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

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A B S T R A C T

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 β2-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.

Introductions

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. 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. Among T helper (Th) 2 cytokines, interleukin (IL)-5 is a well-recognized mediator in eosinophilic inflammation, and the in vitro effects on eosinophils include prolongation of eosinophil survival and modification of their functions. Recent studies suggested that in asthmas with persistent sputum eosinophilia, treatment with anti-IL-5 mAb reduced asthma exacerbations and the requirement of systemic
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 β2 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 patients12–13 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.5,15 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 CXC chemokine receptor (CXCR) 3 and β2 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.

β2-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 β2-adrenoreceptor agonists are capable of suppressing eosinophilic inflammation remains unknown. Some reports suggest that β2-adrenoreceptor agonists suppress the function of mast cells,24–27 neutrophils,28–30 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 μl of 1 × 105 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 μl of HBSS/gel was then added to the wells. Standards comprised of 100 μl of serially diluted cell suspensions (1 × 104, 3 × 103, 1 × 103, 3 × 102, and 1 × 105 cells/ml) were added to the empty wells. The EPO substrate (1 mM o-phenyleneediamine, 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 μl 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 μl) to SOD control wells and then HBSS/gel to all wells to bring the final volume to 100 μl. The eosinophil density was adjusted to 1.25 × 105 cells/ml of HBSS/gel mixed 4:1 with cytochrome C (12 mg/ml of HBSS/gel), and 100 μl 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 μg/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 mmol-1 cm−1, as nanomoles of cytochrome C reduced per 1.0 × 106 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 × 106 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 ± 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 ± 1.1%, FOR (1000 nM) 15.4 ± 0.6%; $P < 0.05$), although 10 nM or 100 nM formoterol did not suppress adhesion [FOR (10 nM) 19.1 ± 1.2%, FOR (100 nM) 18.0 ± 1.1%]. In contrast, salbutamol (10–1000 nM) did not suppress eosinophil adhesion [Fig. 2B; medium (without salbutamol (SAL)] 22.9 ± 2.2%, SAL (10 nM) 20.4 ± 1.7%, SAL (100 nM) 21.3 ± 1.3%, SAL (1000 nM) 20.6 ± 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 β2-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,15 100 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 ± 1.3%, FOR (1000 nM) 9.4 ± 1.0%; $P < 0.05$]. In contrast, salbutamol did not suppress eosinophil adhesion [Fig. 3B; medium (without SAL) 15.1 ± 1.7%, SAL (1000 nM) 13.3 ± 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. 4 A, B). Formoterol (100–1000 nM) significantly suppressed IP-10-stimulated eosinophil adhesion to ICAM-1 (Fig. 4A; medium (without FOR)
13.9 ± 0.6%, FOR (100 nM) 10.8% ± 1.0%; FOR (1000 nM) 8.5 ± 0.7%; *P < 0.01, respectively. Conversely, salbutamol did not suppress IP-10-stimulated eosinophil adhesion [Fig. 4B; medium (without SAL) 11.0 ± 1.8%, SAL (1000 nM) 9.6 ± 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 O2/C0 generation and degranulation**

Finally, we examined whether formoterol could suppress other functions of eosinophils such as O2/C0 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 O2/C0 generation and EDN release were measured. Formoterol (1000 nM) significantly suppressed IL-5, LTD4, or IP-10-
induced O₂ 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 + 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 O₂ 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 O₂ 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
results suggest that administration of formoterol could be an effective strategy for treating asthma exacerbation via both its bronchodilatory and anti-inflammatory actions. β2-adrenergic receptor agonists, especially short-acting beta agonists, are thought to be an aggravating factor for eosinophilic airway inflammation and airway hyperresponsiveness. However, several studies have suggested that formoterol may have anti-inflammatory properties in vivo. For example, Kelly et al. reported that addition of formoterol to budesonide therapy suppressed the eosinophil count in sputum as compared to budesonide alone. Maneechotesuwon et al. reported that formoterol attenuated neutrophilic airway inflammation in asthma, while Bowden et al. reported that formoterol inhibited neutrophil and eosinophil adhesion to venules of rat trachea. Furthermore, Whelan et al. reported that formoterol, but not salbutamol, suppressed platelet activating factor (PAF)-induced eosinophil accumulation in guinea-pig lung. As β2-adrenergic receptor antagonists are present on inflammatory cells, such as mast cells, monocytes, neutrophils, eosinophils, and T-lymphocytes, formoterol can affect these cells through activation of β2-adrenergic receptors in vitro. Formoterol-stimulated and salbutamol-stimulated eosinophils in greater degree than salbutamol, thus suppressing eosinophil degranulation.50 As for neutrophils, formoterol increased cAMP levels in neutrophils to a greater extent than salbutamol,26 which may inhibit neutrophilic airway inflammation in asthma. Furthermore, increased levels of IP-10 correlate with disease severity during RV-induced asthma. 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. 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. Furthermore, RV infection increases ICAM-1 expression on epithelial cells, and eosinophil adhesion to ICAM-1 could activate eosinophil functions. Moreover, cysteine leukotrienes are also increased in the airway during virus-induced asthma. For example, van Schaik et al. reported increased production of cysteine leukotrienes in virus-induced wheezing. Matsuda et al. reported that parainfluenza virus 3 infection was associated with a significant increase in sputum cysteine leukotrienes during the acute phase of mild asthma exacerbation. Therefore, IP-10, ICAM-1, and cysteine leukotrienes are all up-regulated in viral or RV-induced asthma, thereby activating eosinophil functions 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. 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. 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. Furthermore, Anderson et al. reported that formoterol suppressed the expression of adhesion molecules (CR3; CD11b/CD18) of neutrophils. 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 μM after inhalation of 90 μg of formoterol. Gravett et al. speculated that the local concentration of formoterol is approximately 10 nM (assuming a maximal single dose of 24 μg, lung deposition of 18.6%, and a tidal volume of 500 ml). Although the actual concentration in the airway is more difficult to ascertain, the concentration of more than 1 μM in the inflammation cites 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, Ottolino et al. reported that salmeterol, but not salbutamol, suppressed super oxide generation by neutrophils. 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. However, the relationship between the elevation of cAMP and the inhibitory effect by salmeterol is not
clear, 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 eosinophil inflammation.

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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 KN participated in the data analyses. MN participated in direction of the study and edited the manuscript. All authors read and approved the final manuscript.

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