Eosinophils as a Direct Target of the Anti-Inflammatory Effect of Salmeterol: Demonstration with Indium-111-Labeled Eosinophils

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Key Words

Eosinophils · Guinea pig · Salmeterol · Indium-111 · Skin

Abstract

Objective: The β_2 -adrenoceptor agonist salmeterol inhibits the accumulation of eosinophils at the site of allergic inflammation, but the cellular target is uncertain. This study was undertaken to determine whether eosinophils themselves are the target of this inhibitory action. **Methods:** Purified guinea pig peritoneal eosinophils were labeled with indium-111 oxine, pre-incubated with salmeterol or drug vehicle before being injected intravenously into guinea pigs, which have previously been immunized with ovalbumin (OA). Four hours after intradermal injection of OA or platelet-activating factor (PAF), the accumulation of labeled eosinophils at the injection sites was determined by measuring the radioactivity in punched-out skin sites. In vitro adhesion to plastic plate was also studied by measuring the eosinophil peroxidase content of adhering cells. **Results:** About 6% of the injected ^{111}In -eosinophils were circulating 10 min after injection and remained relatively steady for over 4 h. In animals given vehicle-treated ^{111}In -eosinophils an up to 3.6-fold increase in the accumulation of the labeled cells at the skin sites of OA or PAF injection was seen. Pre-

treatment of ^{111}In -eosinophils with salmeterol ($1\text{ }\mu\text{M}$) – a concentration giving about 65% inhibition of in vitro adhesion – had no effect on the basal (PBS-induced) skin accumulation of the injected cells. However, it inhibited the net accumulation induced by OA ($0.01\text{--}1\text{ }\mu\text{g}/\text{site}$) and PAF ($0.01\text{--}1\text{ nmol}/\text{site}$) by 58.8–100%. At $1\text{ }\mu\text{M}$, salmeterol itself had no significant effect on the viability and circulation of ^{111}In -eosinophils. **Conclusion:** These results provide evidence for a direct inhibitory effect of salmeterol on eosinophils and suggest that this may account for a significant part of its clinical anti-inflammatory properties.

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Introduction

Eosinophil accumulation in tissues is the hallmark of allergic diseases such as asthma, allergic rhinitis and atopic dermatitis [1, 2], and several studies have emphasized the crucial roles these cells play in the pathophysiology of the diseases [3, 4]. Activated eosinophils release several cationic proteins such as the major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin and eosinophil peroxidase, which are directly toxic to many tissues [5]. In asthma, for example, it has been suggested

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that the release of these substances in the bronchial tissues orchestrate further inflammation that leads to the desquamation of bronchial epithelium and the subsequent bronchial hyperreactivity [6, 7]. Thus, the ability to inhibit allergen-induced eosinophilic inflammation is now regarded as an important property required of a good anti-allergic or anti-asthma drug [8].

Salmeterol is along-acting β_2 -adrenoceptor agonist that is employed as a bronchodilator in the treatment of bronchial asthma. Apart from the prolonged bronchodilation it provides, its effectiveness in asthma also appears to stem from its possession of significant anti-inflammatory effects. Systemic administration of salmeterol, but not the conventional shorter-acting β_2 -adrenoceptor agonists – salbutamol, has been reported to suppress allergen- and mediator-induced bronchial or skin eosinophil recruitment in sensitized guinea pigs [9, 10]. A similar effect against allergen-induced late-phase responses and bronchial hyperreactivity has also been reported in asthmatics [11, 12].

The *in vivo* mechanism of such anti-inflammatory effects is still uncertain, but often attributed to the possible inhibition of T-cell generation of eosinophil-chemotactic cytokines, especially interleukin 5 (IL-5) and granulocyte macrophage colony-stimulating factor (GM-CSF) [13]. However, since eosinophils are known to possess β_2 -adrenoceptors [14, 15] and that salmeterol can inhibit eosinophil activation *in vitro* [16–18], a direct inhibition of the eosinophils themselves could also be a possible *in vivo* mechanism.

The purpose of this study was, therefore, to determine whether eosinophils themselves are a target of the inhibitory effect of salmeterol *in vivo*, especially when eosinophilic inflammation is induced by an allergen.

Materials and Methods

Immunization of Guinea Pigs

Male Dunkin-Hartley guinea pigs (250–300 g) were actively immunized by an intraperitoneal injection of 1 ml of a mixture containing 10 μ g ovalbumin (OA) and 5 mg aluminum hydroxide gel in saline. This injection was repeated after 7 days and between days 21 and 28 after the first injection; the animals were used for the experiments on allergen-induced eosinophilic skin inflammation.

Induction and Purification of Guinea Pig Peritoneal Eosinophils

Peritoneal eosinophilia was induced in male guinea pigs (400–450 g) by repeated intraperitoneal injection (twice weekly for a total of 4–6 injections) of 0.5 ml of horse serum. Peritoneal lavage was then performed with 5×10 ml Ca^{2+} - and Mg^{2+} -free Hank's balanced salt solution (HBSS), pH = 7.4, containing sodium citrate (13 mM) and 0.3% bovine serum albumin (BSA). Cells were recovered by cen-

trifugation, washed once in the same buffer, and resuspended at a concentration of 10^7 cells/ml for purification.

Eosinophils were purified by gradient centrifugation over Percoll (densities: 1.080, 1.085, and 1.090 g/ml). After centrifugation at 900 g on Beckman (GS-6R) centrifuge for 20 min at room temperature, eosinophils were collected as pellets. After hypotonic lysis of the contaminating erythrocytes, the eosinophils were washed twice and resuspended in the reaction buffer (HBSS containing 2 mM Ca^{2+} and 1 mM Mg^{2+} , pH = 7.4) for adherence experiments or in phosphate-buffered saline (PBS) for ^{111}In oxine labeling. Eosinophil preparations of greater than 95% purity were used and the viability (determined by trypan blue exclusion) always exceeded 98%.

*Measurement of Eosinophil Adherence *in vitro**

Peritoneal eosinophils purified from horse serum-treated guinea pigs were resuspended at a concentration of 5×10^5 cells/ml in the reaction buffer. Fifty-microliter aliquots were then added to each well of a 96-well plate (Maxisorp, Nunc, Roskilde, Denmark) and incubated for 15 min with salmeterol or vehicle before stimulating adherence with platelet-activating factor (PAF) (0.001–10 μM). After incubation for 30 min the plate was then washed 3 times with PBS (200 μl /well) at room temperature and the adherent cells lysed with 150 μl of 0.1% Triton X-100. Fifty-microliter aliquots were then taken for the measurement of eosinophil peroxidase (EPO) activity by the *o*-phenylenediamine method [19]. The EPO in adhered cells as a percentage of the amount in the total cells added represented the percentage cell adherence [20].

Labeling of Eosinophils and the Measurement of ^{111}In -Labeled Eosinophils in Guinea Pig Skin

Purified eosinophils were labeled by incubating 2×10^7 cells (in 1 ml PBS) with ^{111}In oxine (100 μCi in 10 μl) for 15 min at room temperature. The labeled cells were washed twice and subsequently incubated for 10 min with salmeterol (1 μM) or drug vehicle (dimethylsulfoxide (DMSO) diluted in buffer). After two further washes, the cells were finally resuspended at a concentration of 5×10^6 cells/ml in PBS. Five million ^{111}In -eosinophils (1.5 – 2.0×10^7 counts per minute (CPM)) were then injected intravenously into each OA-immunized guinea pig, under pentobarbitone anesthesia (30 mg/kg *i.p.*). In experiments to evaluate the influence of drug treatment of ^{111}In -eosinophils on their subsequent circulation *in vivo*, 400 μl of venous blood was withdrawn at intervals after their injection and the radioactivity counted. The percentage of circulating ^{111}In -eosinophils at each point in time was determined according to the following formula (assuming blood volume of 70 ml/kg body weight):

$$\% \text{ circulating cells} = \frac{2.5 \times [\text{count in 0.4 ml blood}] \times [\text{blood volume of animal (ml)}]}{\text{total count injected}} \times 100$$

For experiments on skin accumulation of ^{111}In -eosinophils, the intravenous injection of the cells was followed 10 min later by intradermal injection of 100 μl of various concentrations of OA and PAF or saline in the dorsal shaved skin in a randomized site plan. After 4 h the animals were killed, the skin removed, cleaned of subcutaneous tissues and the injected sites (10 mm diameter) punched out. The radioactivity of the samples as an index of accumulated ^{111}In -eosinophils was then determined in a Packard Cobra II, well-type γ counter, adjusted to include both 171- and 245-keV γ energies. The number of eosinophils infiltrating the injected sites was then calculated by dividing the sample count with the count per injected cell (usually

Fig. 1. Inhibition by salmeterol of the in vitro adherence of guinea pig eosinophils to plastic plates. Adherence was induced with $1 \mu\text{M}$ PAF and the mean net uninhibited adherence was $23.6 \pm 5.7\%$ of the total cells added. Spontaneous adherence was always less than 5%. Values are mean net percentage inhibition of adherence $\pm$ standard deviation, $n = 4$. * $p < 0.05$; ** $p < 0.01$.

2.8–7.0 CPM). Preliminary data showed that circulating non-eosinophil-associated radioactivity during 4 h of the experiment was less than 1%; consequently, no correction was made to subsequent experimental data.

Reagents

The following reagents were obtained from Sigma Chemical Co., St. Louis, Mo., USA: Horse serum, BSA, HBSS, OA, trypan blue, DMSO, *o*-phenylenediamine, PAF and all the inorganic salts. ^{111}In oxine was obtained from Amersham PLC, UK, while salmeterol was a generous gift of Glaxo Wellcome, PLC Greenford, Middx, UK.

Statistics

Values are given as means $\pm$ standard deviation for the number (n) of independent experiments. The concentration producing 50% inhibition of adhesion was calculated by the non-linear regression analysis using GraphPad InPlot (GraphPad Software Inc., USA): Statistical significance was determined by the paired or unpaired t -tests as appropriate. For multiple comparisons, one-way analysis of variance (ANOVA) was applied, followed by Dunnett's multiple comparison test. A p value < 0.05 was considered significant.

Results

Effect of Salmeterol on Eosinophil Adherence to Plastic Plate

In order to determine the appropriate concentration of salmeterol to be used for pre-treating ^{111}In -eosinophils before injection into guinea pigs, the effect of different

Fig. 2. Time course of the circulation of ^{111}In -eosinophils after intravenous injection in guinea pigs. Labeled cells were pretreated for 15 min with salmeterol ($1 \mu\text{M}$) or vehicle and washed twice before injection. Values are means $\pm$ standard deviation, $n = 5$ in each case.

concentrations of the drug on PAF-induced adherence of eosinophils to plastic plates was studied. As shown in figure 1, salmeterol produced a concentration-dependent inhibition of PAF-induced adherence. The concentration that achieved 50% inhibition (IC_{50} value) was $0.4 \mu\text{M}$. The concentration of $1 \mu\text{M}$, which inhibited adherence by approximately 65% but without any significant toxicity (as judged by trypan blue exclusion), was chosen for the subsequent in vivo experiments.

Effect of in vitro Pre-Treatment of ^{111}In -Eosinophil with Salmeterol on Their Subsequent Circulation in vivo

In order to study how the pre-treatment of ^{111}In -eosinophils with salmeterol would affect their subsequent accumulation in the skin, it was necessary to first assess the effect of such treatment on eosinophil circulation per se. As shown in figure 2, when immunized guinea pigs were injected intravenously with vehicle-treated (and twice washed) ^{111}In -eosinophils, approximately 6% of the injected cells were circulating 10 min after the injection. This level of circulating cells remained relatively steady for 4 h. When ^{111}In -eosinophils were pre-treated with salmeterol ($1 \mu\text{M}$) and washed twice before injection, there was no significant difference in the level of circulating labeled cells at all time points studied. However, a slightly higher percentage of the injected cells were circulating in

Fig. 3. The effect of in vitro pretreatment of ¹¹¹In-eosinophils with salmeterol or vehicle on their subsequent skin accumulation in OA-immunized guinea pigs in response to intradermal injection of various concentrations of OA, PAF or PBS. Labeled cells were pretreated for 10 min with salmeterol (1 µM) or vehicle and washed twice before injection intravenously into the animals. Values are means ± standard deviation, n = 5 for vehicle and 6 for salmeterol. ^a p < 0.05, ^b p < 0.01, ^c p < 0.001 (compared with PBS-injected site, by ANOVA followed by Dunnet's test). * p < 0.05, ** p < 0.01 (compared with corresponding vehicle-pretreated, by t-test).

the first 1 h after injection. Imaging of the whole animal showed that the bulk of the injected radioactivity was localized in the liver within the first 10 min and later in the spleen as well (data not shown).

Effect of in vitro Pre-Treatment of ¹¹¹In-Eosinophils with Salmeterol on Their Subsequent Skin Accumulation in vivo

In animals given vehicle-treated ¹¹¹In-eosinophils, PAF (0.01–1.0 nmol/site) and OA (0.01–1.0 µg/site) induced dose-dependent accumulation of the labeled cells in the skin. Compared with PBS-injected sites, the two highest concentrations of PAF and all concentrations of OA produced statistically significant increases of ¹¹¹In-eosinophil accumulation (p < 0.05–0.01) (fig. 3). At the highest concentrations of PAF and OA, the increases were 2.9-fold (1,850 ± 371 vs. 630 ± 84 cells/skin site), and 3.6-fold (2,293 ± 384 vs. 630 ± 84 cells/skin site), respectively, p < 0.01 in both cases.

Pre-treatment of eosinophils with salmeterol (1 µM) had no significant effect on the background accumulation of ¹¹¹In-eosinophils at PBS-injected sites, but significantly reduced the accumulation at the sites injected with PAF or OA. For example, at the sites injected with the highest

concentration of PAF (1 nmol/site), ¹¹¹In-eosinophil accumulation was reduced from 1,850 ± 371/skin site for vehicle-treated cells to 1,316 ± 339/skin site for salmeterol-treated cells, p < 0.05, n = 5–6 (fig. 3). For the highest concentration of OA, the corresponding values were 2,293 ± 384/skin site for vehicle-treated and 1,416 ± 472/skin site for salmeterol-treated cells, p < 0.01. Thus, salmeterol appeared to be slightly more effective in inhibiting OA-induced than PAF-induced ¹¹¹In-eosinophil accumulation. When the net accumulation was considered, salmeterol almost completely abolished the effect of the two lower concentrations of both PAF and OA. At the highest concentration of PAF, salmeterol achieved a reduction of 58.0 ± 5.2% while for OA it was 69.1 ± 8.1%.

Discussion

Previous reports have shown that salmeterol possesses in vivo anti-inflammatory effects in animal models of late allergic reactions and in human allergic diseases [9–12]. However, the cellular target of this action has remained uncertain. To determine if eosinophils are a target of this action, we have employed the guinea pig model of allergic skin inflammation. In this model, the injection of antigen into the skin of a previously immunized animal resulted in a skin reaction with heavy eosinophilic infiltration. The use of ¹¹¹In-eosinophils allowed for precise quantitation of eosinophils infiltrating the sites of allergic skin reaction. The results obtained clearly showed that when ¹¹¹In-eosinophils were pre-treated in vitro with salmeterol, washed and then injected intravenously into immunized animals, the ability of the cells to accumulate at allergen-challenged skin sites was either completely abolished or significantly reduced. A similar effect was also seen if skin reaction was induced with the eosinophil chemotactic factor PAF. These results therefore show that salmeterol can act directly on eosinophils to inhibit their ability to respond to chemotactic stimuli generated by the antigen-antibody interaction. In the only other similar study, Teixeira and Hellewell [21] recently reported similar results although they employed only soluble mediators and zymosan-activated plasma. Thus, the present results represent the first demonstration of the effect of salmeterol against eosinophil accumulation induced by antigen-antibody interaction.

In experiments of this nature, it is often crucial to ensure that neither the labeling of the cells nor their treatment with the drug affected their ability to circulate and function normally in vivo. It is also important to ensure

that experiments are performed during a steady-state circulation of the injected cells. Previous studies have shown that guinea pigs labeled with ^{111}In oxine fully retained viability as well as their *in vivo* responsiveness to various stimuli [22, 23]. In the present experiments, we first studied the concentration-dependent inhibition of eosinophil adherence *in vitro* and on the basis of the results, the concentration of $1\ \mu\text{M}$ salmeterol, which produced about 65% inhibition of adherence while having no effect on cell viability, was chosen for the subsequent *in vivo* experiments. Next we verified that ^{111}In -eosinophils could circulate long enough for the *in vivo* experiments to be performed. Although only about 6% of the injected eosinophils were circulating 10 min after injection, this level remained relatively constant over the next 4 h – a sufficient time for the subsequent experiments. Whole body imaging (data not shown) showed that the bulk of the injected radioactivity was trapped in the liver and spleen soon after injection. The reason for this enhanced trapping is unclear, but may be a consequence of the purification/washing processes or the fact that the cells were derived from the peritoneum rather than the blood. A similar rapid extravasation of purified guinea pig ^{111}In -eosinophils has been previously reported [21, 24]. Finally, it was verified that the pre-treatment of ^{111}In -eosinophils with the chosen concentration of salmeterol did not significantly affect their subsequent circulation *in vivo*. This excluded the possibility that the inhibition of skin accumulation noted with salmeterol-treated ^{111}In -eosinophils was a result of a lower number of circulating cells. On the contrary, there was a small early transient increase in the percentage of salmeterol-treated cells circulating compared with the vehicle-treated cells.

When salmeterol is given systemically, its ability to inhibit allergen-induced eosinophil infiltration into tissues is likely to result from at least two mechanisms. Firstly, it could act indirectly by inhibiting the release of eosinophil chemotactic factors (e.g., PAF, IL-5 and C5a) generated by a variety of pro-inflammatory cells as a consequence of the antigen-antibody interaction. The drug can do this by activating the β_2 -receptors present on most inflammatory cells. The second mechanism, which the present study suggests, is that the drug acts directly on eosinophils preventing them from responding to the endogenously generated chemotactic stimuli. Although the exact biochemical effect of salmeterol on eosinophils was not specifically sought in the current study, it is known that eosinophils of guinea pigs and humans possess functional β_2 -adrenoceptors on their surfaces [14, 15]. The activation of these receptors by salmeterol would be

expected to cause an increase in the intracellular levels of adenosine 3',5'-cyclic monophosphate (cAMP) – a powerful inhibitor of leukocyte responses [17, 25]. Thus, in view of the current and previous data [18], it is likely that the inhibition of adherence is crucial to the observed reduction in the *in vivo* cellular infiltration. Adherence to the vascular endothelium is the first step in the process of exsavasation of blood leukocytes into tissues. A direct inhibition by salmeterol of the ability of eosinophils to adhere, perhaps as a result of the down-regulation of the expression of surface adhesion molecules (e.g. VLA4 and CD11/CD18), may underlie the reduced skin accumulation by these cells.

Conclusion

The *in vitro* treatment of eosinophils with salmeterol strongly reduced their ability to accumulate in the skin in response to both antigen-antibody interaction and to a direct chemoattractant – PAF. This provides evidence for a direct inhibitory effect of salmeterol on eosinophils and suggests that this may account for a significant part of its clinical anti-inflammatory property. The exact mechanism remains unclear but may likely involve the inhibition of the ability of eosinophils to adhere to the vascular endothelium in the process of extravasation.

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