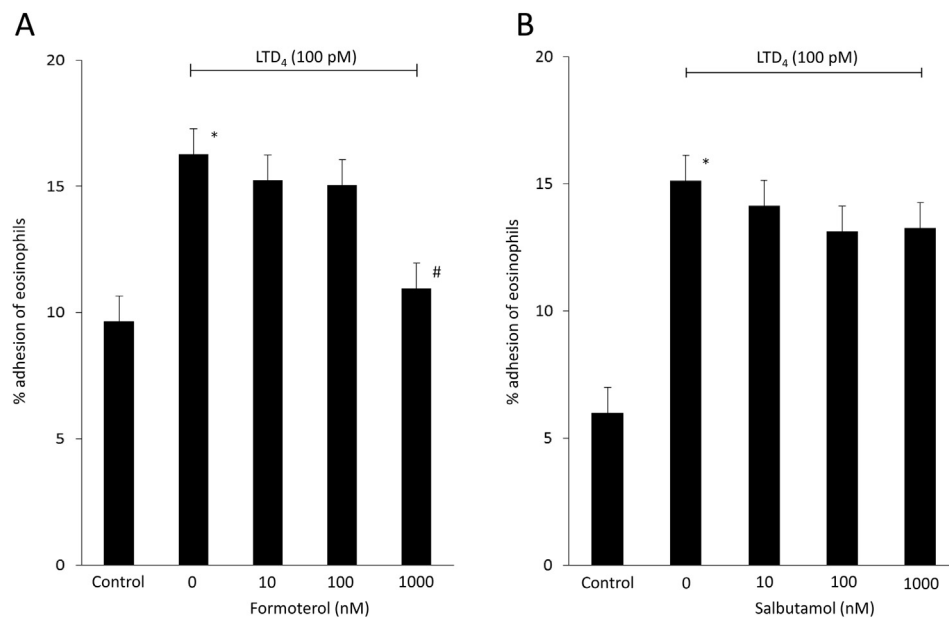


Fig. 3. Effect of formoterol or salbutamol on eosinophil adhesiveness to ICAM-1 enhanced by LTD₄. Eosinophils were pre-incubated with formoterol (10 nM/100 nM/1000 nM) (A) or salbutamol (10 nM/100 nM/1000 nM) (B) for 15 min, and then stimulated with LTD₄ (100 pM). The adhesiveness of eosinophils to rh-ICAM-1 was assessed using residual EPO assays. Data are shown as means $\pm$ SEM of 6 experiments using cells from different donors. * $P < 0.05$ vs. eosinophil adhesion without LTD₄ (control). # $P < 0.05$ vs. eosinophil adhesion by LTD₄ without formoterol.

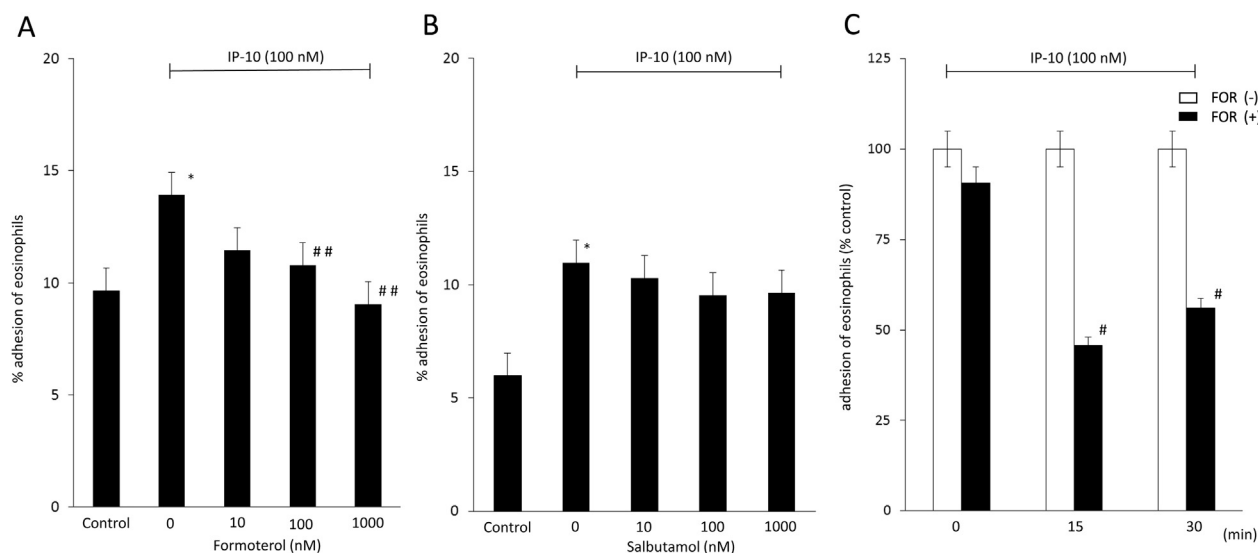


Fig. 4. Effect of formoterol or salbutamol on eosinophil adhesiveness to ICAM-1 enhanced by IP-10. Eosinophils were pre-incubated with formoterol (10 nM/100 nM/1000 nM) (A) or salbutamol (10 nM/100 nM/1000 nM) (B) for 15 min, and then stimulated with IP-10 (100 nM). The adhesiveness of the eosinophils to rh-ICAM-1 was assessed using residual EPO assays. Data are shown as means $\pm$ SEM of 6 experiments using cells from different donors. * P < 0.05 vs. eosinophil adhesion without IP-10 (control). ## P < 0.01 vs. eosinophil adhesion by IP-10 without formoterol. (C) Effect of pre-incubation time of formoterol on eosinophil adhesion. Eosinophils were pre-incubated with formoterol (1000 nM) for 0 (no pre-incubation), 15, or 30 min, and then stimulated with IP-10 (100 nM). The adhesiveness of eosinophils to rh-ICAM-1 was assessed (n = 4). The adhesion of eosinophils observed in response to IP-10 without formoterol treatment was used as a control (100%). # P < 0.05 vs. eosinophil adhesion by IP-10 without formoterol.

induced O_2^- generation (Fig. 5A; IL-5 3.4 ± 0.4 nmol/million cells, IL-5 + FOR 2.4 ± 0.3 nmol/million cells, LTD₄ 1.2 ± 0.1 nmol/million cells, LTD₄ + FOR 0.5 ± 0.1 nmol/million cells, IP-10 1.1 ± 0.1 nmol/million cells, IP-10 + FOR 0.5 ± 0.1 nmol/million cells; P < 0.05, respectively) or EDN release (Fig. 5B; IL-5 247.4 ± 7.1 ng/ml, IL-5 + FOR 134.9 ± 6.2 ng/ml, P < 0.01; LTD₄ 69.4 ± 4.6 ng/ml, LTD₄ + FOR 33.2 ± 2.8 ng/ml, P < 0.05; IP-10 68.8 ± 5.4 ng/ml, IP-10 + FOR 26.1 ± 1.9 ng/ml, P < 0.05). Therefore, formoterol could suppress overall functions of eosinophils including O_2^- generation and degranulation, enhanced by IL-5, LTD₄, or IP-10.

Discussion

In this study, we found that formoterol, but not salbutamol, suppressed the adhesion of eosinophils to ICAM-1 following activation by IL-5, LTD₄, or IP-10. However, in the absence of activator(s), neither formoterol nor salbutamol suppressed eosinophil adhesion. We also found that formoterol suppressed the IL-5, LTD₄, or IP-10-induced eosinophil O_2^- generation or EDN release. As IL-5, cysLTs, and IP-10 are involved in development of asthma exacerbation through eosinophilic inflammation and viral infection, our

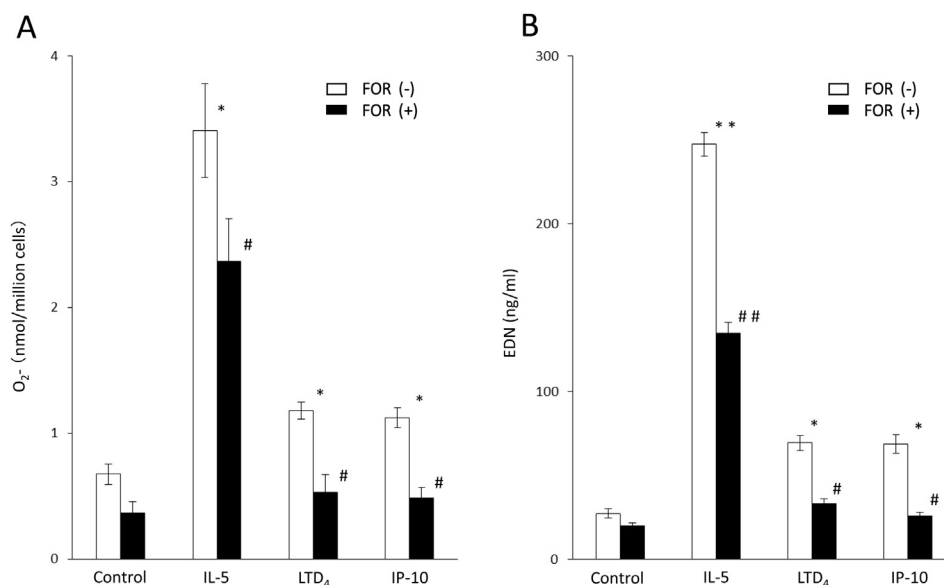


Fig. 5. Effect of formoterol on eosinophil O_2^- generation and EDN release. Eosinophils were incubated with formoterol (1000 nM) and stimulated with IL-5 (100 pM), LTD₄ (100 pM), or IP-10 (100 nM). (A) The generation of eosinophil O_2^- was examined based on the SOD-inhibitable reduction of cytochrome C. The absorbance of the cell suspensions in the wells was measured at 550 nm, followed by repeated measurements over the next 240 min. The maximum value during the incubation time was examined to evaluate the effects of various factors on eosinophil O_2^- generation. (B) EDN concentrations of cell-free supernatants were measured by ELISA. Data are shown as means $\pm$ SEM of 4 experiments using cells from different donors. * P < 0.05 and ** P < 0.01 vs. control. # P < 0.05 and ## P < 0.01 vs. without formoterol.

results suggest that administration of formoterol could be an effective strategy for treating asthma exacerbation via both its bronchodilation and anti-inflammatory actions.

β 2-adrenoreceptor agonists, especially short-acting beta agonists, are thought to be an aggravating factor for eosinophilic airway inflammation and airway hyperresponsiveness.^{38,41–44} However, several studies have suggested that formoterol may have anti-inflammatory properties *in vivo*.^{45–49} For example, Kelly *et al.* reported that addition of formoterol to budesonide therapy suppressed the eosinophil count in sputum as compared to budesonide alone.⁴⁵ Maneechotesuwan *et al.* reported that formoterol attenuated neutrophilic airway inflammation in asthma,⁴⁶ while Bowden *et al.* reported that formoterol inhibited neutrophil and eosinophil adhesion to venules of rat trachea.⁴⁷ Furthermore, Whelan *et al.* reported that formoterol, but not salbutamol, suppressed platelet activating factor (PAF)-induced eosinophil accumulation in guinea-pig lung.⁴⁸ As β 2-adrenoreceptor receptors are present on inflammatory cells, such as mast cells, monocytes, neutrophils, eosinophils, and T-lymphocytes,^{24,49} formoterol can affect these cells through activation of β 2-adrenergic receptors *in vitro*. For example, formoterol suppresses the release of histamine from mast cells,²⁴ and inhibits the function of neutrophils.^{26–28} However, there is little information about the effect of formoterol on eosinophil function. In this context, Eda *et al.* reported that formoterol at concentrations greater than 1 μ M inhibited PAF-induced eosinophil chemotaxis and degranulation.³¹ In the present study, we found that 1 μ M formoterol inhibited eosinophil adhesiveness enhanced by IL-5, LTD₄, or IP-10 (Fig. 2–4). Furthermore, 1 μ M formoterol also suppressed IL-5, LTD₄, or IP-10-induced eosinophil O₂⁻ generation or EDN release (Fig. 5). Therefore, 1 μ M formoterol appears to down-modulate the effector functions of eosinophils. As for the involvement of β 2-adrenergic receptor, Bowden *et al.* reported that ICI-118551, a β 2-adrenergic receptor antagonist, restored the formoterol-induced suppression of eosinophil or neutrophil adhesion to rat trachea.⁴⁷ Therefore, we think formoterol suppressed the function of eosinophils through β 2-adrenergic receptor although we have not examined the effect of its antagonist in this study.

The exact mechanisms by which formoterol, but not salbutamol, suppressed eosinophil adhesiveness or other functions remain unknown. One possibility is that the differences in cyclic adenosine 3', 5'-monophosphate (cAMP) levels in eosinophils may affect their functions. Kita *et al.* reported that pretreatment with a cAMP analog or cAMP phosphodiesterase-inhibitors strongly inhibited immunoglobulin-induced human eosinophil degranulation.⁵⁰ They also reported that cAMP accumulation is inversely correlated with eosinophil degranulation, and that the time course of cAMP accumulation closely paralleled that of inhibition of eosinophil degranulation.⁵⁰ As for neutrophils, formoterol increased cAMP more than salbutamol,^{27,28} and suppressed neutrophil functions, including O₂⁻ generation, LTB₄ generation, and elastase release.^{27,28} Furthermore, increased cAMP levels in neutrophils inhibited their adhesion.⁵¹ Formoterol also increased cAMP levels to a greater degree than salbutamol in cultured rat cardiomyocytes.⁵² Therefore, formoterol would increase the cAMP level in eosinophils to a greater degree than salbutamol, thus suppressing eosinophil functions, although we could not evaluate cAMP levels in formoterol-stimulated and salbutamol-stimulated eosinophils in this study. Other possibility is that greater intrinsic efficacy and higher receptor binding affinity of formoterol^{53,54} may affect the eosinophil adhesion or other functions. In this study, we compared the effect of formoterol with that of salbutamol at same concentrations. Considering the difference of intrinsic efficacy and higher receptor binding affinity, higher concentration of salbutamol may suppress the eosinophil functions.

Viral infections, in particular rhinovirus (RV), is a major cause of asthma exacerbation.¹⁵ Recent evidence suggests that IP-10 is involved in RV-induced asthma exacerbation.^{17–19} For example, RV infection induces bronchial epithelial cells to produce IP-10 *in vitro* and *in vivo*,¹⁷ and serum IP-10 concentrations are specifically increased in RV-induced asthma.¹⁹ Furthermore, increased levels of IP-10 correlate with disease severity during RV-induced exacerbation. These data suggest that IP-10 is likely to play a role in the pathogenesis of virus-induced asthma exacerbation. We previously reported that IP-10 up-regulates the effector functions of eosinophils such as adhesiveness, O₂⁻ generation, degranulation, and cytokine/chemokine production.²⁰ ICAM-1 is an adhesion molecule that plays an important role in inflammatory cell recruitment, as well as being a cellular receptor for the majority of RV-As and all RV-Bs.⁵⁵ Furthermore, RV infection increases ICAM-1 expression on epithelial cells,^{56,57} and eosinophil adhesion to ICAM-1 could activate eosinophil functions.^{58,59} Moreover, cysLTs are also increased in the airway during virus-induced asthma.^{60,61} For example, van Schaik *et al.* reported increased production of cysLTs in virus-induced wheezing.⁶⁰ Matsuse *et al.* reported that parainfluenza virus 3 infection was associated with a significant increase in sputum cysLTs during the acute phase of mild asthma exacerbation.⁶¹ Therefore, IP-10, ICAM-1, and cysLTs are all up-regulated in viral or RV-induced asthma, thereby activating eosinophil function in the airway. In this study, formoterol was shown to suppress the adhesiveness of eosinophils enhanced by IP-10 or LTD₄ and by ICAM-1 (Fig. 3A, 4A). Furthermore, budesonide/formoterol maintenance and reliever therapy was observed to reduce the risk of severe cold-related exacerbations, but not the incidence of colds, in asthmatics.⁶² Taken together, administration of formoterol could be a novel strategy for treating virus-induced asthma by suppressing the function of eosinophils activated by IP-10, LTD₄, and ICAM-1.

In this study, we have not examined the effect of formoterol on the expression of adhesion molecules such as CD11b/CD18. However, we previously reported that LTD₄ increased the expression of CD11b/CD18 on the eosinophil surface, and thus augmented the eosinophil adhesion to ICAM-1.¹⁰ Furthermore, Anderson *et al.* reported that formoterol suppressed the expression of adhesion molecules (CR3; CD11b/CD18) of neutrophils.²⁷ Taken together, we think that formoterol suppressed the eosinophil adhesion to ICAM-1 by suppressing the CD11b/CD18 expression on the eosinophil surface.

One limitation in interpreting the findings of our study is that there is little information about formoterol concentration in plasma or airways. Campestrini *et al.* reported that the plasma concentration of formoterol reached approximately 300 pM after inhalation of 90 μ g of formoterol.⁶³ Gravett *et al.* speculated that the local concentration of formoterol is approximately 10 nM (assuming a maximal single dose of 24 μ g, lung deposition of 18.6%, and a tidal volume of 500 ml).²⁶ Although the actual concentration in the airway is more difficult to ascertain, the concentration of more than 1 μ M in the inflammation sites seems to be hard to achieve after formoterol treatment during asthma attack.

We have not examined the effect of salmeterol, another long-acting beta agonist, in this study. However, some reports suggested that salmeterol might also have an anti-inflammatory property. For example, Ottonello *et al.* reported that salmeterol, but not salbutamol, suppressed super oxide generation by neutrophils.³⁰ Ezeamuzie *et al.* reported that salmeterol, but not salbutamol, suppressed IL-5 or PAF-induced activation of effector functions of eosinophils such as adhesion, super oxide generation, and degranulation.³² However, the relationship between the elevation of cAMP and the inhibitory effect by salmeterol is not

clear,³⁰ suggesting mechanisms other than increased cAMP levels may play roles in salmeterol-mediated suppression of neutrophil or eosinophil function.

In conclusion, we found that when eosinophils were activated by IL-5, LTD₄, or IP-10, formoterol, but not salbutamol, suppressed the adhesion of eosinophils to ICAM-1. Furthermore, formoterol suppressed the IL-5, LTD₄, or IP-10-induced eosinophil O₂⁻ generation or degranulation. Therefore, administration of formoterol may be a novel strategy for treating asthma exacerbation via its inhibitory effect on eosinophilic inflammation.

Acknowledgments

The authors thank Ms. Akemi Yokote for her excellent technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology (Japan, Grant nos. 205591191, 21790783, and 24791004).

Conflict of interest

MN received honoraria from MSD, Torii Pharmaceutical and AstraZeneca. The rest of the authors have no conflict of interest.

Authors' contributions

TN carried out the experiments, analyzed the data, and drafted the manuscript. KN participated in direction of the study, analyzed the data, and wrote the manuscript. TK and YU carried out the eosinophil experiments. TS and HN participated in the data analyses. MN participated in direction of the study and edited the manuscript. All authors read and approved the final manuscript.

References

- Gleich GJ. Mechanisms of eosinophil-associated inflammation. *J Allergy Clin Immunol* 2000;**105**:651–63.
- Weller PF. Human eosinophils. *J Allergy Clin Immunol* 2000;**100**:283–7.
- Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002;**360**:1715–21.
- Teran LM. Chemokines and IL-5: major players of eosinophil recruitment in asthma. *Clin Exp Allergy* 1999;**29**:287–90.
- Tai PC, Sun L, Spry CJ. Effects of IL-5, granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-3 on the survival of human blood eosinophils in vitro. *Clin Exp Immunol* 1991;**85**:312–6.
- Yamaguchi Y, Hayashi Y, Sugama Y, Miura Y, Kasahara T, Kitamura S, et al. Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs in vitro survival. IL-5 as an eosinophil chemotactic factor. *J Exp Med* 1988;**167**:1737–42.
- Nair P, Pizzichini MM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E, et al. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N Engl J Med* 2009;**360**:985–93.
- Haldar P, Brightling CE, Hargadon B, Gupta S, Monteiro W, Sousa A, et al. Mepolizumab and exacerbations of refractory eosinophilic asthma. *N Engl J Med* 2009;**360**:973–84.
- Ortega HG, Liu MC, Pavord ID, Brusselle GG, FitzGerald JM, Chetta A, et al. Mepolizumab treatment in patients with severe eosinophilic asthma. *N Engl J Med* 2014;**371**:1198–207.
- Nagata M, Saito K, Tsuchiya T, Sakamoto Y. Leukotriene D₄ upregulates eosinophil adhesion via the cysteinyl leukotriene 1 receptor. *J Allergy Clin Immunol* 2002;**109**:676–770.
- Saito K, Nagata M, Kikuchi I, Sakamoto Y. Leukotriene D₄ and eosinophil transendothelial migration, superoxide generation, and degranulation via beta2 integrin. *Ann Allergy Asthma Immunol* 2004;**93**:594–600.
- Currie GP, Lee DK, Haggart K, Bates CE, Lipworth BJ. Effects of montelukast on surrogate inflammatory markers in corticosteroid-treated patients with asthma. *Am J Respir Crit Care Med* 2003;**167**:1232–8.
- Bjerner L, Bisgaard H, Bousquet J, Fabbri LM, Greening AP, Haahtela T, et al. Montelukast and fluticasone compared with salmeterol and fluticasone in protecting against asthma exacerbation in adults: one year, double blind, randomised, comparative trial. *BMJ* 2003;**327**:891.
- Bisgaard H, Zielen S, Garcia-Garcia ML, Johnston SL, Gilles L, Menten J, et al. Montelukast reduces asthma exacerbations in 2- to 5-year-old children with intermittent asthma. *Am J Respir Crit Care Med* 2005;**171**:315–22.
- Friedlander SL, Busse WW. The role of rhinovirus in asthma exacerbations. *J Allergy Clin Immunol* 2005;**116**:267–73.
- Johnston SL, Pattemore PK, Sanderson G, Smith S, Campbell MJ, Josephs LK, et al. The relationship between upper respiratory infections and hospital admissions for asthma: a time-trend analysis. *Am J Respir Crit Care Med* 1996;**154**:654–60.
- Spurrell JC, Wiehler S, Zaheer RS, Sanders SP, Proud D. Human airway epithelial cells produce IP-10 (CXCL10) in vitro and in vivo upon rhinovirus infection. *Am J Physiol Lung Cell Mol Physiol* 2005;**289**:L85–95.
- Korpi-Steiner NL, Valkenaar SM, Bates ME, Evans MD, Gern JE, Bertics PJ. Human monocytic cells direct the robust release of CXCL10 by bronchial epithelial cells during rhinovirus infection. *Clin Exp Allergy* 2010;**40**:1203–13.
- Wark PA, Bucchieri F, Johnston SL, Gibson PG, Hamilton L, Mimica J, et al. IFN-γ-induced protein 10 is novel biomarker of rhinovirus induced asthma exacerbation. *J Allergy Clin Immunol* 2007;**120**:586–93.
- Takaku Y, Nakagome K, Kobayashi T, Hagiwara K, Kanazawa M, Nagata M. IFN-γ-inducible protein of 10 kDa upregulates the effector functions of eosinophils through β2 integrin and CXCR3. *Respir Res* 2011;**12**:138.
- 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.
- Rabe KF, Atienza T, Magyar P, Larsson P, Jorup C, Lalloo UG. Effect of budesonide in combination with formoterol for reliever therapy in asthma exacerbations: a randomised controlled, double-blind study. *Lancet* 2006;**368**:744–53.
- O'Byrne PM, Bisgaard H, Godard PP, Pistolesi M, Palmqvist M, Zhu Y, et al. Budesonide/formoterol combination therapy as both maintenance and reliever medication in asthma. *Am J Respir Crit Care Med* 2005;**171**:129–36.
- Johnson M. Effects of beta2-agonists on resident and infiltrating inflammatory cells. *J Allergy Clin Immunol* 2002;**110**:S282–90.
- Butchers PR, Vardey CJ, Johnson M. Salmeterol: a potent and long-acting inhibitor of inflammatory mediator release from human lung. *Br J Pharmacol* 1991;**104**:672–6.
- Gravett CM, Theron AJ, Steel HC, Tintinger GR, Cockeran R, Feldman C, et al. Interactive inhibitory effects of formoterol and montelukast on activated human neutrophils. *Eur Respir J* 2010;**36**:1417–24.
- Anderson R, Theron AJ, Steel HC, Durandt C, Tintinger GR, Feldman C. The beta-2-adrenoreceptor agonists, formoterol and indacaterol, but not salbutamol, effectively suppress the reactivity of human neutrophils in vitro. *Mediat Inflamm* 2014;**2014**:105420.
- Tintinger GR, Anderson R, Theron AJ, Ramafi G, Ker JA. Comparison of the effects of selective and non-selective beta-adrenoreceptor agonists on the pro-inflammatory activities of human neutrophils in vitro. *Inflammation* 2000;**24**:239–49.
- Kawashima A, Suzuki T, Nishihara F, Kobayashi T, Takaku Y, Nakagome K, et al. Effect of formoterol on eosinophil trans-basement membrane migration induced by interleukin-8-stimulated neutrophils. *Int Arch Allergy Immunol* 2013;**161**(Suppl 2):10–5.
- Otonello L, Morone P, Dapino P, Dallegri F. Inhibitory effect of salmeterol on the respiratory burst of adherent human neutrophils. *Clin Exp Immunol* 1996;**106**:97–102.
- Eda R, Sugiyama H, Hopp RJ, Okada C, Bewtra AK, Townley RG. Inhibitory effects of formoterol on platelet-activating factor induced eosinophil chemotaxis and degranulation. *Int Arch Allergy Immunol* 1993;**102**:391–8.
- Ezeamuzie CI, al-Hage M. Differential effects of salbutamol and salmeterol on human eosinophil responses. *J Pharmacol Exp Ther* 1998;**284**:25–31.
- Myo S, Zhu X, Myou S, Meliton AY, Liu J, Boetticher E, et al. Additive blockade of beta 2-integrin adhesion of eosinophils by salmeterol and fluticasone propionate. *Eur Respir J* 2004;**23**:511–7.
- Hashimoto T, Kitamura N, Kobayashi N, Suko M, Kaminuma O, Mori A. Effect of formoterol on allergen-induced cytokine synthesis by atopic asthmatics. *Int Arch Allergy Immunol* 2007;**143**(s1):106–9.
- Machida K, Inoue H, Matsumoto K, Tsuda M, Fukuyama S, Koto H, et al. Activation of PI3K-Akt pathway mediates antiapoptotic effects of beta-adrenergic agonist in airway eosinophils. *Am J Physiol Lung Cell Mol Physiol* 2005;**288**:L860–7.
- Yukawa T, Ukena D, Kroegel C, Chanez P, Dent G, Chung KF, et al. Beta 2-adrenergic receptors on eosinophils. Binding and functional studies. *Am Rev Respir Dis* 1990;**141**:1446–52.
- Muñoz NM, Rabe KF, Vita AJ, McAllister K, Mayer D, Weiss M, et al. Paradoxical blockade of beta adrenergically mediated inhibition of stimulated eosinophil secretion by salmeterol. *J Pharmacol Exp Ther* 1995;**273**:850–4.
- Yamaguchi T, Soma T, Takaku Y, Nakagome K, Hagiwara K, Kanazawa M, et al. Salbutamol modulates the balance of Th1 and Th2 cytokines by mononuclear cells from allergic asthmatics. *Int Arch Allergy Immunol* 2010;**152**(s1):32–40.
- Kobayashi T, Takaku Y, Yokote A, Miyazawa H, Soma T, Hagiwara K, et al. Interferon-β augments eosinophil adhesion inducing activity of endothelial cells. *Eur Respir J* 2008;**32**:1540–7.
- Soma T, Takaku Y, Kobayashi T, Hagiwara K, Kanazawa M, Uematsu K, et al. Inhibitory effect of budesonide alone and in combination with formoterol on IL-5 and RANTES production from mononuclear cells. *Int Arch Allergy Immunol* 2008;**146**(s1):22–7.
- Gauvreau GM, Jordana M, Watson RM, Cockcroft DW, O'Byrne PM. Effect of regular inhaled albuterol on allergen-induced late responses and sputum eosinophils in asthmatic subjects. *Am J Respir Crit Care Med* 1997;**156**:1738–45.
- Swystun VA, Gordon JR, Davis EB, Zhang X, Cockcroft DW. Mast cell tryptase release and asthmatic responses to allergen increase with regular use of salbutamol. *J Allergy Clin Immunol* 2000;**106**:57–64.

43. Lindqvist A, Karjalainen EM, Laitinen LA, Kava T, Altraja A, Pulkkinen M, et al. Salmeterol resolves airway obstruction but does not possess anti-eosinophil efficacy in newly diagnosed asthma: a randomized, double-blind, parallel group biopsy study comparing the effects of salmeterol, fluticasone propionate, and disodium cromoglycate. *J Allergy Clin Immunol* 2003;**112**:23–8.
44. Mcivor RA, Pizzichini E, Turner MO, Hussack P, Hargreave FE, Sears MR. Potential masking effects of salmeterol on airway inflammation in asthma. *Am J Respir Crit Care Med* 1998;**158**:924–30.
45. Kelly MM, O'Connor TM, Leigh R, Otis J, Gwozd C, Gauvreau GM, et al. Effects of budesonide and formoterol on allergen-induced airway responses, inflammation, and airway remodeling in asthma. *J Allergy Clin Immunol* 2010;**125**:349–56.
46. Maneechotesuwan K, Essilfie-Quaye S, Meah S, Kelly C, Kharitonov SA, Adcock IM, et al. Formoterol attenuates neutrophilic airway inflammation in asthma. *Chest* 2005;**128**:1936–42.
47. Bowden JJ, Sulakvelidze I, McDonald DM. Inhibition of neutrophil and eosinophil adhesion to venules of rat trachea by beta 2-adrenergic agonist formoterol. *J Appl Physiol* 1994;**77**:397–405.
48. Whelan CJ, Johnson M, Vardey CJ. Comparison of the anti-inflammatory properties of formoterol, salbutamol and salmeterol in guinea-pig skin and lung. *Br J Pharmacol* 1993;**110**:613–8.
49. Barnes PJ. Beta-adrenoceptors on smooth muscle, nerves and inflammatory cells. *Life Sci* 1993;**52**:2101–9.
50. Kita H, Abu-Ghazaleh RI, Gleich GJ, Abraham RT. Regulation of Ig-induced eosinophil degranulation by adenosine 3',5'-cyclic monophosphate. *J Immunol* 1991;**146**:2712–8.
51. Bloemen PG, van den Tweel MC, Henricks PA, Engels F, Kester MH, van de Loo PG, et al. Increased cAMP levels in stimulated neutrophils inhibit their adhesion to human bronchial epithelial cells. *Am J Physiol* 1997;**272**:L580–7.
52. Freyss-Beguín M, Griffaton G, Lechat P, Picken D, Quennedey MC, Rouot B, et al. Comparison of the chronotropic effect and the cyclic AMP accumulation induced by beta 2-agonists in rat heart cell culture. *Br J Pharmacol* 1983;**78**:717–23.
53. Hanania NA, Sharafkhaneh A, Barber R, Dickey BF. Beta-agonist intrinsic efficacy: measurement and clinical significance. *Am J Respir Crit Care Med* 2002;**165**:1353–8.
54. Johnson M. Pharmacology of long-acting beta-agonists. *Ann Allergy Asthma Immunol* 1995;**75**:177–9.
55. Greve JM, Davis G, Meyer AM, Forte CP, Yost SC, Marlor CW, et al. The major human rhinovirus receptor is ICAM-1. *Cell* 1989;**56**:839–47.
56. Papi A, Johnston SL. Rhinovirus infection induces expression of its own receptor intercellular adhesion molecule1 (ICAM-1) via increased NF- κ B-mediated transcription. *J Biol Chem* 1999;**274**:9707–20.
57. Grünberg K, Sharon RF, Hiltermann TJ, Brahim JJ, Dick EC, Sterk PJ, et al. Experimental rhinovirus 16 infection increases intercellular adhesion molecule-1 expression in bronchial epithelium of asthmatics regardless of inhaled steroid treatment. *Clin Exp Allergy* 2000;**30**:1015–23.
58. Nagata M, Sedgwick JB, Bates ME, Kita H, Busse WW. Eosinophil adhesion to vascular cell adhesion molecule-1 activates superoxide anion generation. *J Immunol* 1995;**155**:2194–202.
59. Nagata M, Sedgwick JB, Kita H, Busse WW. Granulocyte macrophage colony-stimulating factor augments ICAM-1 and VCAM-1 activation of eosinophil function. *Am J Respir Cell Mol Biol* 1998;**19**:158–66.
60. van Schaik SM, Tristram DA, Nagpal IS, Hintz KM, Welliver 2nd RC, Welliver RC. Increased production of IFN-gamma and cysteinyl leukotrienes in virus-induced wheezing. *J Allergy Clin Immunol* 1999;**103**:630–6.
61. Matsuse H, Kondo Y, Saeki S, Nakata H, Fukushima C, Mizuta Y, et al. Naturally occurring parainfluenza virus 3 infection in adults induces mild exacerbation of asthma associated with increased sputum concentrations of cysteinyl leukotrienes. *Int Arch Allergy Immunol* 2005;**138**:267–72.
62. Reddel HK, Jenkins C, Quirce S, Sears MR, Bateman ED, O'Byrne PM, et al. Effect of different asthma treatments on risk of cold-related exacerbations. *Eur Respir J* 2011;**38**:584–93.
63. Campestrini J, Lecaillon JB, Godbillon J. Automated and sensitive method for the determination of formoterol in human plasma by high-performance liquid chromatography and electrochemical detection. *J Chromatogr B Biomed Sci Appl* 1997;**704**:221–9.