Differential Effects of Salbutamol and Salmeterol on Human Eosinophil Responses

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ABSTRACT

In the treatment of bronchial asthma, salmeterol is believed to have a greater anti-inflammatory activity than salbutamol. To determine whether the comparative effects of these drugs on eosinophil function are the basis of their differential anti-inflammatory properties, we studied the effect of the two drugs on interleukin-5 (IL-5) and 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (PAF)-induced O$_2^-$ release and adherence to fibronectin-coated plates, as well as the C5a- and N-formylmethionyl-leucyl-phenylalanine (FMLP)-induced degranulation of purified human blood eosinophils in vitro. Salmeterol significantly inhibited IL-5-induced O$_2^-$ release in a concentration-dependent manner with an IC$_{50}$ of 2.2 × 10$^{-6}$ M (95% CI, 1.6–2.7 × 10$^{-6}$ M) and a maximal inhibition of about 70%. In contrast, salbutamol had no significant effect even at 10$^{-5}$ M. Both drugs significantly inhibited PAF-induced O$_2^-$ generation, but salmeterol was approximately 20 times more potent than salbutamol. Salmeterol also significantly inhibited adherence induced by both IL-5 and PAF, whereas salbutamol had no significant effect on adherence induced by both agents. Both drugs failed to block C5a-induced eosinophil peroxidase release, whereas for FMLP-induced release, salbutamol, but not salmeterol, produced significant inhibition. Unlike salbutamol, all the actions of salmeterol were independent of beta-2 adrenoceptors. These results confirm that human eosinophils can be modulated directly by beta-2 adrenoceptor agonists, but that salmeterol and salbutamol have differential effects which depend on both the stimulus used and the response being measured and that the reported greater in vivo anti-inflammatory effect of salmeterol may reflect its superior ability to inhibit eosinophil O$_2^-$ release and adherence, rather than degranulation.

Beta-2-selective adrenoceptor agonists are the key bronchodilators used in the reversal of the acute bronchospasm of bronchial asthma. Salmeterol and salbutamol are among the most widely used members of this group. In some respects, however, the pharmacodynamic characteristics of these two drugs differ. Unlike salbutamol, which is a short-acting bronchodilator, salmeterol produces a sustained bronchodilatory effect in asthmatic patients that may last for more than 12 h (Lotvall et al., 1992; Ball et al., 1991). This prolonged effect has been attributed to its interaction with an “exosite” around the beta-2 adrenoceptor domain which restricts its dissociation from the receptors (Johnson, 1990). Another characteristic of salmeterol which differs from salbutamol, and which may potentially contribute to the reported clinical superiority of this drug over salbutamol and other conventional beta-2 adrenoceptor agonists (Di Lorenzo et al., 1995), is its possession of significant anti-inflammatory effect. Unlike the conventional beta-2 adrenoceptor agonists, the drug suppressed allergen-induced bronchial eosinophil recruitment in sensitized guinea pigs (Sanjar et al., 1991), as well as allergen-induced late-phase responses and hyperreactivity in asthmatics (Twentyman et al., 1990; Dahl et al., 1990).

Bronchial inflammation is now recognized as an invariable component of bronchial asthma and several reports have shown that bronchial eosinophil infiltration and activation play a critical role in the pathogenesis of the disease (Frigas and Gleich 1986; Wardlaw et al., 1988; Barnes 1989). The eosinophil contains several granule-derived cationic proteins such as EPO, ECP, EDN and MBP, which when secreted by the accumulated bronchial eosinophils cause airway epithelial damage, resulting in the development of bronchial hyperreactivity that is characteristic of asthma (Motijima et al., 1989; Laitinen et al., 1985).

Data from several clinical studies suggest that the eosinophils might be one of the important targets for the anti-

ABBREVIATIONS: IL-5, interleukin-5; PAF, 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine; C5a, complement fragment 5a; O$_{2}^{\bullet-}$, superoxide anion; EPO, eosinophil peroxidase; FMLP, N-formylmethionyl-leucyl-phenylalanine; ECP, eosinophil cationic protein; MBP, major basic protein; EDN, eosinophil-derived neurotoxin; PBS, phosphate-buffered saline; LTB$_{4}$, leukotriene B$_{4}$; BSA, bovine serum albumin; SOD, superoxide dismutase; DMSC, dimethyl sulfoxide; CB, cytochalasin B; PLC, phospholipase C; PKA, protein kinase A; PKC, protein kinase C; IC$_{50}$, concentration achieving 50% inhibition; HEPES, N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulphonic acid]; OPD, O-phenylenediamine; ICI 118551, erythro-(-)-1-(7-methylindan-4-yloxy)-3-isopropylamino butan-2-ol hydrochloride; CI, confidence interval; ANOVA, analysis of variance.

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inflammatory action of salmeterol. Pedersen and his colleagues (1993) showed that the increases in serum levels of ECP and eosinophil protein-X after antigen challenge of asthmatics were significantly inhibited by pretreatment with inhaled salmeterol. Di Lorenzo and his colleagues (1995) also showed that salmeterol treatment produced a decrease in serum levels of ECP in allergen-challenged asthmatics without modifying the blood eosinophil count. These studies indicate that the action of the drugs may more likely involve the inhibition of degranulation or the suppression of their mobilization to the potential sites for degranulation in the bronchial tissues rather than a general suppression of peripheral blood eosinophilia.

Eosinophils possess membrane beta-2 adrenocort receptor agonists (Yukawa et al., 1990), but whether beta-2 adrenocort receptor agonists are able to directly modulate eosinophil function via these receptors is still controversial (Masuyama and Ishikawa, 1985). Rabe and his colleagues (1993) reported that salbutamol and formoterol, but not salmeterol, inhibited LTB4-induced hydrogen peroxide generation from guinea pig eosinophils, and that such effect was propranolol-sensitive, which suggests that it is beta adrenergic mediator. A similar effect was reported in FMLP-induced EPO release from human blood eosinophils (Munoz et al., 1995). In both studies, salmeterol was even shown to act as a beta-2 adrenoceptor antagonist. This is an apparent paradox, because, in vivo both in man and experimental animals, it is salmeterol rather than salbutamol that is credited with the anti-inflammatory action that is manifested both as the suppression of bronchial eosinophil infiltration (Whelan and Johnson, 1992; Whelan et al., 1993) and degranulation (Pedersen et al., 1993; Di Lorenzo et al., 1995). Furthermore, the other long-acting beta-2 adrenocort receptor agonists, formoterol and eformoterol, inhibit eosinophil functions in vitro (Anderson 1991; Rabe et al., 1993). These apparently conflicting reports, and particularly the discrepancy between the in vivo and in vitro effects of beta-2 adrenocort receptor agonists on eosinophil function, require clarification. Furthermore, the analysis of the in vitro effects of these drugs may have been complicated by the use of different stimuli and the measurement of different responses.

This study compared the direct effects of salmeterol and salbutamol on various eosinophil responses, stimulated in a variety of ways, to determine whether their comparative actions on these cells is the basis for their differential in vivo anti-inflammatory properties. We also sought to clarify, by pharmacological analysis, if any such effect is mediated via beta-2 adrenocort receptors.

**Methods**

**Isolation of human peripheral blood eosinophils.** Fresh blood was obtained from consenting healthy or mildly atopic adults who had taken no medications in the past 72 h. Eosinophils were purified by a slight modification of the immunomagnetic method (Hansel et al., 1991). Three parts of sodium citrate-anticoagulated (13 mM final) blood was mixed with one part of 1% (w/v of 0.9% saline) hydrated methylcellulose solution to sediment the erythrocytes during 30 min at room temperature. The leukocyte-rich supernatant was collected and centrifuged at 200 \( \times g \) for 10 min at room temperature. After aspirating off the platelet-rich supernatant, the pelleted leukocytes were washed in “wash buffer” (Ca\(^{2+}\)- and Mg\(^{2+}\)-free, 10 mM HEPES-buffered Hanks’ balanced salt solution containing 0.25% BSA) and resuspended in the same buffer at approximately 10% of the original blood volume. A 2-mL aliquot was then layered on a two-step Percoll gradient (1.080 and 1.090 g/mL) and centrifuged at 900 \( \times g \) on Beckman (GS-6R) centrifuge for 20 min at room temperature. The upper layers (mononuclear cells and Percoll) were discarded and the pellet (granulocytes) were recovered and washed twice in the same buffer by centrifugation at 250 \( \times g \) for 10 min at 4°C. After a hypotonic lysis of contaminating erythrocytes with ice-cold distilled water, and readjusting the tonicity with the same volume of double-strength saline, the cells were washed, counted and resuspended at a concentration of \( 2 \times 10^6 \) cells/ml in the wash buffer. For the eosinophil purification, 1.25 mL of the granulocyte suspension was then mixed with 5 mL mouse anti-human CD16 monoclonal antibody in a sterile siliconized test tube and incubated on ice for 1 h with frequent gentle rotation. Cells were then washed twice in wash buffer and after the final wash, the cells were pelleted by centrifugation and then resuspended in 500 \( \mu \)L of prewashed immunomagnetic beads precoated with affinity-purified sheep anti-mouse IgG (2 \( \times 10^6 \) coated beads) and incubated in ice for 1 h with frequent tube rotation. The immunomagnetically labeled neutrophils were then removed by magnetic extraction. The purified eosinophils were then recovered by centrifugation and resuspended in reaction buffer (wash buffer containing 2 mM Ca\(^{2+}\) and 1 mM Mg\(^{2+}\)) for experiments. The eosinophil purity was assessed by differential count of Wright-Giemsa stained cytocentrifuge. The final cell preparation routinely consisted of more than 98% pure eosinophils. Viability was determined by trypan blue exclusion and always exceeded 98%.

**Superoxide anion release.** Superoxide anion \((O_2^-)\) generation was determined by the SOD-inhibitable reduction of ferricytochrome c (Sedgwick et al., 1988). Prewarmed eosinophils were resuspended at a concentration of 5 \( \times 10^6 \) cells/ml and 50- \( \mu \)L aliquots dispensed into each well of the 96-well microplate containing 50 \( \mu \)L of cytochrome c (100 \( \mu \)M, final) and 50 \( \mu \)L reaction buffer. After preincubation for 10 min at 37°C, the mixture, 50 \( \mu \)L of the stimulus was then added and the mixture (total 200 \( \mu \)L) incubated at 37°C for 1 h. Corresponding wells containing 0.25 \( \mu \)M (final) SOD were included to assess specific superoxide formation. In experiments in which the effect of drugs was assessed, 50 \( \mu \)M of the drugs (4 times the required concentration in reaction buffer) was added in place of the buffer and incubated with the cells for 10 min before the addition of the stimulus. In some experiments in which the reversal of the effect of the drugs was attempted, the reversing agent was added as 5 \( \mu \)L of a 40 times the required concentration. After incubation of the reaction mixtures, 150 \( \mu \)L was transferred to a fresh plate and the absorbance read at 550 nm on the Titertek Multiscan (Flow Labs, Rickmansworth, U.K.) plate reader. The amount of O2\(^-\) generated was estimated as the nanomoles of ferricytochrome c reduced per 10\(^6\) cells per hour with the extinction coefficient of 2.1 \( \times 10^{-4}\) M\(^{-1}\) cm\(^{-1}\).

**Adherence.** Immunoplates (Maxisorp, Nunc Roskilde, Denmark) were coated with 50 \( \mu \)L of human fibronectin (40 \( \mu \)g/ml) for 1 h at 37°C and washed three times before use. In many experiments, the measurement of adherence and O2\(^-\) were done in the same experiment. In these cases, cells were incubated with the stimuli and cytochrome c as described above, and at the end of incubation, the supernatant was removed for the measurement of O2\(^-\) release. The plate was then washed three times with PBS at room temperature and the adherent cells lysed with 150 \( \mu \)L of 0.1% Triton X-100. Fifty-microliter aliquots were then taken for the measurement of EPO as described below. The EPO in adhered cells as a percentage of the original cell content represented the percentage cell adherence (Sedgwick et al., 1992).

**EPO release.** Prewarmed eosinophils were used at a concentration of 10\(^6\) cells/ml. Fifty microliters of prewarmed cell suspension containing 5 \( \times 10^6 \) cells was dispensed into each well of a microplate. Then, 100 \( \mu \)L of the reaction buffer containing 5 \( \mu \)g/ml CB was added, and after 10 min preincubation, the cells were stimulated with 50 \( \mu \)L of human recombinant C5a or FMLP. The mixture was further incubated for 1 h at 37°C. As determined in pilot experiments, this time
was sufficient for the virtual completion of the degranulation process. At the end of the incubation period, reaction was stopped by cooling on ice, and after centrifugation at 900 x g, 50-μl aliquots of the supernatant as well as Triton X-100-lysed cells (for total content determination) were taken for the determination of the released enzymes. EPO activity was measured by the OPD method as reported previously (Kroegel et al., 1989). OPD substrate solution containing 0.4 mg/ml OPD and 0.4 mg/ml urea hydrogen peroxide in PBS-citrate buffer (pH 4.5) was prepared from SIGMA FAST OPD tablets (Sigma, St. Louis, MO). One hundred microliters of this substrate was added to 50 μl of the samples in a microplate and incubated for 30 min at 37°C. In experiments in which the effect of drugs was assessed, 50 μl of the drugs (4 times the required concentration in reaction buffer) was added in place of the buffer and incubated with the cells for 5 min before the addition of the stimulus. In some experiments in which the reversal of the effect of the drugs was attempted, the reversing agent was added as 5 μl of a 40 times the required concentration. Reaction was then stopped with 50 μl of 4 M H2SO4 and the reaction plate was read at 490 nm. Values were expressed as percentage of total content, with the amount obtained in half the same number of cells, after lysis, as 50%.

Chemicals and biochemical reagents. The following reagents and materials were purchased from Sigma Chemical Co., St. Louis, MO: PAF, recombinant human C5a, recombinant human IL-5, FMLP, Percoll, ferricytochrome c (from horse heart), Tris buffer, HEPES, SOD (from bovine erythrocytes), BSA, salbutamol, OPD, DMSO and cytochalasin B. Salmeterol was a generous gift of Glaxo Research and Development Ltd, Greenford, UK and ICI 118,551 was purchased from Tocris Cookson Ltd, Bristol, UK. Mouse monoclonal anti-human CD16 antibody (clone FcR gran1) was a generous gift of Dr. M. de Haas, CLB, Plesmanlaan, Amsterdam, whereas the magnetic beads (coated with sheep anti-mouse IgG) were supplied by Dynal AS, Oslo, Norway. All the inorganic salts were obtained from British Drug House (BDH, Poole, UK) and were of analytical grade.

Stock PAF solution (4 x 10^-2 M) was made in DMSO + ethanol (1:1, v/v) and diluted directly in buffer. Salmeterol was first dissolved in one drop of glacial acetic acid and then diluted down in buffer. The pH of the reaction mixture containing 10^-6 M salmeterol was 7.3, which was the same as the reaction buffer. The final concentration of all organic solvents present at the highest drug concentrations did not exceed 0.025%, concentrations that had no effect on eosinophil responses. All the other drugs and reagents were first dissolved in distilled water and diluted down in reaction buffer.

Statistical analysis. Experimental data are presented as mean ± standard deviation from the number (n) of independent experiments. The concentrations producing 50% inhibition of response (IC50 values) were calculated with use of the concentration-effect curves by nonlinear regression analysis by GraphPad InPlot (GraphPad Software Inc., Philadelphia, PA). Statistical significance (P) was determined by the paired t test and analysis of variance (ANOVA) as appropriate (InStat, GraphPad, Software Inc., San Diego, CA).

Results

Effect on O2^- release. As shown in figure 1, a and b, PAF and IL-5 produced a concentration-dependent generation of O2^- from purified eosinophils. Release generally occurred in the concentration range 10^-10 to 10^-6 M for PAF and 0.01 to 100 ng/ml for IL-5, and maximal releases (at the highest concentrations tested) were 28.7 ± 5.2 and 36.6 ± 6.6 nmol reduced ferricytochrome c/10^6 cells/h, respectively. The concentrations of the stimuli that gave comparable effects (10^-6 M for PAF and 30 ng/ml for IL-5) were chosen for the study of the inhibitory effects of the drugs.

Salbutamol and salmeterol showed clear differential effects on O2^- release induced by both PAF and IL-5 (fig. 2, a and b). Salmeterol strongly inhibited IL-5-induced O2^- release in a concentration-dependent manner with an IC50 of 2.2 x 10^-6 M (95% CI, 1.6-2.7 x 10^-6 M) and a maximal inhibition of approximately 70%. In contrast, salbutamol had no significant inhibitory effect even at 10^-5 M (fig. 2a). One PAF-induced O2^- generation (fig. 2b), both drugs produced significant inhibition, but salmeterol was approximately 2.2 times more potent than salbutamol [IC50 values of 3.2 x 10^-7 M (95% CI, 2.1-4.3 x 10^-7 M) and 6.3 x 10^-6 M (95% CI, 4.7-8.1 x 10^-6 M)], respectively. At the highest concentration used (10^-5 M), salmeterol also achieved a higher maximal inhibition (~75%) than salbutamol (~54%). Higher concentrations were not tested because pilot studies showed that such concentrations of salbutamol exhibited significant oxygen radical scavenging effect, whereas for salmeterol some nonspecific membrane effect was suspected.

To determine whether the effects of these drugs were mediated via beta-2 adrenoceptors, the ability of the potent and selective beta-2 adrenoceptor antagonist ICI 118551 was studied. Results in figure 3a show that the inhibitory effects of 10^-6 M salmeterol on IL-5- and PAF-induced O2^- releases were unaffected by pretreatment of the cells with ICI 118551 (10^-7 M). In contrast, the drug completely reversed the inhibitory effect of salbutamol (10^-6 M) on PAF-induced O2^- release (fig. 3b).

Effect on adherence. In the same concentration ranges at which IL-5 and PAF stimulated O2^- release, they also stimulated cell adherence to fibronectin-coated plastic surface. Maximal adherence was in the range 30 to 38% for IL-5 and 16 to 24% for PAF (fig. 4, a and b). As shown in figure 5a,
both salmeterol and salbutamol only weakly inhibited IL-5-induced adherence, but at the highest concentration used (10^{-3} M), the maximal inhibition by salmeterol (49.8±7.6%), but not salbutamol (24.6±4.4%) was statistically significant, (P<.05). PAF-induced adherence was potently inhibited with an IC_{50} of 5.5±10^{-3} M (95% CI, 3.1–7.9×10^{-3} M) and maximal inhibition of approximately 85%. Again salbutamol failed to significantly affect this response (fig. 5b). These inhibitory effects of salmeterol were not significantly reversed by ICI 118551, as shown in figure 6.

Effect on EPO release. As shown in figure 7, in the presence of 5 μg/ml of CB, both C5a and FMLP induced substantial release of EPO from purified eosinophils in a concentration-dependent manner. C5a was more than 2 orders of magnitude more potent than FMLP and also induced higher maximal release. At 10^{-3} M, for example, C5a induced a mean release of 34.5±4.2% total EPO content compared with 21.9±4.7% for FMLP. No significant EPO release occurred in the absence of CB.

With use of roughly equipotent concentrations of FMLP and C5a (10^{-6} M and 10^{-8} M, respectively), which gave EPO releases in the range 16 to 30%, the inhibitory actions of the two drugs were studied. As shown in figure 8a, concentrations of both drugs up to 10^{-5} M failed to significantly block the C5a-induced EPO release. When EPO release was induced by FMLP (fig. 8b), the release was significantly inhibited by salbutamol with a mean IC_{50} value of 7.1×10^{-7} M (95% CI, 4.4–9.8×10^{-7}) and maximal inhibition of 59.3±6.4% at the concentration of 10^{-5} M. In contrast salmeterol, up to a concentration of 10^{-5} M, had no significant effect.

In five independent experiments, the pretreatment of the cells with ICI 118551 significantly blocked the inhibitory effect of salbutamol on FMLP-induced EPO release (data not shown).

Discussion

The clinical superiority of salmeterol and the other long-acting beta-2 adrenoceptor agonists over conventional short-acting ones, such as salbutamol, has been attributed not only to the longer-lasting bronchodilation that they provide, but also to their possession of some anti-inflammatory actions (Sanjar et al., 1991; Twentyman et al., 1990; Di Lorenzo et al., 1995). In this study, we attempted to determine whether the difference in the anti-inflammatory actions of salmeterol and salbutamol is a reflection of their differential ability to suppress the responses of activated eosinophils. Thus, we have compared the ability of the two drugs to inhibit IL-5- and PAF-induced O_{2}^{-} release and adherence to fibronectin-coated microplates, as well as C5a- and FMLP-induced release of EPO from blood eosinophils in vitro. We also sought to clarify if any such effects were mediated via the beta-2 adrenoceptors presumably present on these cells.

Our results show a major difference in the actions of these two drugs. For example, salmeterol significantly inhibited
both $O_2^-$ release and adherence induced by IL-5, whereas salbutamol had no significant effect on both responses. Salmeterol was also a potent inhibitor PAF-induced $O_2^-$ release and adherence, whereas salbutamol significantly inhibited PAF-induced $O_2^-$ release but not adherence. Thus the action of salbutamol may not only be stimulus-dependent but may also be response-dependent. Further evidence of stimulus-related differential effect of these drugs was provided by the results of degranulation experiments. Salbutamol, but not salmeterol inhibited FMLP-induced EPO release, whereas both drugs failed to inhibit the release of the same product when induced by C5a.

These actions of salmeterol appear to be exerted independent of beta-2 adrenoceptors because they were not significantly reversed by the potent and selective beta-2 adrenoceptor antagonist ICI 118551. In contrast, the only two responses inhibited by salbutamol (PAF-induced $O_2^-$ release and FMLP-induced EPO release) were both reversed by ICI 118551, which suggests that these effects were mediated via beta-2 adrenoceptors. The striking ability of salbutamol to inhibit $O_2^-$ release induced by PAF, but not that induced by IL-5, is intriguing, but may reflect differences in the signal transduction pathways used by the two stimuli. PAF-induced activation of human eosinophil respiratory burst is believed to depend more on PKC activation (Bach et al., 1992; Shute, 1993) than activation induced by IL-5, which is more dependent on PKA (Koenderman et al., 1992). Thus, the inhibition of PAF-induced $O_2^-$ release by salbutamol is likely to be a consequence of beta-2 adrenoceptor-mediated cAMP-dependent activation of PKA, which is believed to decouple PAF receptors from PLC activation (Kita et al., 1991). Furthermore, in human eosinophils, IL-5, unlike PAF, does not induce a significant Ca$^{++}$ mobilization (van der Bruggen et al., 1993), and has a much slower time course of $O_2^-$ generation compared with PAF (Zeck-Kapp et al., 1995). It is, therefore, not unlikely that these differences may contribute to the differential effects of the two drugs. Salbutamol, which potently inhibited PAF-induced $O_2^-$ release, may have failed to affect the adherence induced by the same agent because shape change in eosinophils (prelude to adherence), unlike $O_2^-$ release, is independent of both PKC activation and increase in intracellular Ca$^{++}$ (Kernen et al., 1991).

Rabe and his co-workers (1993) reported previously that salbutamol and eformoterol, but not salmeterol, inhibited LTB$_4$-induced $H_2O_2$ generation from guinea pig eosinophils.
and that such effect was propranolol-sensitive. The lack of effect of salmeterol in that study may reflect species differences because we have also found that salmeterol is a relatively weak inhibitor of LTB4-induced O2− release in eosinophils from this species (submitted for publication). Incidentally, there seem to be no reports in the literature on the action of these drugs on the release of oxygen radicals from human eosinophil in response to IL-5 or PAF. There are also no reports, to our knowledge, on the comparative in vitro effect of these drugs on eosinophil adherence induced by endogenous mediators such as IL-5 and PAF. However, our results from adherence studies are consistent with other reports that show that salmeterol, but not the short-acting salbutamol, inhibited PAF-induced eosinophil accumulation in guinea pig lungs (Whelan and Johnson, 1992; Whelan et al., 1993).

The mechanism whereby salmeterol inhibits human eosinophil responses remains a matter of speculation. It is certainly not mediated via beta-2 adrenoceptors because none of the observed effects of this drug were reversed by ICI 118551. Such beta-2 adrenoceptor-independent actions of salmeterol on pro-inflammatory cells have been observed by other workers (Johnson, 1990; Baker and Fuller, 1990). It is possible, however, that the reported extrareceptor mechanism of action of this drug, which probably arises from its lipophilic properties (Rhodes et al., 1992), may be important.

The reason for the inability of both drugs to significantly inhibit EPO release induced by C5a + CB is presently unknown, but may reflect the rather atypical nature of the degranulation of eosinophils by this stimulus. Although, after 1 h of incubation of eosinophils with 10−8 M C5a in the presence of CB, the cell viability remained similar to that of FMLP-treated cells (around 95%), we noticed that after further 2-h incubation a more rapid drop in viability to about 75% (90% for FMLP + CB) was seen with the former. This suggests that unlike the degranulation induced by FMLP + CB (Munoz et al., 1993), C5a + CB-induced eosinophil degranulation may slowly compromise the integrity of the cell. Indeed, Zeck-Kapp and colleagues (1995) have shown that human eosinophils exposed to C5a + CB tended to develop microchannels in their membrane to which the secretory vesicles were associated.

Our results on the ability of salbutamol, but not salmeterol, to inhibit FMLP-induced EPO release are essentially similar to those recently reported by Munoz and his co-workers (1995). This is difficult to explain, however, because the other long-acting beta-2 adrenoceptor agonists, formoterol and eformeterol, have been credited with the inhibition of eosinophil functions in vitro (Anderson, 1991; Rabe et al., 1993). Salmeterol itself has, in fact, been reported to inhibit antigen-induced degranulation of mast cells present in the human lung fragments in vitro (Butchers et al., 1991).

Data from several clinical studies have provided evidence that the anti-inflammatory actions of salmeterol may involve the inhibition of eosinophil degranulation, because the increases in the serum levels of ECP and eosinophil protein-X after antigen challenge of asthmatic patients were significantly reduced by pretreatment with salmeterol, but not salbutamol (Pedersen et al., 1993; Di Lorenzo et al., 1995). Because salmeterol exhibited significant inhibitory effect against adherence induced by PAF and IL-5, two pro-inflammatory mediators that are crucial in the pathophysiology of asthma, it is possible that the reported in vivo anti-inflammatory action of salmeterol may well depend on the prevention of eosinophil extravasation and infiltration into the bronchial tissues rather than inhibition of eosinophil degranulation. In such a scenario, the reduction of the bronchial eosinophil infiltration would reduce the number of eosinophils available for antigen and immune complex-mediated degranulation in the lung tissues, thus explaining the reduction in eosinophil granule products in the serum as has been demonstrated clinically. On the other hand, the failure of salmeterol to inhibit C5a- and FMLP-induced EPO release does not necessarily rule out the possible inhibition of anti-

**Fig. 7.** Release of EPO from human eosinophils induced by human recombinant C5a and FMLP, both in the presence of 5 μg/ml CB. Spontaneous releases (0–5.5%) have been subtracted from all values. Values are means ± S.D. of five experiments each. The corresponding dashed lines represent agonist-induced releases in the absence of CB.

**Fig. 8.** The effect of salmeterol (SM) and salbutamol (SB) on the release of EPO from human eosinophils induced by 10−8 M C5a (a) or 10−6 M FMLP (b), both in the presence of 5 μg/ml CB. The uninhibited releases (100%) were in the range 16 to 33% for both stimuli. Cells were preincubated with SM or SB for 5 min before stimulation. Values are means ± S.D. of six independent experiments. *P < .05; **P < .01.
gen-induced eosinophil degranulation in vivo because of the obvious stimulus-dependent nature of drug effect on eosinophils. Our attempt to verify this was hampered by the inconsistent nature of antigen-induced degranulation of human eosinophils in vitro. In fact salbutamol, which in this and other studies [Munoz et al., 1993] have been shown to clearly inhibit FMLP-induced EPO release from eosinophils, lacked such effect on sIgA- and IgG-induced degranulation (Kita et al., 1991).

In summary, our results confirm that human eosinophils can be directly modulated by beta-2 adrenoceptor agonists, but that salbutamol and salbutamol have differential effects on these cells which depend on the stimulus used and the response being measured. Furthermore, although salbutamol produced its effects via the beta-2 adrenoceptors, salmeterol acted completely independent of these receptors. The results thus suggest that the reportedly greater in vivo anti-inflammatory effect of salmeterol than salbutamol may derive more from salmeterol’s differential or superior inhibition of IL-5 and PAF-induced O$_2^-$ release and adherence (prelude to bronchial eosinophil infiltration) than from its inhibition of eosinophil degranulation.

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References


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