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Inhibition by fenoterol of human eosinophil functions including β_2-adrenoceptor-independent actions

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SUMMARY

Agonists at β_2 adrenoceptors are used widely as bronchodilators in treating bronchial asthma. These agents also may have important anti-inflammatory effects on eosinophils in asthma. We examined whether widely prescribed β_2-adrenoceptor agonists differ in ability to suppress stimulus-induced eosinophil effector functions such as superoxide anion (O_2^-) generation and degranulation. To examine involvement of cellular adhesion in such responses, we also investigated effects of β_2 agonists on cellular adhesion and on CD11b expression by human eosinophils. O_2^- was measured using chemiluminescence. Eosinophil degranulation and adhesion were assessed by a radioimmunoassay for eosinophil protein X (EPX). CD11b expression was measured by flow cytometry. Fenoterol inhibited platelet-activating factor (PAF)-induced O_2^- generation by eosinophils significantly more than salbutamol or procaterol. Fenoterol partially inhibited PAF-induced degranulation by eosinophils similarly to salbutamol or procaterol. Fenoterol inhibited phorbol myristate acetate (PMA)-induced O_2^- generation and degranulation by eosinophils, while salbutamol or procaterol did not. Fenoterol inhibition of PMA-induced O_2^- generation was not reversed by ICI-118551, a selective β_2-adrenoceptor antagonist. Fenoterol, but not salbutamol or procaterol, significantly inhibited PAF-induced eosinophil adhesion. Fenoterol inhibited O_2^- generation and degranulation more effectively than salbutamol or procaterol; these effects may include a component involving cellular adhesion. Inhibition also might include a component not mediated via β_2 adrenoceptors.

INTRODUCTION

Eosinophils and their products, such as toxic granule proteins and oxygen radicals, are thought to play important roles in the pathogenesis of allergic diseases such as bronchial asthma [1,2], although some studies in asthmatic patients have not supported major involvement of eosinophils [3,4]. Eosinophil products cause airway epithelial damage that results in development of the bronchial hyperreactivity that is characteristic of asthma [5,6]. Allergic inflammation of the lung involves accumulation of eosinophils. Eosinophilic infiltration from the blood vessels into tissues involves multiple steps including adhesion, chemotaxis, and transmigration through the endothelial cell layer and the basement membrane [7]. These processes are induced by various inflammatory mediators and chemokines, including platelet-activating factor (PAF) [8], leukotriene (LT) B4 [9], RANTES [10] and eotaxin [11]. Adhesion molecules including β_2 integrins, particularly $\alpha M\beta_2$ (CD11b/CD18), also mediate these processes in eosinophil recruitment [7]. For example, eosinophil adhesion induced by PAF, granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin (IL)-5 was inhibited by anti-CD18 monoclonal antibody (MoAb) or anti-CD11b MoAb [12–14]. Furthermore, recent studies suggest that cellular adhesion through a β_2 integrin is an important step for eosinophil activation, including degranulation and superoxide production, and that the effector functions of eosinophil induced by GM-CSF, PAF or immobilized IgG are mediated by $\alpha M\beta_2$ [12,14,15].

Agonists at β_2 adrenoceptors are used widely as bronchodilators to reverse acute bronchospasm in asthma. Such agonists also were shown to decrease reactivity and effects of inflammatory cells. These drugs can inhibit mast cell degranulation [16], early and late cutaneous reactions to antigen as well as certain other allergic reactions [17,18], plasma leakage in response to various inflammatory stimuli [19–21], oxidant production [22–25] and LTC4 production [26].

Previous clinical studies suggest that eosinophils might be an important target for the anti-inflammatory action of β_2 agonists, whereas other studies suggest β_2 agonists contribute to the prolonged eosinophil survival through the inhibition of apoptosis

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[27]. In allergen-challenged asthmatic patients, pretreatment with salmeterol, a commonly used, long-acting β_2 agonist, has been reported to inhibit elevations in serum concentrations of eosinophil cationic protein (ECP) and eosinophil protein X (EPX) [28,29]. Furthermore, β_2 agonists have been shown to inhibit eosinophil function *in vitro* [28,30–32], and the anti-inflammatory effects of some β_2 agonists are mediated via β_2 receptors [32,33]. However, the anti-inflammatory effects on leucocytes as well as the mechanisms underlying the effects are likely to differ between individual β_2 agonists [33–35]. For example, the anti-inflammatory effects of salbutamol are mediated via β_2 receptors on cell surfaces, while salmeterol, a commonly used long-acting β_2 agonist, has an anti-inflammatory effect that is independent of β_2-receptors [33].

Among β_2 agonists, high-dose preparations of fenoterol are associated with increased mortality, most probably from acute effects related to their overuse in life-threatening asthmatic attacks [36]. Previous studies have indicated that fenoterol has greater intrinsic β_2-receptor activity and less β_2 selectivity than other β agonists such as albuterol and terbutaline [37–39]. Compared to other agonists, these pharmacological properties of fenoterol result in greater inotropy and chronotropy, more intense electrophysiologic effects, and greater hypokalemic effects upon repeated inhalation than those seen with other agonists [40–42]. Furthermore, because fenoterol has greater intrinsic activity, its maximum cardiac and hypokalaemic effects exceed those of albuterol irrespective of the dose inhaled [41]. However, important details of pharmacological properties of fenoterol that may be related to deaths in asthma remain unknown.

Based on the above findings, we hypothesized that pharmacological anti-inflammatory effects of fenoterol on eosinophil function also differ from those of other β_2 agonists. We therefore investigated the effects of fenoterol and other β_2 agonists on human eosinophil function.

MATERIALS AND METHODS

Reagents

PAF C-16, obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA) was dissolved in ethanol, and diluted to 40 mM. Phorbol myristate acetate (PMA), procaterol, fenoterol, salbutamol and ICI-118551 all were purchased from Sigma Chemical (St Louis, MO, USA). Procaterol, salbutamol, fenoterol and ICI-118551 were dissolved in distilled water at concentrations of 10 or 100 mM. PMA was dissolved in dimethylsulphoxide (DMSO) at concentrations of 5 mg/ml. All drugs were stored at –20°C and were diluted later with each reaction medium to the desired concentration immediately before use. Hanks's balanced salt solution (HBSS, 0·98% w/v; Nissui Pharmaceutical, Tokyo, Japan) was made up in double-distilled water, and the pH was adjusted to 7·40 with 0·15 M disodium phosphate. A chemiluminescence probe for superoxide radicals representing a *Cypridina* luciferin analogue (2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo-[1,2-a] pyrazin-3-one, or MCLA) was obtained from Tokyo Kasei (Tokyo, Japan), dissolved in doubly distilled water and stored at –80°C until needed. The concentration of MCLA solution was based on the following: ε430 nm = 9600 $M^{-1}cm^{-1}$, as described previously [43,44]. Phycoerythrin (PE)-conjugated anti-CD11b mouse monoclonal antibody (MoAb) and mouse IgG2a, control immunoglobulin, were purchased from Becton Dickinson (San Jose, CA, USA). Superoxide dismutase (Sigma; purified from bovine erythrocytes, 3400 units/mg protein) was dissolved in distilled water and diluted to 1·8 mg/ml, and then stored at –80°C until use.

Cell isolation

Eosinophil purification was performed using a magnetic cell separation system (MACS; Becton Dickinson) as described previously with minor modifications [45]. Briefly, heparinized blood, obtained from normal healthy volunteers and diluted with an equal volume of phosphate-buffered saline (PBS), was layered over a Histopaque solution (density 1·083 g/ml; Sigma) in a 50-ml Falcon plastic tube (Becton-Dickinson Labware, Lincoln Park, NJ, USA), and centrifuged at 700 ***g*** for 30 min at 4°C. The supernatant and the mononuclear cells at the interface were removed carefully by aspiration. The inside wall of the tube was wiped with sterile gauze to remove attached mononuclear cells. Erythrocytes in the sediment were removed by two cycles of hypotonic lysis. Isolated granulocytes were washed with piperazine-*N, N′*-bis (2-ethanesulphonic acid) (PIPES) buffer (25 mM PIPES, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, and 5·4 mM glucose at pH 7·4) with 1% fetal bovine serum (FBS; JRH Biosicences, Lenexa, KS, USA) and an equal volume of anti-CD16 MoAb conjugated with magnetic particles (Miltenyi Biotec, Bergisch Gladbach, Germany) was added to the cell pellet. After 60 min of incubation on ice, 5 ml of PIPES with 1% FBS was added to the cell/antibody mixture. Resuspended cells were loaded onto a separation column positioned in the magnetic field of the MACS. Eosinophils were eluted three times with 5 ml of PIPES buffer with 1% FBS. The purity of eosinophils counted after Randolph staining was more than 98%. Purified eosinophils were washed in PIPES buffer with 1% FBS and suspended in each reaction medium.

Superoxide anion (O_2^-) generation

O_2^- generation by eosinophils was measured by MCLA-dependent chemiluminescence, as described previously [43,44], using a luminescence reader (BLR-102, or 301, Aloka, Tokyo, Japan). Polyethylene test tubes (Aloka) were coated with 250 μl of 2·5% human serum albumin (HSA, Sigma, St Louis, MO, USA) dissolved in PBS at pH 7·40 and kept overnight at 4°C. On the next day, the tubes were washed three times with PBS and then used immediately. Eosinophils were washed with HBSS and resuspended in the same medium at 10^6 cells/ml. Stimulants were diluted in the same medium at the desired concentration. Assay mixture contained $2·5 \times 10^5$ cells together with β_2 agonist or a corresponding volume of medium (HBSS) as a control; 3 μM MCLA; 1 μM PAF; and HBSS to result in a total volume of 2·0 ml. When cells were stimulated by PMA, assay mixture contained $7·0 \times 10^4$ cells with β_2 agonist or HBSS; 9 μM MCLA; 100 ng/ml PMA; and HBSS. Components except for MCLA and stimulant were preincubated in HSA-coated tubes for 30 min at 37°C. After MCLA was added, incubation continued for another 5 min. Then O_2^- production was started by addition of stimulant and measured for 30 min. Additionally, to examine effects of β_2 antagonists on O_2^- generation inhibited by β_2 agonist, cells were preincubated with ICI-118551 for 15 min at 37°C before the effects of β_2 agonists on O_2^- generation were tested. When cells were stimulated by PMA, the incubated mixture tube in the luminescence reader was agitated constantly by rotation during measurement of luminescence to exclude the effect of cellular adhesion. The same mixture without stimulant, β_2 agonist or β_2 antagonist was incubated and assayed as a control. The amount of O_2^- generation (counts) was

calculated as the area under the chemiluminescence curve (AUC) and corrected by subtracting control readings. Luminescence was completely inhibited by 0·5 μM superoxide dismutase (data not shown).

Degranulation

Eosinophil degranulation was assessed by quantitating eosinophil protein X (EPX), one of the eosinophil granule proteins. Ninety-six-well, flat-bottom tissue culture plates (Costar 3595, Corning Incorporated, Corning, NY, USA) were coated with 50 μl of 2·5% HSA dissolved in PBS (pH 7·4) at 37°C for 2 h and washed three times with the same medium before use. The standard mixture contained 5×10^4 cells together with β_2 agonist or a corresponding volume of medium (RPMI) as a control; 1 μM PAF or 1 ng/ml PMA; and RPMI to result in a total volume of 200 μl. Components except for stimulant were preincubated for 30 min at 37°C in 5% CO_2. Cellular degranulation was then started by addition of stimulant. Additionally, to examine effects of β_2 antagonists on O_2^- generation inhibited by β_2 agonist, cells were preincubated with ICI-118551 for 15 min at 37°C in 5% CO_2 before the effects of β_2 agonists on cellular degranulation were tested. After incubation for 4 h at 37°C in 5% CO_2, supernatants from wells were collected and frozen at −20°C until assayed.

The concentrations of EPX in the sample supernatants were measured by double-antibody competition radioimmunoassay (RIA) as reported elsewhere [46]. Total cellular EPX contents were measured in parallel samples from cells lysed with 0·5% NP-40 detergent. All experiments were conducted in duplicate. Percent degranulation was calculated as a ratio of EPX content in each sample to total available EPX.

Adhesion assay

Eosinophils adhesion was determined by measuring the EPX contents of adherent cells by RIA, as reported previously [14]. As described above, eosinophils were stimulated on HSA-coated tissue culture plates. The standard mixture contained 5×10^4 cells together with β_2 agonist or a corresponding volume of medium (RPMI) as a control; 1 μM PAF; and RPMI to result in a total volume of 200 μl. Components except for stimulant were preincubated for 30 min at 37°C in 5% CO_2. Then cellular degranulation was started by PAF. One hour after stimulation, the supernatant fluids were collected for assay of EPX, and the plates were rinsed gently with warm PBS to remove non-adherent cells. Adherent cells were then lysed with 0·5% NP-40 and the EPX contents in the lysates were measured by RIA. Percentage of adhesion was calculated as a ratio of EPX content in adherent eosinophils to total available EPX after incubation according to the following equation:

% adhesion = (EPX in lysates of adherent cells after incubation) × 100/(total EPX in lysates of cells before incubation – EPX released into supernatants during incubation)

Flow cytometry

Expression of CD11b on eosinophils was determined on a flow cytometer (Epics XLII, Beckman Coulter, Tokyo, Japan). Purified eosinophils were washed with RPMI with 2% FBS and resuspended in the same medium at 10^6 cells/ml. Eosinophils ($2·5 \times 10^5$ cells/sample), preincubated with β_2 agonist for 30 min at 37°C in 5% CO_2 in polypropylene test tubes were started by the addition of the stimulant at 37°C for 15 min, after which the reaction was stopped by placing the tubes in an ice bath. Then eosinophils were centrifuged at 300 ***g*** at 4°C, and pellets were then stained with a saturating amount of mouse PE-conjugated anti-CD11b MoAb or isotype-matched control for 30 min at 4°C in darkness. Cells were washed twice with PBS at 300***g*** at 4°C, resuspended in 500 μl of PBS and kept at 4°C until analysis. Expression of CD11b on eosinophils was expressed as mean fluorescence intensity (MFI). Cell viability was assessed simultaneously by staining the cell with small amount of propidium iodide (1 μg/ml) shortly before the analysis.

O_2^- generation via the hypoxanthine–xanthine oxidase system

The standard reaction mixture contained 2×10^{-7} M MCLA, 5×10^{-5} M hypoxanthine, 6·5 units of xanthine oxidase (XOD), β_2 agonist and 50 mM Tris-HCl buffer containing 0·1 mM ethylenediamine tetraacetic acid (EDTA) at pH 7·8, in a total volume of 3·0 ml. Chemiluminescence measurement was initiated by adding MCLA to the reaction mixture without XOD; 2 min later, XOD was added. Chemiluminescence was then measured with a luminescence reader (BLR-301; Aloka) at 37°C, as reported previously [23]. Percentage of inhibition by the β_2 agonist of XOD-dependent luminescence without β_2 agonist was calculated. The results are expressed as a percentage relative to results in controls with no addition of drugs.

Statistical analysis

Normalized values from the number of independent trials indicated in the figure legend were expressed as the mean ± s.e.m. Statistical significance of the difference between various treatment groups was determined with paired or unpaired Student's *t*-tests.

RESULTS

Effect of β_2 agonists on stimulus-induced O_2^- generation and degranulation by human eosinophils

First we tested the effect of β_2 agonists on O_2^- generation, a major effector function of eosinophils, as induced by PAF (Fig. 1). As for effective concentrations, inhibitory effects of fenoterol, salbutamol, or procaterol on PAF-induced O_2^- generation all became apparent at 10^{-7} M. At 10^{-5} M, fenoterol maximally inhibited PAF-induced O_2^- generation by eosinophils (by approximately 80%). In contrast, salbutamol or procaterol partially inhibited PAF-induced O_2^- generation by eosinophils less completely at 10^{-5} M, by approximately 45%. Thus, fenoterol at 10^{-5} M inhibited PAF-induced O_2^- generation by eosinophils significantly more than salbutamol or procaterol. At the highest concentration used in this study (10^{-5} M), no drug had any effect on cell viability as determined by trypan blue and propidium iodide staining (data not shown).

We also investigated the effect of β_2 agonists on degranulation and other effector functions of eosinophils induced by PAF. At all concentrations studied, fenoterol showed significant partially inhibition of PAF-induced degranulation by eosinophils, as did salbutamol or procaterol at 10^{-5} M (Fig. 2). No significant difference was seen between the three drugs at 10^{-5} M in inhibition of PAF-induced eosinophil degranulation (data not shown). Figure 3a shows the effects of β_2 agonists on PMA-induced O_2^- generation by eosinophils. Fenoterol at 10^{-5} M inhibited PMA-induced O_2^- generation, with a maximal inhibition of

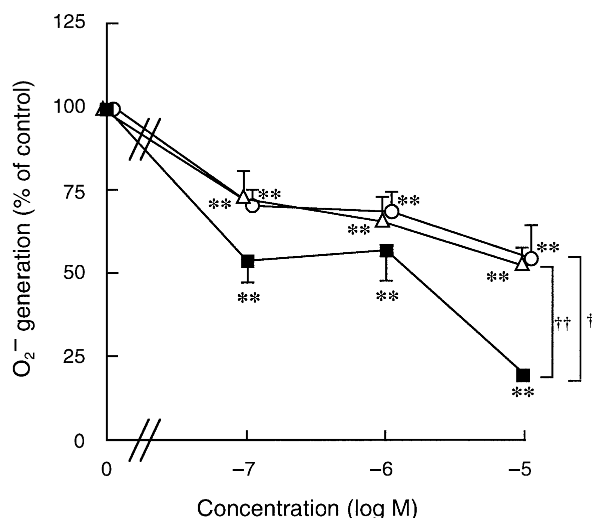

Fig. 1. Effects of β_2 agonists on PAF-induced O_2^- generation in human eosinophils. Purified human eosinophils ($2{\cdot}5 \times 10^5$ cells) were incubated in a polyethylene tube. MCLA-dependent chemiluminescence was measured with a luminescence reader. Standard assay mixtures contained $2{\cdot}5 \times 10^5$ cells, β_2 agonist (fenoterol, salbutamol or procaterol) and HBSS in a total volume of 2·0 ml. After cells were preincubated with β_2 agonist for 30 min at 37°C, 3 μM MCLA was added. Then O_2^- generation was initiated by addition of 1 μM PAF. Amounts of O_2^- generation for 30 min were calculated from the area under the chemiluminescence curve and were expressed as counts. Data were normalized to values obtained without β_2-agonist pretreatment (taken as 100%). Values represent the mean ± s.e.m. from four to six experiments. O_2^- generation in response to PAF in controls with no drug pretreatment was $43{\cdot}8 \pm 6{\cdot}9 \times 10^5$ counts ($n = 6$). Significant differences between cells treated and those not treated with β_2 agonists are shown as $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$. Significant differences from cells treated with β_2 agonists at 10^{-5} M are shown as $^\dagger P < 0{\cdot}05$ and $^{\dagger\dagger}P < 0{\cdot}01$. ■, Fenoterol; △, salbutamol; ○, procaterol.

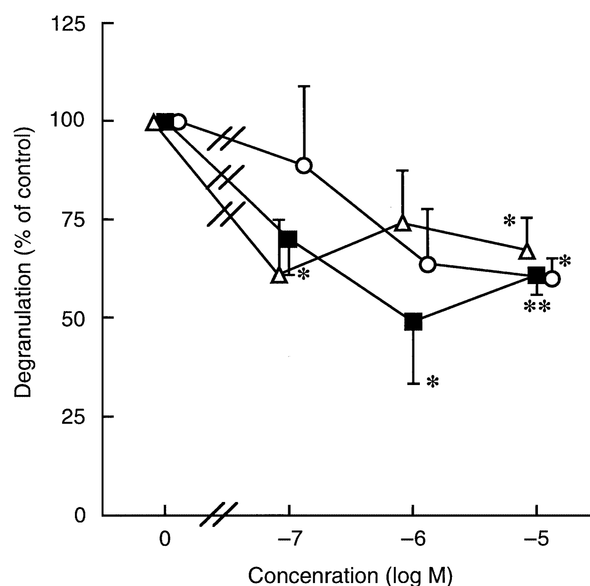

Fig. 2. Effects of β_2 agonists on eosinophil degranulation induced by PAF. Purified eosinophils (5×10^4 cells) were prepared in HSA-coated, 96-well culture plates, and were preincubated with β_2 agonist for 30 min at 37°C. Then cellular degranulation was initiated by adding PAF (1 μM). After 4 h of incubation at 37°C in an atmosphere including 5% CO_2, supernatants in each well were collected and EPX content was measured by RIA. Percentage degranulation was calculated as the ratio of EPX content of supernatant to total cellular EPX content. Data were normalized to values obtained without β_2-agonist pretreatment (taken as 100%). Values represent the mean ± s.e.m. from five experiments. Degranulation in controls without drugs pretreatment was amounted to: medium alone, $100{\cdot}82 \pm 25{\cdot}85$; and PAF, $555{\cdot}8 \pm 141{\cdot}4$ ng/10^6 cells ($n = 6$). Total EPX content was $2300{\cdot}1 \pm 136{\cdot}0$ ng/10^6 cells ($n = 6$). Significant differences from cells without β_2 agonists are shown as $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$. ■, Fenoterol; △, salbutamol; ○, procaterol.

approximately 25%. In contrast, salbutamol or procaterol at 10^{-5} M did not show a significant inhibitory effect. Fenoterol showed any oxygen radical scavenging effect at 10^{-5} M (control, $100 \pm 0{\cdot}0$ *versus* fenoterol, $98{\cdot}3 \pm 1{\cdot}0$%; $n = 3$). Furthermore, when we tested the effect of β_2 agonist on PMA-induced degranulation by eosinophils (Fig. 3b), fenoterol at 10^{-5} M significantly inhibited PMA-induced degranulation by up to 65%, while salbutamol or procaterol at 10^{-5} M did not significantly inhibit degranulation. These results show that fenoterol may have a stronger anti-inflammatory effect than the other two β_2 agonists.

Effect of a β_2-receptor antagonist on β_2-agonist inhibition of O_2^- generation and degranulation by stimuli

To determine whether the inhibitory effects of β_2 agonists on O_2^- generation by stimulated eosinophils were mediated via β_2 receptors, we performed superoxide inhibition assays in the presence of a selective β_2-receptor antagonist, ICI-118551. At 10^{-5} M, ICI-118551 only partially reversed the inhibitory effects of all three drugs on PAF-induced O_2^- generation; this reversal was not statistically significant (Fig. 4a). When we performed assays of degranulation inhibition in the presence of ICI-118551, the antagonist failed to reverse the inhibitory effects of fenoterol on PAF-induced degranulation, while ICI-118551 partially (although not significantly) reversed the inhibitory effects of salbutamol or procaterol (data not shown). We similarly used ICI-118551 to determine whether inhibitory effects of fenoterol on PMA-induced O_2^- generation (Fig. 4b) and degranulation (data not shown) by eosinophils were mediated by β_2 adrenoceptors. ICI-118551 at 10^{-5} M could not reverse the inhibitory effects of fenoterol at 10^{-5} M on PMA-induced O_2^- generation and degranulation. These results suggest that β_2 agonists had an inhibitory effect on PAF-induced effector function partially mediated via the β_2 receptor, while inhibitory effects of fenoterol on PMA-induced O_2^- generation and degranulation occurred independently of the β_2 receptor.

Effect of β_2 agonists on PAF-induced adhesion by eosinophils

Adhesion molecules, especially $\alpha M\beta_2$, play critical roles in eosinophil effector functions [12,14,15]. We examined whether β_2 agonists affected eosinophil adhesion induced by PAF (Table 1). Fenoterol at 10^{-5} M significantly inhibited PAF-induced cellular adhesion, while salbutamol and procaterol did not significantly inhibit cellular adhesion at any concentration tested.

Effect of β_2 agonists on PAF-induced CD11b expression by eosinophils

We investigated the effect of β_2 agonists on PAF-induced CD11b expression on eosinophils. The alpha chain of the adhesion

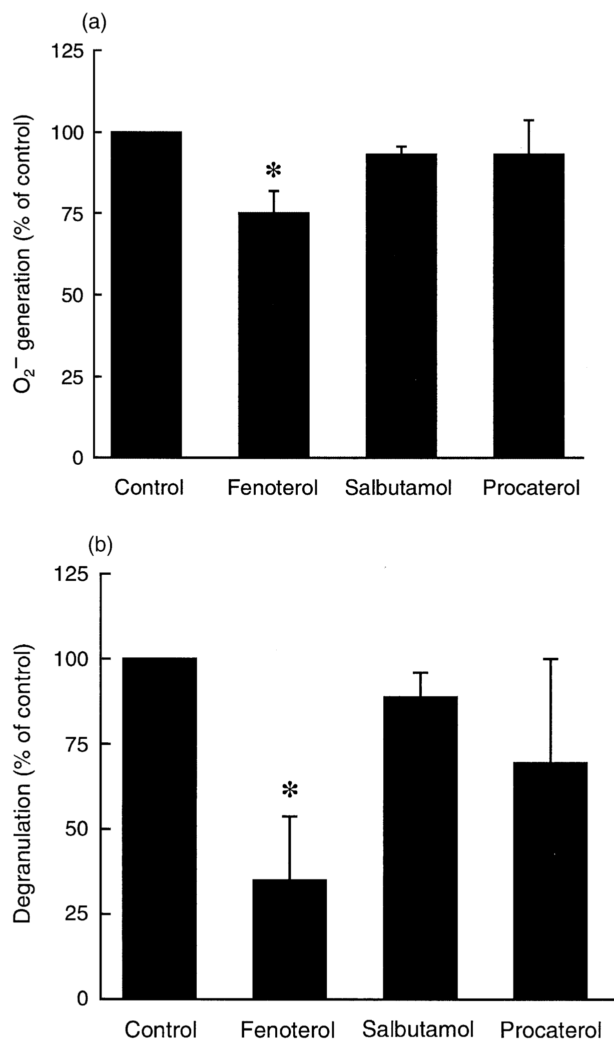

Fig. 3. Effects of β_2 agonists on PMA-induced O_2^- generation and degranulation in human eosinophils. (a) Effects of β_2 agonists on PMA-induced O_2^- generation. Human eosinophils (7×10^4 cells) were preincubated with β_2 agonist (fenoterol, salbutamol, or procaterol) for 30 min at 37°C. MCLA (9 μM) was added, and O_2^- generation was initiated by addition of PMA (100 ng/ml), as described for Fig. 1. Amounts of O_2^- generation for 30 min were calculated from the area under the chemiluminescence curve. Data were normalized to values obtained without β_2-agonist pretreatment (taken as 100%). Values represent the mean ± s.e.m. from four to five experiments. O_2^- generation in controls without drug pretreatment in response to PMA was $471{\cdot}9 \pm 41{\cdot}9 \times 10^5$ counts ($n = 5$). Significant differences from cells not treated with β_2 agonists are shown as $*P < 0{\cdot}05$. (b) Effects of β_2 agonist on PMA-induced eosinophil degranulation. Purified eosinophils (5×10^4 cells) were prepared in HSA-coated 96-well culture plates and preincubated with β_2 agonist for 30 min at 37°C. Then cellular degranulation was initiated by adding PMA (1 ng/ml). Eosinophil degranulation was measured as described for Fig. 2. Data were normalized to values obtained without β_2-agonist pretreatment (taken as 100%). Values represent the mean ± s.e.m. from five experiments. Degranulation in controls with no drug pretreatment was: medium alone $87{\cdot}5 \pm 37{\cdot}5$; and PMA, $358{\cdot}8 \pm 69{\cdot}5$ ng/10^6 cells ($n = 4$). Total EPX content was $2189{\cdot}9 \pm 183{\cdot}1$ ng/10^6 cells ($n = 4$). Significant differences from cells without β_2-agonist exposure are shown as $*P < 0{\cdot}05$.

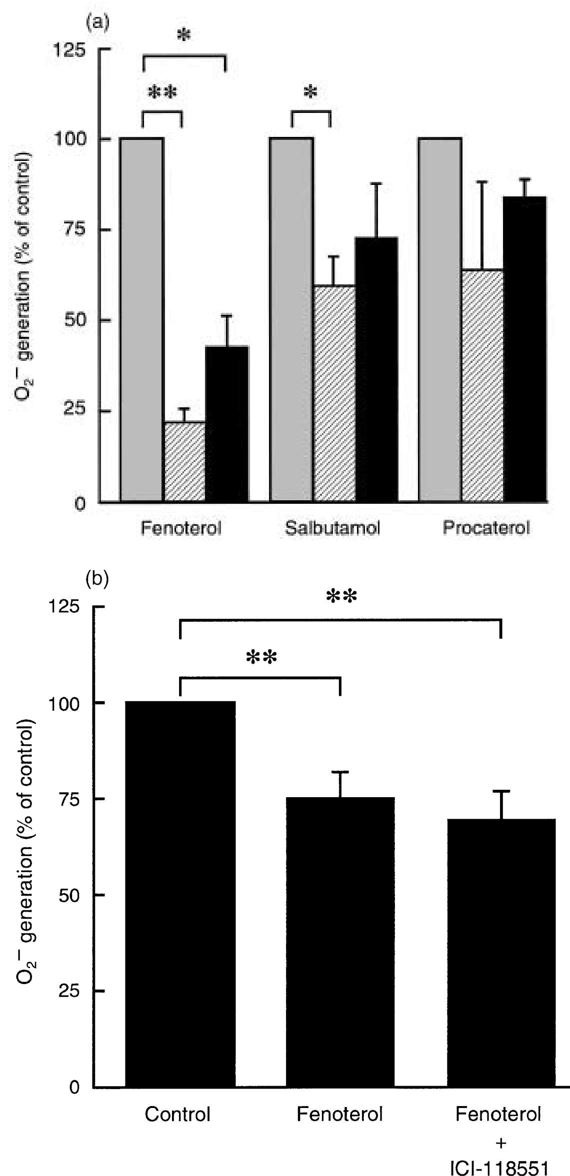

Fig. 4. Effect of ICI-118551 on inhibition of stimulus-induced O_2^- generation and degranulation by β_2 agonist in human eosinophils. (a) Effect of ICI-118551 on PAF-induced O_2^- generation inhibited by β_2 agonist. Human eosinophils ($2{\cdot}5 \times 10^5$ cells) were preincubated with ICI-118551 (10^{-5} M) for 15 min. The inhibitory effect of fenoterol on PAF-induced O_2^- generation was determined as described for Fig. 1. Data were normalized to values obtained without β_2-agonist or β_2-antagonist pretreatment (taken as 100%). Values represent the mean ± s.e.m. from three to four experiments. O_2^- generation in response to PAF in controls with no drug pretreatment was $51{\cdot}37 \pm 6{\cdot}34 \times 10^5$ counts ($n = 4$). Significant differences from cells with out β_2 agonists or β_2 antagonists are shown as $*P < 0{\cdot}05$ and $**P < 0{\cdot}01$. □, PAF alone; ▨, PAF + β_2 agonist; ■, PAF + β_2 agonist + ICI-118551. (b) Effect of ICI-118551 on PMA-induced O_2^- inhibition of generation by fenoterol. Human eosinophils (7×10^4 cells) were preincubated with ICI-118551 (10^{-5} M) for 15 min. Then the inhibitory effect of fenoterol on PMA-induced O_2^- generation was determined as described for Fig. 3. Data were normalized to values obtained without β_2-agonist pretreatment (taken as 100%). Values represent the mean ± s.e.m. from three to four experiments. O_2^- generation in controls with no drug pretreatment in response to PMA was $471{\cdot}9 \pm 41{\cdot}9 \times 10^5$ counts ($n = 5$). Significant differences from cells without β_2 agonists or β_2 antagonists are shown as $**P < 0{\cdot}01$.

Table 1. The effects of β_2-agonist on cellular adhesion induced by PAF

		Adhesion (% of control)/PAF (1 μM)		
		Concentration (log M)		
Control		− 7	− 6	− 5
Fenoterol	100	87·9 ± 20·2	76·0 ± 12·2	51·4 ± 10·5*
Salbutamol	100	90·9 ± 22·7	87·3 ± 22·4	80·2 ± 13·3
Procaterol	100	140·7 ± 30·0	88·0 ± 28·5	74·4 ± 17·0

Purified eosinophils (5×10^4 cells) were prepared in HSA-coated 96-well culture plates and were preincubated with β_2 agonist for 30 min at 37°C. Then cellular adhesion was initiated by adding PAF (1 μM). After 60 min of incubation at 37°C in an atmosphere including 5% CO_2, non-adherent cells were removed by gentle rinse with the warm medium. Adherent cells were then lysed with NP-40, and EPX content in the lysates was measured by RIA. Percentage adhesion was determined by the equation described under Methods. Data were normalized to values obtained without β_2 agonist pretreatment (taken as 100%). Values represent the mean ± s.e.m. from three to four experiments. Percentage of adhesion (mean ± s.e.m.) in control with no drug pretreatment was: medium alone, 0·45 ± 0·10; and PAF, 5·85 ± 0·63% ($n = 5$). Total EPX content was 2244·0 ± 151·8 ng/10^6 cells ($n = 5$). Significant differences from cells without β_2 agonist exposure are shown as *$P < 0{\cdot}01$.

molecule, $\alpha M\beta_{21}$, has been confirmed to be crucial for eosinophil effector functions [12,14,15]. Figure 5a shows a typical histogram of CD11b expression by eosinophils. Eosinophils constitutively expressed CD11b; in addition, PAF up-regulated CD11b expression. Procaterol inhibited PAF-induced CD11b expression on eosinophils. Figure 5b shows the effect of β_2 agonists on PAF-induced CD11b expression by eosinophils. Inhibitory effects of fenoterol, salbutamol and procaterol became apparent at 10^{-7} M, 10^{-6} M and 10^{-7} M, respectively. The inhibitory effect of each drug at 10^{-5} M was approximately 25%.

DISCUSSION

In treatment of bronchial asthma, fenoterol is believed to have greater anti-inflammator effects than salbutamol and procaterol. In the present study, we sought to determine whether fenoterol, considered a β_2-adrenoceptor agonist, and salbutamol or procaterol, considered partial agonists, differed in ability to suppress responses of activated eosinophils. We therefore compared the ability of these drugs to inhibit stimulated O_2^- generation, degranulation, adhesion, and CD11b expression in human eosinophils. In addition, we used antagonists to determine whether effects of β_2-adrenoceptor agonists were mediated via β_2 receptors.

We found that fenoterol inhibited PAF-induced O_2^- generation by eosinophils significantly more than salbutamol or procaterol. In addition, fenoterol significantly inhibited PMA-induced O_2^- generation and degranulation by eosinophils, while salbutamol or procaterol did not. Furthermore, effects of fenoterol against PMA-induced O_2^- generation were not reversed by ICI-118551, a selective β_2-adrenoceptor antagonist. Finally, fenoterol significantly inhibited PAF-induced eosinophil adhesion, while salbutamol or procaterol did not. In contrast, PAF-induced CD11b expression on eosinophils was partially inhibited to the same extent by fenoterol, salbutamol or procaterol. These results indicated collectively that fenoterol may have actions against eosinophil effector functions that differ partly from those of salbutamol or procaterol.

We found that the inhibitory effects of fenoterol at high concentrations on PAF- or PMA-induced O_2^- generation and degranulation by eosinophils were more potent than those of salbutamol or procaterol. We have found recently that at high concentrations fenoterol significantly inhibited both FMLP- and PMA-induced O_2^- generation by human neutrophils, while salbutamol or procaterol partially inhibited O_2^- generation with FMLP but not PMA [47]. Other investigators also have shown that fenoterol inhibited O_2^- generation by neutrophils more effectively than salbutamol [34,35]. Concerning this point, previous reports indicated that a high concentration of fenoterol (at least 2×10^{-5} M) inhibited stimulant-induced increases in O_2^- by a scavenging effect related to the molecular structure of β_2 agonists [48]. However, we found that at concentrations up to 10^{-5} M, fenoterol did not show any O_2^- scavenging, so inhibition by fenoterol had a different in our study basis. We observed that at 10^{-5} M none of the three drugs used in this study affected eosinophil survival *in vitro*, ruling out non-specific toxicity to eosinophils as a cause of impaired cell function.

ICI-118551 partially reversed the inhibitory effects of β_2-agonists on PAF-induced O_2^- generation and degranulation, but this agent could not reverse the inhibitory effects of fenoterol on PMA-induced O_2^- generation. These results suggested that the inhibitory effect of β_2 agonists or PAF-induced effector functions was mediated partially via β_2 receptors, while inhibition of PMA-induced O_2^- generation by fenoterol was independent of the β_2 receptor. Phospholipase (PL) C has been implicated in eosinophil effector functions induced by PAF [49]. PLC carries out hydrolysis of phosphatidylinositol 4, 5-biphosphate to generate an intracellular second messenger, inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol. IP_3 brings about downstream activation such as elevation of intracellular calcium and activation of PKC [50]; the latter activates O_2^- generating enzymes such as NADPH oxidase. On the other hand, β_2-adrenoceptor agonists bind to the receptor and activate the Gs protein. In turn, adenylate cyclase is activated, resulting in elevation of intracellular cAMP. Increased intracellular cAMP activates protein kinase A, which down-regulates PLC activity [51–53]. Thus, inhibition of PAF-induced O_2^- generation by β_2 agonists is thought to result partly from β_2-adrenoceptor-mediated cAMP-dependent activation of PKA [54], which uncouples heterogeneous receptors from PLC activation [52,53]. We examined the effect of β_2 agonists on eosinophil effector function induced by PMA, a direct PKC activator. Fenoterol at 10^{-5} M significantly inhibited PMA-induced O_2^- generation and degranulation by eosinophils. Effects of fenoterol against PMA-induced O_2^- generation were not reversed by ICI-118551, although effects of β_2 agonists on PAF-induced O_2^- generation and degranulation were partially reversed by the antagonist. These results suggest that fenoterol may inhibit PMA-induced O_2^- generation at least partly independently of the β_2 adrenoceptor, by a direct effect on PKC or downstream from that enzyme. We have already demonstrated that fenoterol inhibits O_2^- generation by stimulated human neutrophils via similar direct inhibitory mechanisms [47]. Mitsuyama *et al.* suggested that in human neutrophils cAMP negatively regulated O_2^- generation both by actions upstream from PKC and actions downstream from the G protein subsequent to PKC effects [51]. However, we know of no previous report

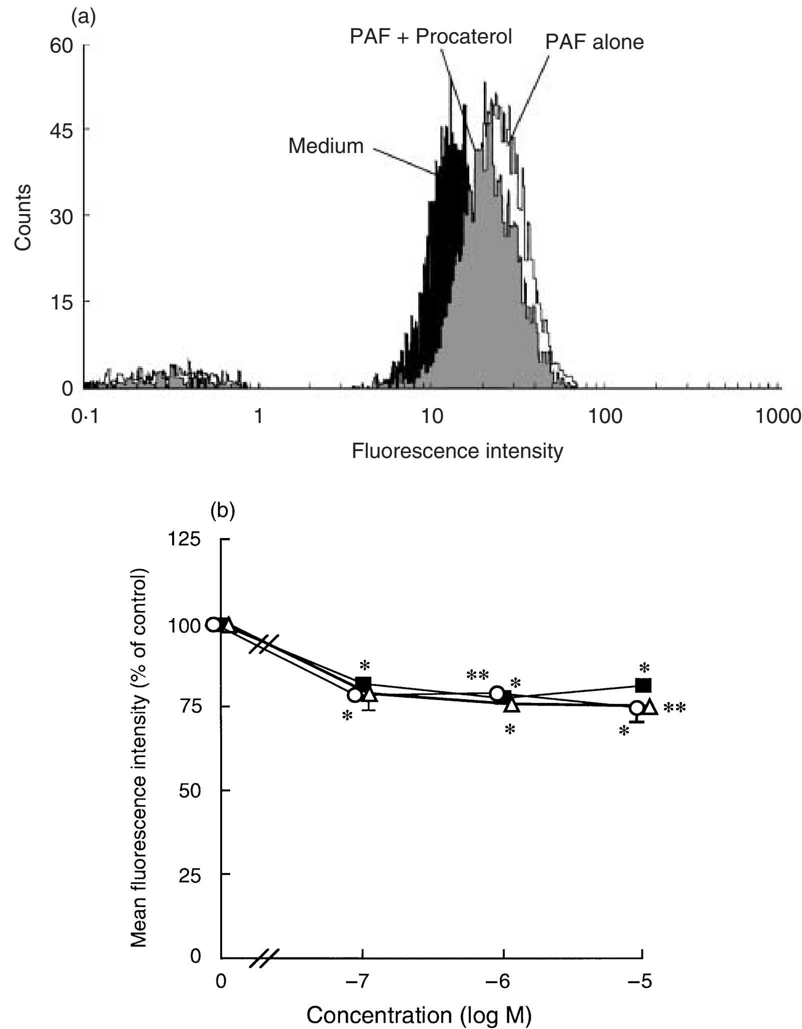

Fig. 5. Effects of β_2 agonist on CD11b expression induced by PAF. Purified eosinophils ($2{\cdot}5 \times 10^5$ cells/sample) were preincubated with β_2 agonist for 30 min at 37°C. Cells then were incubated with PAF (1 μM) for 15 min at 37°C. Cells were stained with mouse PE-conjugated anti-CD11b MoAb for 60 min at 4°C in darkness. CD11b expression on eosinophils was measured by a flow cytometer and expressed as MFI. (a) Histograms showing a typical result of CD11b expression by eosinophils incubated with PAF, PAF plus procaterol or control medium. (b) Effect of β_2 agonists on PAF-induced CD11b expression by eosinophils. Data were normalized to values obtained without β_2-agonist pretreatment (taken as 100%). Values represent the mean ± s.e.m. from three to four experiments. Significant differences from cells without β_2 agonists are shown as $*P < 0{\cdot}05$ and $**P < 0{\cdot}01$. ■, Fenoterol; △, salbutamol; ○, procaterol.

suggesting that fenoterol inhibited PMA-induced eosinophil function independently of the β_2 adrenoceptor by an effect on PKC or downstream from it.

Whether occurring via PKC itself or at a downstream site, mechanism by which fenoterol exerts its β_2-receptor-independent inhibition of O_2^- generation by human neutrophils and eosinophils may be unique to this agent. We know of no previous report suggesting that fenoterol may have such an effect in human leucocytes or in other types of cells, including smooth muscle.

Activation of PKC has been postulated to be an important modulator of cardiac contractile function and hypertrophy [55]. Furthermore, activation of PKC can protect the heart from ischaemic injury, while PKC inhibitors block the cardioprotective effect of ischaemic preconditioning in experimental animals and in human myocytes [55–60]. On the other hand, fenoterol has known inotrophic and chronotrophic effdcts as well as other electrophysiological influences [40–42]; these are enhanced under hypoxic conditions [61]. We have observed that fenoterol inhibited FMLP-induced O_2^- generation from neutrophils via a β_2-receptor-dependent signalling pathway, while at high concentrations fenoterol also inhibits FMLP-induced O_2^- generation independently of β_2 adrenoceptors by a direct effect on PKC or at a downstream site [47]. In this study we found that fenoterol at 10^{-5} M also inhibited PMA-induced O_2^- generation from eosinophils independently of β_2 adrenoceptors, again involving an effect on PKC or at a downstream site.Based on these results we suspect that fenoterol at therapeutic serum concentrations (up to 10^{-7} M) [62] may not have a direct effect on PKC,while an excessive serum concentration resulting from overuse of fenoterol could lead to inhibition of PKC. Furthermore, the inhibition of PKC by fenoterol at high concentrations may interfere with cardiac resistance to ischaemic injury similarly to the effect of a direct PKC inhibitor. These mechanisms may at least, in part, contribute to

cardiac dysfunction associated with overuse of fenoterol in life-threatening asthmatic attacks.

Interestingly, it has been reported that β agonists contribute to the prolonged eosinophil survival through the inhibition of apoptosis [27]. Another possible mechanism for the increased risk of death from asthma may be the prevention of eosinophil clearance by the inhibition of apoptosis resulting in a more severe inflammatory response and asthmatic symptoms.

Accumulating evidence suggests that cell adhesion through β_2 integrin, especially $\alpha M\beta_2$ (CD11b/CD18, Mac-1) plays a critical role in stimulus-induced effector functions of eosinophils such as O_2^- generation and degranulation [12,14,15]. Accordingly, we investigated the effect of β_2-agonists on PAF-induced eosinophil adhesion. Fenoterol at high concentration inhibited PAF-induced eosinophil adhesion significantly, while salbutamol or procaterol did not. Our results from adhesion studies were consistent with other findings that salbutamol failed to inhibit eosinophil adherence induced by PAF [33]. Therefore, the inhibitory effects of fenoterol on PAF-induced O_2^- generation by eosinophils may be mediated partially by inhibition of cellular adhesion, but those of salbutamol or procaterol do not act via such inhibition of cellular adhesion.

At least three mechanisms have been proposed to contribute to leucocyte adhesion [63]: expression of adhesion molecules, clustering of adhesion molecules on the cell surface [64] and conformational change in adhesion molecules [65]. To investigate which component is more important in inhibitory effects of β_2 agonists on eosinophil adhesion, we examined PAF-induced CD11b expression by eosinophils. Fenoterol inhibited CD11b expression in response to PAF; salbutamol or procaterol acted similarly. These results suggest that the latter two mechanisms – clustering or conformational change of adhesion molecules – may be more important in inhibiting PAF-induced cellular adhesion by fenoterol than they are for the other two agonists, as expression effects were similar for all three drugs.

In conclusion, fenoterol at high concentrations inhibits strongly activation of eosinophils by mechanisms likely to differ from those of salbutamol or procaterol. These include inhibition of eosinophil adhesion and a direct effect on PKC or a site downstream from PKC. These pharmacological properties of fenoterol may result in more effective inhibition of inflammation in allergic disease, but increase risk of mortality from overuse of fenoterol in life-threatening asthmatic attacks.

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