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Evidence that the eosinophil is a cellular target for the inhibitory action of salmeterol on eosinophil recruitment in vivo

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Abstract

Systemic administration of agents which elevate cyclic AMP (e.g., phosphodiesterase inhibitors, prostanoids, β-adrenoceptor agonists) effectively modulates eosinophil recruitment in vivo. The present study was undertaken to evaluate whether the eosinophil itself is a cellular target for the inhibitory action of these drugs in vivo. We chose to use the long-acting β_2-adrenoceptor agonist salmeterol to test this hypothesis. Eosinophils were pretreated with salmeterol (10^{-6} M) before washing and testing in two salmeterol-sensitive systems, namely eosinophil aggregation and ^{111}In-eosinophil accumulation in guinea-pig skin. Pretreatment with salmeterol inhibited by 65% and 43% eosinophil aggregation induced by platelet-activating factor (PAF) and human recombinant C5a, respectively. Similarly, ^{111}In-eosinophil accumulation induced by PAF and zymosan-activated plasma, a source of guinea-pig des-Arg-C5a, was inhibited by 55% and 45%, respectively. In contrast, the level of circulating ^{111}In-eosinophils at 1 h was enhanced by 35% in animals which received salmeterol-pretreated ^{111}In-eosinophils. Our results suggest that the eosinophil itself is one of the cellular targets of the inhibitory action of systemically administered salmeterol on eosinophil recruitment in vivo.

Keywords: Salmeterol; Eosinophil; cAMP; β_2-Adrenoceptor agonist; Eosinophil aggregation

1. Introduction

There is good evidence linking eosinophil accumulation and activation in tissues with the pathophysiology of allergic diseases such as asthma, allergic rhinitis and atopic dermatitis (Weller, 1991; Butterfield and Leiferman, 1993). Recently, Foster et al. (1996) provided convincing evidence of a role for interleukin-5 and interleukin-5-driven eosinophilia in determining airway hyperresponsiveness in a murine 'model' of allergic asthma. Thus, it is considered that drugs which modulate eosinophil recruitment into tissue may be of benefit in the therapy of allergic diseases.

Agents which elevate cyclic AMP (e.g., phosphodiesterase inhibitors, prostanoids, β-adrenoceptor agonists) are effective modulators of eosinophil recruitment in vivo (Teixeira et al., 1995b). For example, we have previously shown that rolipram, an inhibitor of phosphodiesterase 4 isoenzymes, markedly inhibited eosinophil accumulation induced by various chemoattractants/mediators and in a passive cutaneous anaphylactic reaction in guinea-pig skin (Teixeira et al., 1994b). More recently, we and others have shown that two β_2-adrenoceptor agonists, salbutamol and salmeterol, inhibited eosinophil recruitment in allergic- and mediator-induced inflammation in guinea-pig skin (Whelan et al., 1993; Teixeira et al., 1995c). Similarly, phosphodiesterase 4 inhibitors and β_2-adrenoceptor agonists have shown to markedly suppress eosinophil recruitment following allergen challenge of the lung and the eye of sensitized animals (Fugner, 1989; Newsholme and Schwartz, 1993; Underwood et al., 1993). However, it is unclear whether systemic administration of these agents inhibit eosinophil recruitment by inhibiting the activation/generation of mediators by intermediate cells, such as mast cells and endothelial cells, or by inhibiting eosinophils themselves.

The present study was undertaken to evaluate whether the eosinophil itself is a cellular target for the inhibitory effects of a cyclic AMP-elevating agent on eosinophil recruitment in vivo. We chose to use salmeterol to test this hypothesis. Salmeterol is a long-acting β_2-adrenoceptor agonist which has been shown to bind to and activate guinea-pig β_2-adrenoceptors with a long half-life, even after the tissue has been repeatedly washed (Ball et al., 1991). When given systemically or

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i.d. with inflammatory stimuli, salmeterol causes an effective inhibition of eosinophil recruitment into guinea-pig skin (Whelan et al., 1993; Teixeira et al., 1995c) and in vitro pretreatment with salmeterol has been shown to suppress eosinophil activation, including eosinophil homotypic aggregation and chemotaxis (Koenderman et al., 1992; Teixeira et al., 1996a). Purified guinea-pig eosinophils were pretreated with salmeterol, washed twice and eosinophil aggregation in vitro and eosinophil recruitment in vivo in response to various intradermal inflammatory stimuli assessed.

2. Material and methods

2.1. Harvesting and purification of guinea-pig eosinophils

Guinea-pig eosinophils were prepared as previously described (Faccioli et al., 1991; Teixeira et al., 1994b). Briefly, eosinophils were harvested from the peritoneal cavity of horse serum-treated guinea pigs and purified on a discontinuous Percoll gradient. Preparations of purity greater than 95% were used for the experiments described herein. Purified eosinophils were then washed once in phosphate-buffered saline (PBS, Ca^{2+}- and Mg^{2+}-free, pH 7.4). For the in vivo experiments, eosinophils were labelled with ^{111}In as previously described (Teixeira et al., 1994b). After the first wash, ^{111}In-eosinophils (5×10^6 eosinophils/ml) were incubated with salmeterol (10^{-6} M) or vehicle for 15 min and washed twice in PBS before infusion into recipient animals (see below). For the in vitro experiments, eosinophils (5×10^6 eosinophils/ml) were incubated with salmeterol (10^{-6} M) or vehicle for 15 min and then washed twice. This concentration of salmeterol (10^{-6} M) has been previously shown to have the maximal inhibitory effect on eosinophil aggregation induced by platelet-activating factor (PAF) and C5a (Teixeira et al., 1996a).

2.2. Eosinophil aggregation

Aggregation experiments were carried out as previously described (Teixeira et al., 1995a, 1996b). Briefly, control or salmeterol-treated eosinophils were resuspended (5×10^6 cells/ml) in PBS containing $CaCl_2$ and $MgCl_2$ (final concentrations 1.0 mM and 0.7 mM, respectively) and kept on ice until use. Aliquots (300 μl) of cells were then dispensed into siliconized cuvettes which were then placed into a dual channel platelet aggregometer (Chronolog 440 VS) linked to a dual pen recorder (Chronolog 707). The cells were incubated for 5 min at 37°C with continuous stirring at 700 r.p.m. and then stimulated with PAF (10^{-7} M) or C5a (10^{-7} M). The reference cuvette contained buffer alone. Responses were allowed to develop for at least 5 min and the results expressed as the percentage of maximal aggregation induced by 10^{-6} M phorbol myristate acetate (PMA). Eosinophil aggregation was tested at least half an hour after the last wash.

2.3. Preparation of zymosan-activated plasma

Zymosan-activated plasma was used as a source of guinea-pig des-Arg-C5a. Zymosan-activated plasma was prepared by incubating heparinized (10 IU/ml) plasma obtained from naive guinea pigs (Harlan, Bicester, UK, 350–400 g) with zymosan (5 mg/ml) for 30 min at 37°C. Zymosan was then removed by centrifugation (2×10 min at $3000 \times g$) and stored in aliquots at −20°C.

2.4. Measurement of ^{111}In-eosinophil accumulation in guinea-pig skin

Control or salmeterol-treated ^{111}In-labelled eosinophils were injected i.v. (2.5×10^6 cells per animal) into recipient guinea pigs (350–400 g) sedated with Hypnorm (0.15 ml i.m.). After 5 min, duplicate i.d. injections of zymosan-activated plasma and PAF were given in 0.1 ml volumes into the dorsal shaved skin following a randomized injection plan. Inflammatory responses (^{111}In-labelled eosinophil accumulation per skin site) were assessed 1 h after i.d. injection of mediators. At this time, a blood sample was obtained by cardiac puncture, the animals were killed with an overdose of sodium pentobarbitone, the dorsal skin was removed, cleaned free of excess blood and the sites punched out with a 17 mm punch. The samples were counted in an automatic 5-head gamma counter (Canberra Packard, Pangbourne, UK). The percentage of circulating ^{111}In-eosinophils at 1 h were calculated according to the following formula (assuming a blood volume of 70 ml of blood/kg weight):

$$\frac{(\text{counts in 1 ml of blood}) \times (\text{blood volume of the animal (in ml)})}{\text{total counts injected}}$$

2.5. Reagents

Dimethylsulphoxide (DMSO) and zymosan were purchased from Sigma (Poole, UK). Hanks' solutions, HEPES buffer and horse serum were purchased from Life Technologies (Paisley, UK). Percoll was purchased from Pharmacia (Milton

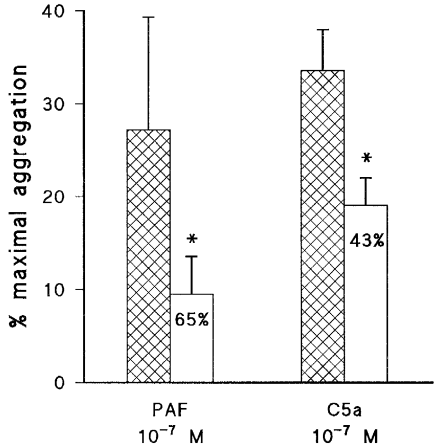

Fig. 1. Effect of salmeterol pretreatment on eosinophil aggregation induced by PAF and hrC5a. Eosinophils were incubated with vehicle (hatched bars) or salmeterol (10^{-6} M, open bars) for 15 min, washed twice and eosinophil aggregation assessed as changes in light transmission after addition of PAF (10^{-7} M) or C5a (10^{-7} M). Results are shown as percent maximal aggregation in response to 10^{-6} M PMA and are the mean ± S.E.M. of 4–5 observations. * $P < 0.05$ when compared to vehicle-treated cells.

Keynes, UK) and PAF (C16) from Bachem (Saffron Walden, UK). $^{111}InCl_3$ was obtained from Amersham International (Amersham, UK). Salmeterol (Ball et al., 1991) was synthesized and kindly supplied by Ciba Geigy (Basel, Switzerland) as a racemic mixture of *R* and *S* isomers. Salmeterol was dissolved in 100% DMSO and diluted further in saline. The concentration of DMSO used for eosinophil pretreatment was less than 1%. Human recombinant C5a (hrC5a) was a gift from Dr. Oostrum (Ciba Geigy, NJ, USA).

2.6. Statistical analysis

Data were analyzed using Student's *t*-test or analysis of variance where appropriate (*P* value assigned using Newman-Keuls' post test) using the statistical program Instat (GraphPad Software V2.03). Results were considered significant when $P < 0.05$ and data are shown as the mean ± S.E.M. of *n* experiments.

3. Results

3.1. Effects of salmeterol pretreatment on eosinophil aggregation

We have previously shown salmeterol to inhibit PAF- and hrC5a-induced eosinophil aggregation by approximately 80 and 50%, respectively (Teixeira et al., 1996a). In the present series of experiments, a maximally effective concentration of

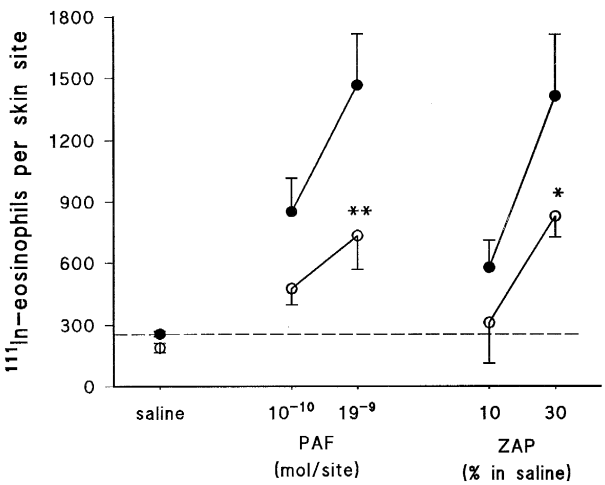

Fig. 2. Effect of salmeterol pretreatment on ^{111}In-eosinophil accumulation induced by PAF and zymosan-activated plasma in guinea-pig skin. ^{111}In-eosinophils were incubated with vehicle (closed circles) or salmeterol (10^{-6} M, open circles) for 15 min, washed twice and ^{111}In-eosinophil accumulation per skin site assessed 1 h after i.d. administration of PAF (10^{-10} and 10^{-9} mol/site) and zymosan-activated plasma (10 and 30% dilution in saline). Each animal received 2.5×10^6 ^{111}In-eosinophils i.v. just prior the i.d. injections. Results are the mean ± S.E.M. of 6 pairs of animals. * $P < 0.05$ and * * $P < 0.01$ when compared to control animals.

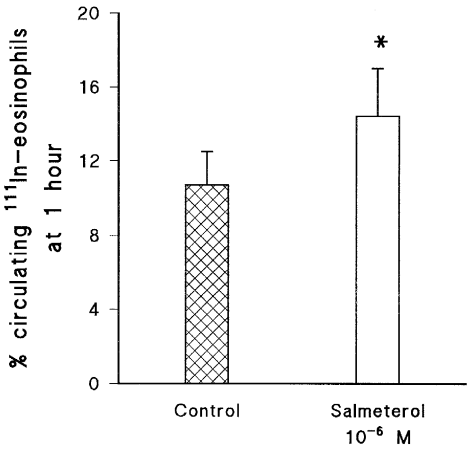

Fig. 3. Effect of salmeterol pretreatment on the levels of circulating ^{111}In-eosinophil at 1 h. ^{111}In-eosinophils were incubated with control vehicle or salmeterol (10^{-6} M) for 15 min, washed twice and each animal received 2.5×10^{6} ^{111}In-eosinophils i.v. just prior the i.d. injections. At 1 h, animals were given an overdose of pentobarbitone and blood obtained via a cardiac puncture. Results are the mean ± S.E.M. of 6 pairs of animals. * $P < 0.05$ when compared to control animals.

salmeterol (10^{-6} M) was used to assess whether inhibition still occurred after treatment of eosinophils for 15 min with salmeterol followed by two washes. As shown in Fig. 1, pretreatment with salmeterol inhibited eosinophil aggregation induced by PAF (10^{-7} M) and hrC5a (10^{-7} M) by 65 and 43%, respectively. These results are in agreement with previous studies in guinea-pig tissues, suggesting that multiple washings of the preparation interfered little with the binding and inhibitory capacity of salmeterol up to 7 h after pretreatment (Ball et al., 1991).

3.2. Effects of salmeterol pretreatment on eosinophil recruitment in vivo

The same concentration of salmeterol (10^{-6} M) was then used to assess whether pretreatment with salmeterol would inhibit mediator-induced accumulation of ^{111}In-eosinophils. Fig. 2 shows the effects of salmeterol pretreatment on ^{111}In-eosinophil accumulation induced by PAF (10^{-10} and 10^{-9} mol/site) and zymosan-activated plasma (10% and 30% dilution in saline). At the concentration used, salmeterol significantly inhibited ^{111}In-eosinophil accumulation by the top dose of PAF and zymosan-activated plasma by 55% and 45%, respectively. Pretreatment with salmeterol also inhibited ^{111}In-eosinophil accumulation induced by hrC5a (control eosinophils: hrC5a 10^{-10} mol/site, 2587 ± 967 ^{111}In-eosinophils per site; salmeterol pretreated: hrC5a, 1114 ± 416 ^{111}In-eosinophils per site, $n = 4$, $P < 0.05$).

Next we evaluated whether the inhibitory effect of salmeterol pretreatment on ^{111}In-eosinophil accumulation was due to a decrease in the number of circulating ^{111}In-eosinophils. Contrary to its inhibitory effects on cell recruitment, pretreatment with salmeterol resulted in a significant increase in the number of ^{111}In-eosinophils circulating at 1 h (Fig. 3).

4. Discussion

Systemic treatment with salmeterol and other shorter-acting β_2-adrenoceptor agonists has been shown to inhibit the recruitment of eosinophils induced by allergen challenge of sensitized animals (e.g., Fugner, 1989; Sugiyama et al., 1992; Whelan et al., 1993; Teixeira et al., 1995c). In these complex in vivo experimental systems, it is unclear which cell or cells are the targets for the inhibitory action of the β_2-adrenoceptor agonist. There is a substantial amount of data demonstrating an inhibitory effect of β_2-adrenoceptor agonists on mediator release from mast cells (e.g., Church and Hiroi, 1987; Butchers et al., 1991) and, indeed, inhibition of mast cells may be an important cellular target for the inhibitory effects of salmeterol on eosinophil accumulation in vivo (discussed in Teixeira et al., 1995c). However, salmeterol inhibits eosinophil accumulation induced by PAF, a mast cell-independent inflammatory mediator, in guinea-pig skin (Teixeira et al., 1995c and see Fig. 2) suggesting that inhibition of a cell type other than the mast cell is also likely to occur following systemic treatment with a β_2-adrenoceptor agonist. Another putative cellular target for the inhibitory action of systemic β_2-adrenoceptor agonist treatment is the endothelial cell. Recent observations have shown that elevation of cyclic AMP in endothelial cells with dibutyryl cyclic AMP and 3-isobutyl-1-methylxanthine is associated with a reduced capacity to express the cell adhesion molecules vascular cell adhesion molecule-1 (VCAM-1) and E-selectin following stimulation with tumour necrosis factor-α (Pober et al., 1993). This is an interesting observation inasmuch as cell adhesion molecules appear to be essential for the migration of eosinophils in vivo (Teixeira et al., 1995b). Whether β_2-adrenoceptor agonists affect the induction of

endothelial cell adhesion molecules and whether this contributes to their inhibitory activity in vivo, particularly over a short time period as used in our study, is currently under investigation in our laboratory.

Clearly, another target for β_2-adrenoceptor agonists in vivo could be the eosinophil and there is considerable evidence to suggest that eosinophil function is inhibited by β_2-adrenoceptor agonists in vitro. We have shown previously that systemic treatment with salmeterol (0.1 mg/kg) effectively inhibits ^{111}In-eosinophil accumulation induced by various mediators and in allergic reactions in guinea-pig skin (Teixeira et al., 1995c). In addition, we have shown that salmeterol effectively inhibits mediator-induced eosinophil aggregation, a CD18-dependent functional response (Teixeira et al., 1996a). Since salmeterol is known to bind to and inhibit β_2-adrenoceptors in guinea-pig tissues with a long duration of action (over 7 h) despite continuous superfusion with drug-free solution (Ball et al., 1991), this drug was used to pretreat the eosinophils before washing and testing in the two salmeterol-sensitive systems described above, namely eosinophil aggregation and ^{111}In-eosinophil accumulation in guinea-pig skin. Our in vitro results showed that salmeterol pretreatment was still inhibitory even after the cells had been washed twice. If the salmeterol-pretreated ^{111}In-eosinophils were then injected into recipient guinea pigs and their accumulation in response to PAF, zymosan-activated plasma and hrC5a followed for 1 h, a significant inhibition of ^{111}In-eosinophil accumulation was observed. It is important to note that only the ^{111}In-eosinophils, but not the recipient animals, were pretreated with salmeterol at any time. Thus, inhibition of eosinophil function (as demonstrated by inhibition of eosinophil aggregation) by salmeterol was also associated with inhibition of eosinophil accumulation in vivo. Together, these results suggest that the eosinophils themselves are a cellular target for the inhibitory effects of systemic administration of salmeterol in vivo.

High concentrations of salmeterol ($\geq 10^{-5}$ M but effective inhibition only at 10^{-4} M) have been previously shown to have anti-inflammatory effects on human alveolar macrophages independent of an action on β-adrenoceptors (Backer and Fuller, 1990). The concentration of salmeterol used in our study (10^{-6} M) is considerably lower than that shown to affect cellular function in a β-adrenoceptor-independent manner. Moreover, we have previously shown that the inhibitory effects of salmeterol on eosinophil aggregation in vitro (Teixeira et al., 1996a) and eosinophil migration in guinea-pig skin (Teixeira et al., 1995c) is reversed by previous treatment with the β-blocker propranolol. Thus, it is likely that the inhibitory effects of salmeterol were mediated by an action on β_2-adrenoceptors.

The systemic administration of β_2-adrenoceptor agonists in man can decrease the levels of circulating blood eosinophils (Ohman et al., 1972) and this decrease can account, at least partially, for the inhibitory effects of a β_2-adrenoceptor agonist on eosinophil recruitment following allergen challenge in human skin (Ting et al., 1983). We have previously demonstrated that a decrease in the number of circulating ^{111}In-eosinophils was associated with diminished accumulation of these cells in guinea-pig skin (Teixeira et al., 1994a). Thus, it was important to assess whether the levels of circulating ^{111}In-eosinophils fell following pretreatment of these cells with salmeterol. In contrast to its inhibitory effects on ^{111}In-eosinophil recruitment, pretreatment with salmeterol actually enhanced by approximately 35% the levels of circulating ^{111}In-eosinophils. It will be of interest to investigate the mechanisms by which salmeterol pretreatment enhances the levels of circulating ^{111}In-eosinophils in vivo.

It is unclear how salmeterol switches off the ability of eosinophils to accumulate in sites of cutaneous inflammation. Salmeterol has been shown to potently inhibit eosinophil chemotaxis in vitro even after long-term incubation with drug-free medium (Koenderman et al., 1992), but it is uncertain whether this is important for the in vivo situation. However, another interesting possibility has arisen from the ability of salmeterol to inhibit eosinophil aggregation in vitro. We have recently shown that drugs which elevate cyclic AMP (e.g., salmeterol, prostaglandin E_1 and rolipram) inhibit eosinophil aggregation induced by both PAF and C5a (Teixeira et al., 1996a). Interestingly, the ability of these drugs to inhibit eosinophil aggregation was associated with their ability to decrease the upregulation of the β_2 integrin CD18 on the eosinophil surface (Teixeira et al., 1996a) and, presumably, the affinity of CD18 for its ligand(s). As shown in Fig. 1, salmeterol pretreatment also inhibited eosinophil aggregation induced by both PAF and C5a. Inasmuch as CD18 is important for eosinophil migration in vivo (Teixeira et al., 1994a), down-regulation of agonist-induced CD18 expression and, more importantly, of CD18 binding affinity by salmeterol may play a role in the inhibitory effects of this drug in vivo. In agreement with this proposal, Derian et al. (1995) have recently demonstrated that cyclic AMP-elevating agents inhibit the adhesion of neutrophils to vascular endothelium via inhibition of the expression of CD11/CD18 on neutrophils, although an effect on eosinophils has not been reported.

In conclusion, our results suggest that the eosinophil itself is one of the cellular targets of the inhibitory action of systemically administered salmeterol on eosinophil recruitment in vivo. It is thus possible, but not proven, that the ability of other cyclic AMP-elevating agents (e.g., phosphodiesterase inhibitors) to block eosinophil migration in vivo is also at least partially dependent on their ability to inhibit the eosinophil directly.

Acknowledgements

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