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Additive blockade of β_2-integrin adhesion of eosinophils by salmeterol and fluticasone propionate

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ABSTRACT: Migration of human eosinophils is regulated by integrin expression, conformational change, and activation of cytosolic phospholipase A_2 ($cPLA_2$). Corticosteroids have been shown to inhibit $cPLA_2$ hydrolysis in human eosinophils. The objective of this study was to determine the mechanisms of fluticasone propionate (FP) alone or in combination with salmeterol (SM) in blocking adhesion mediated by β_2-integrin in human eosinophils.

Human eosinophils were isolated by negative magnetic selection. β_2-integrin-mediated eosinophil adhesion was measured by residual eosinophil peroxidase activity. Eosinophils were pretreated for 12 h to 24 h with FP and with or without SM for 30 min.

Both SM alone and FP alone inhibited eosinophil adhesion in concentration- and time-dependent manner. SM alone modestly (~30%) inhibited interleukin (IL)-5-induced eosinophil adhesion. Blockade of IL-5-induced eosinophil adhesion caused by 10^{-7} M FP at 24 h was augmented by 10^{-7} M SM from 41.5% to 72.5%. Similar blockade was also observed for eotaxin-induced eosinophil adhesion. Neither SM, FP, nor FP+SM blocked either: 1) upregulation of CD11b surface expression; or 2) phosphorylation of $cPLA_2$.

Blockade of β_2-integrin-mediated eosinophil adhesion by fluticasone propionate is augmented by salmeterol. Decreased adhesion results from augmented blockade of nuclear translocation of cytosolic phospholipase A_2 caused by addition of salmeterol to fluticasone.
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Eosinophilic infiltration of tissues from the blood vessels involves multiple steps including directed chemotaxis, adhesion, and diapedesis [1]. Eosinophil adhesion by the β_2-integrin, CD11b/CD18 (Mac-1), to immunoglobulin supergene, intercellular adhesion molecule I (ICAM-1), is an essential early step for cell migration in allergic inflammation as occurs in asthma [2–4]. Blockade of the CD11b subunits inhibits human eosinophil adhesion [5–7] *in vitro* and causes the inhibition of eosinophil infiltration into airways *in vivo* [8–10].

Recent investigation has suggested that salmeterol (SM), a long-acting β_2-selective adrenoceptor agonist might also have an independent inhibitory effect on the inflammatory response in asthma [11]. SM has been reported to reduce adherence of eosinophils in mucosal blood vessels in rat airways [12] and eosinophil adhesion to fibronectin induced by both interleukin (IL)-5 and platelet activating factor *in vitro* [13, 14]. Other β_2 adrenoceptor agonists (fenoterol, salbutamol and procaterol) have been reported to inhibit CD11b upregulation in eosinophils caused by platelet activating factor [15], but another long-acting β_2-adrenergic receptor agonist, formoterol, did not inhibit the surface upregulation of CD11b [16].

For editorial comments see page 501.

Inhaled glucocorticoids also have been reported to diminish eosinophil infiltration in asthma; however, the mechanism by which glucocorticoids attenuate eosinophil adhesion is not established [17–19]. The current authors have reported previously that cytosolic phospholipase A_2 ($cPLA_2$) activation is essential for β_1- and β_2-integrin-dependent adhesion of eosinophils [20, 21]. Blockade of $cPLA_2$ *in vivo* also blocked airway infiltration of eosinophils and attenuated airway responsiveness to methacholine [22]. An essential regulatory step in eosinophil adhesion is phosphorylation of extracellular signal regulated kinase (ERK) 1/2. ERK 1/2 then phosphorylates $cPLA_2$ at the serine^{505} position. Phosphorylation at this site converts the enzyme into its active hydrolytic form. Hydrolysis of membrane lipid into lysophospholipids that mediate eosinophil adhesion further requires the translocation of $cPLA_2$ to the nuclear membrane [23].

This investigation was undertaken to determine the potential role of β_2-adrenoceptor stimulation and treatment with corticosteroid in blocking β_2-integrin-mediated eosinophil adhesion caused by IL-5 and eotaxin-1 in human eosinophils *in vitro*. Studies were conducted to determine the mechanism by which eosinophil adhesion caused by either IL-5 or eotaxin is blocked individually and additively by SM

and fluticasone propionate (FP). The current authors' data demonstrate that SM and FP, even in combination, do not block IL-5- or eotaxin-mediated upregulation of Mac-1 and do not block $cPLA_2$ phosphorylation. Instead it was found that β_2-adrenoceptor stimulation after prior incubation with the corticosteroid, FP, blocks the translocation of activated $cPLA_2$ to the perinuclear membrane, thereby blocking integrin-mediated adhesion caused by IL-5 and eotaxin.

Materials and Methods

Isolation of human eosinophils

Eosinophils were isolated from mildly atopic human volunteers according to a protocol approved by the University of Chicago Institutional Review Board. Informed consent was obtained from all volunteers in this study before participation. Atopy was defined as the presence of a positive radioallergosorbent test (RAST) score for at least two of three allergen extracts (*Dermatophagoides farinae*, timothy grass, or giant ragweed) and through a questionnaire utilised in the National Heart, Lung and Blood Institute Asthma Genetics Project. The study included a total of 16 individuals aged 20–45 yrs, seven males and nine females. For this study, only the NIH questionnaire was used, as the objective was to obtain eosinophils from donors not having asthma or taking asthma or allergy drugs. None of the subjects had received any medication for at least 1 month before the study. This is in compliance with the University of Chicago and US government guideline Health Insurance Portability and Accountability Act (HIPPA) for studies in which donors are not participating subjects.

Human peripheral blood eosinophils were isolated by a method modified from HANSEL *et al.* [24]. The method is based on Percoll centrifugation (density 1.089 $g \cdot mL^{-1}$) to isolate granulocytes, hypotonic lysis of red blood cells, and, finally, immunomagnetic depletion of neutrophils by the magnetic cell separation system using anti-CD16-coated MACS particles (Miltenyi Biotec, Sunnyvale, CA, USA). Eosinophil purity of 98% was routinely obtained, as assessed by Wright-Giemsa staining.

Treatment with fluticasone propionate and salmeterol

Eosinophils were resuspended in Roswell Park Memorial Institute (RPMI) buffer (10% foetal bovine serum, 250 $U \cdot mL^{-1}$ of penicillin and 250 $\mu g \cdot mL^{-1}$ streptomycin, 10 $pg \cdot mL^{-1}$ IL-5). For the time-dependent effect of SM on eosinophil adhesion, aliquots of 10^4 eosinophils were pre-incubated with buffer or 10^{-6} M SM for 5–60 min before adhesion assay. For the concentration-dependent effect of SM on eosinophil adhesion, eosinophils were pre-incubated with 10^{-10}–10^{-6} M SM for 30 min. For the combinatorial effect of FP and SM, aliquots of eosinophils were incubated at 37°C with buffer or 10^{-10}–10^{-6} M FP for 12–24 h, and then were incubated for an additional 30 min with buffer or SM at 10^{-10} and 10^{-7} M. Duplicated samples were prepared for adhesion assay. Recombinant human IL-5 (10 $pg \cdot mL^{-1}$) was added to maintain eosinophil viability during the experiment period, but had no effect on cellular adhesion [20, 25].

Eosinophil adhesion assay

Eosinophil adhesion was assessed as residual eosinophil peroxidase (EPO) activity of adherent cells [20, 25]. Briefly, 96-well microplates were coated with 100 µl of bovine serum albumin (BSA, 10 $\mu g \cdot mL^{-1}$) dissolved in Hank's balanced salt solution (HBSS) and incubated at 4°C overnight and blocked with 200 µL/well of heat-inactivated foetal bovine serum for 60 min at 37°C. Eosinophils (1×10^4/100 µl) were pre-incubated with different concentrations of FP or SM (GlaxoSmithKline, Uxbridge, UK) for indicated times at 37°C. Cells then were added to BSA-coated wells with or without stimulators and allowed to settle for 10 min on ice. Plates were rapidly warmed to 37° C and incubated for 30 min. After 3×washing with HBSS, 100 µl of RPMI buffer was added to the reaction wells, and serial dilutions of original cell suspensions were added to the empty wells to generate a standard curve. 100 µl EPO substrate (1mM hydrogen peroxide, 1 mM *O*-phenylenediamine, and 0.1% triton X-100 in Tris buffer, pH 8.0) then was added to the wells. After 30 min incubation at room temperature, 50 µl of 4 M H_2SO_4 was added to stop the reaction. Absorbance was measured at 490 nM in a microplate reader (Thermomax; Molecular Devices, Menlo Park, CA). All assays were performed in duplicate. Stimulated eosinophil adhesion to plated BSA was β_2-integrin dependent, as anti-CD11b or anti-CD18 blocking antibody suppressed this adhesion substantially [25].

Immunofluorescence microscopy

After stimulation of cells, 5×10^5 cells were prepared from cytospin in a Shandon Cytospin Model 3 (Shandon, Pittsburgh, PA, USA) at 200×*g* for 2 min. Slides were then foil-wrapped and stored at 4°C until further use. For analysis, slides of eosinophils were fixed for 20 min in 2% paraformaldehyde in PBS and treated with 0.4% *p*-benzoquinone (Sigma, St Louis, MO, USA) in PBS for 10 min. Slides then were permeabilised using 0.1% saponin in PBS for 15 min. Following permeabilisation, slides were blocked using 2% human immunoglobulin (Ig)G (Reagent Grade I-4506; Sigma) for 60 min at room temperature. After a washing step, slides were incubated with monoclonal antibody (mAb) directed against $cPLA_2$ (Santa Cruz Biotech, Santa Cruz, CA, USA) for 60 min at room temperature. Binding of mAb to $cPLA_2$ was detected by incubating slides with 20 $\mu g \cdot mL^{-1}$ BODIPY FL-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR, USA) for 60 min at room temperature. After the final wash, 10 µL of anti-bleaching agent (0.4% n-propyl gallate (Sigma)) in 3:1 glycerol:TBS was used before coverslip application. Negative controls were carried out by replacing the primary antibody with PBS or mouse isotype control. Slides were viewed by fluorescence microscopy on a Zeiss Axoplan microscope (magnification ×1000; Carl Zeiss, Thornwood, NY, USA).

Western blot analysis of cytosolic phospholipase A_2 expression and phosphorylation

Eosinophils ($2 \times 10^6 \cdot group^{-1}$) were pre-incubated in the presence or absence of FP for 24 h and SM for 30 min. Then cells were stimulated with either 10 $ng \cdot mL^{-1}$ IL-5 or 100 $ng \cdot mL^{-1}$ eotaxin (R&D system, Minneapolis, MN, USA) for 30 min, and the reaction was stopped by brief centrifugation. The pellets were lysed in 80 µl of boiling buffer (50 mM Tris-HCl, 2 mM EDTA, 2 mM EGTA, 2 mM Na_3VO_4, 50 mM NaF, 2.5 mM DFP, 1% SDS, proteinase inhibitor cocktail), sonicated, and boiled for 5 min. Afterward, 14 µl of 6×loading buffer was added, and the mixture was boiled for 5 min and then saved at -80°C. Samples were subjected to SDS-PAGE, using 7.5% acrylamide gels under

reducing condition (15 mA·gel^{-1}). Electrotransfer of proteins from the gels to polyvinylidene fluoride membrane was achieved using a semi-dry system (400 mA, 60 min). The membrane was blocked with 2% BSA for 60 min, then incubated with 1:1000 anti-phosphorylated $cPLA_2$ (Cell Signaling Technology, Beverly, MA, USA) or 1:400 anti-$cPLA_2$ antibody diluted in Tris-buffered saline plus Tween-20 (TBS-T) for 60 min. The membranes then were washed three times for 20 min with TBS-T. Goat anti-rabbit Ig conjugated with horseradish peroxidase was diluted 1:3000 in TBS-T and incubated with polyvinylidene fluoride membrane for 60 min. The membranes were again washed three times with TBS-T and assayed by an enhanced chemiluminescence system (Amersham, Arlington Hts, IL, USA).

Analysis of surface CD11b expression by immunofluorescence flow cytometry

Eosinophils were pre-incubated with 10^{-7} M FP for 24 h and 10^{-7} M SM for 30 min, then stimulated with or without 10 ng·mL^{-1} IL-5 or 100 ng·mL^{-1} eotaxin, for 30 min. Thereafter, eosinophils were centrifuged at 400×*g* for 10 min, and the pellets were resuspended in PBS/2% BSA. Aliquots of 5×10^5 eosinophils were incubated with 10 µL of mAb CD11b (Bear 1) or isotype-matched control antibody for 30 min at 4°C. After 2 washes, the cells were incubated with an excess of fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin for 30 min at 4°C. The cells were washed twice, resuspended in 1% paraformaldehyde and kept at 4°C until analysed. Flow cytometry was performed on a FACScan (Becton Dickinson, Mountain View, CA, USA). Fluorescence intensity was determined on at least 5,000 cells from each sample.

Determination of eosinophil viability after incubation with fluticasone propionate

To determine if FP affected eosinophil viability, trypan blue exclusion was assessed in eosinophils incubated with various concentration of FP. Aliquots of 10^4 eosinophils were incubated for 12–24 h at 37°C with 10^{-10}–10^{-6} M FP in the presence of 10 pg·mL^{-1} of IL-5. Eosinophils then were centrifuged at 400×*g* and pellets were resuspended in 10 µl of HBSS. An equal volume of 0.01% trypan blue was added, and viable eosinophils were counted in a haemacytometer.

Statistical analysis

All values are expressed as the mean±SEM. Differences between groups was assessed by paired t-test. Where more than two groups were compared, differences among groups were assessed by one-way analysis of variance (ANOVA). Where differences were found, comparisons among groups were made by Fisher's least-protected difference test. Eosinophil adhesion was normalised as a per cent of the response in stimulated control in the same subjects using the following equation: adhesion (%)=100×(test sample data–negative control data)/(positive control data–negative control data). The combinatorial effect shown by SM and FP were designated as: synergistic when inhibition with two drugs combined > inhibition by FP alone + inhibition by SM alone. Additive inhibition was defined when the combined effect of FP and SM together > inhibition by either FP alone or SM alone.

Results

Effect of salmeterol on stimulated eosinophil adhesion

Eosinophil adhesion to plated BSA was elicited by either 10 ng·mL^{-1} of IL-5 or 100 ng·mL^{-1} of eotaxin, the optimal concentrations established previously [20, 26]. Nonstimulated eosinophil adhesion to plated BSA was 2.1±0.3% (mean±SEM). IL-5 caused 23.8±2.2% eosinophil adhesion to plated BSA, while eotaxin caused 17.5±3.3% adhesion ($p<0.01$ *versus* control, both comparisons). SM (at supramaximal concentration 10^{-6} M) inhibited both IL-5- and eotaxin-induced eosinophil adhesion to plated BSA in a time-dependent manner; maximum inhibition was obtained by 30–60 min (fig. 1a). Accordingly a 30 min incubation time for SM was chosen for subsequent experiments. Concentration-responses curves were also generated after pre-incubation with SM (10^{-10} M–10^{-6} M) for 30 min. Statistically significant blockade of eosinophil adhesion was achieved with SM alone only at concentrations $\geqslant10^{-7}$ M. IL-5-induced eosinophil adhesion was ~29% blocked by 10^{-7} M SM to 71.1±10.3% ($p<0.05$) of stimulated control

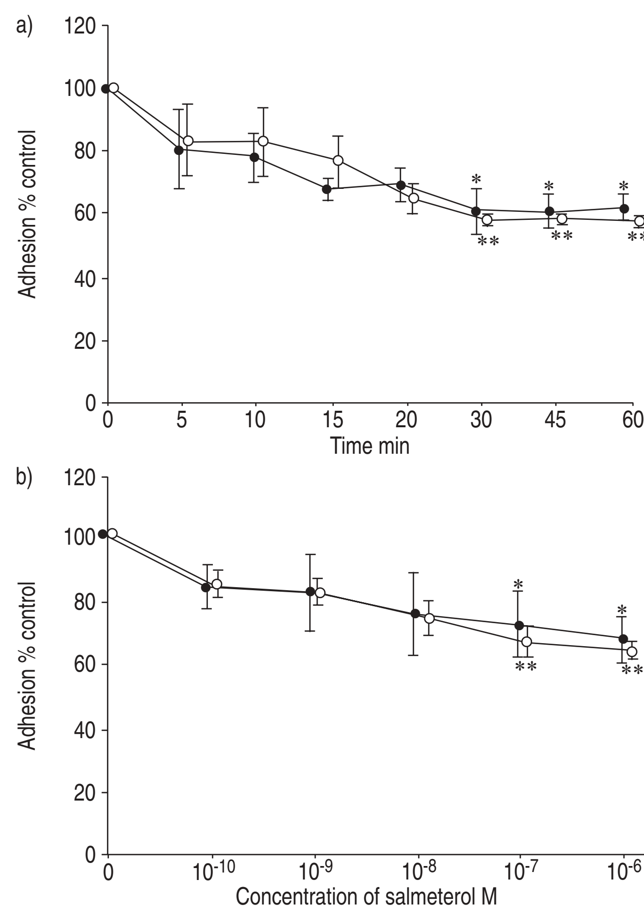

Fig. 1. – a) Time-dependent inhibition of salmeterol (SM) on stimulated adhesion of eosinophils to plated bovine serum albumin (BSA), a surrogate for intercellular adhesion molecule I (ICAM-1) [25]. Eosinophils were pre-incubated with 1 µM SM, a supramaximal concentration, to develop a time-course for maximal activity. At indicated times, cells were activated with either interleukin (IL)-5 (●) or eotaxin (○). b) Concentration-dependent inhibition of SM on stimulated adhesion of eosinophils to plated BSA. Eosinophils were pre-incubated with SM for 30 min, then stimulated by either IL-5 (●) or eotaxin (○). Eosinophil adhesion was measured by residual eosinophil peroxidase activity (n=5). Results are presented as the mean±SEM. *: $p<0.05$ and **: $p<0.01$ compared with positive control.

and to 67.5±4.6% ($p<0.01$) of stimulated control in cells activated by eotaxin (fig. 1b).

Effect of combination of salmeterol and fluticasone propionate on stimulated eosinophil adhesion

Eosinophils were pretreated with FP (10^{-10} M–10^{-6} M) for 12 h or 24 h and then with 10^{-10} M or 10^{-7} M SM for 30 min. Blockade of eosinophil adhesion caused by IL-5 with FP alone was augmented by SM as early as 12 h and increased substantially at 24 h (fig. 2a, b). The combinatorial inhibition of eosinophil adhesion caused by IL-5 for eosinophils treated with SM (10^{-10} M or 10^{-7} M) + FP (10^{-10}–10^{-6} M for 12 h) was additive (fig. 2a). After 24 h pretreatment with FP, SM and FP also produced additive inhibition. However, inhibition was synergistic for 10^{-10}–10^{-8} M FP+10^{-7} M SM (fig. 2b).

Nearly identical results were obtained for blockade of adhesion caused by eotaxin (fig. 2c, d). Progessive augmentation of inhibition of adhesion was elicited over full concentration range of FP with 10^{-10} SM and substantially augmented for the same concentrations of FP by co-treatment with 10^{-7} M SM (fig. 2c, d). As for IL-5, the effects observed at 12 h were substantially amplified at each concentration of FP after 24 h incubation (fig. 2c, d). In all circumstances, the blockade of adhesion by co-treatment with FP+SM was either synergistic (10^{-10}–10^{-9} M FP at 24 h with 10^{-7} M SM) or additive (all other combinations).

At the end of each experiment, the effect of incubation with FP on cell viability was assessed. FP had no effect on eosinophil viability at 12 or 24 h as measured by trypan blue exclusion or propidium iodide. Viability at the beginning and end of each experiment was ⩾98%. Accordingly, blockade of adhesion by FP was not related to diminished viability of the cells during treatment.

Effects of fluticasone propionate and salmeterol on CD11b surface expression

Studies were conducted to determine if blockade of Mac-1 (CD11b/CD18) upregulation accounted for the blockade of eosinophil adhesion caused by FP, SM or the additive combined effects of FP+SM. For these studies, large concentrations of both SM (10^{-7} M) and FP (10^{-7} M) were used to examine the effect of CD11b surface expression as measured by FACScan. Neither FP nor SM alone blocked the upregulation of surface CD11b caused by IL-5 (fig. 3a) or eotaxin (fig. 3b). The combination of FP+SM even at large concentrations also did not inhibit the upregulation of CD11b surface expression caused by IL-5 or eotaxin (fig. 3).

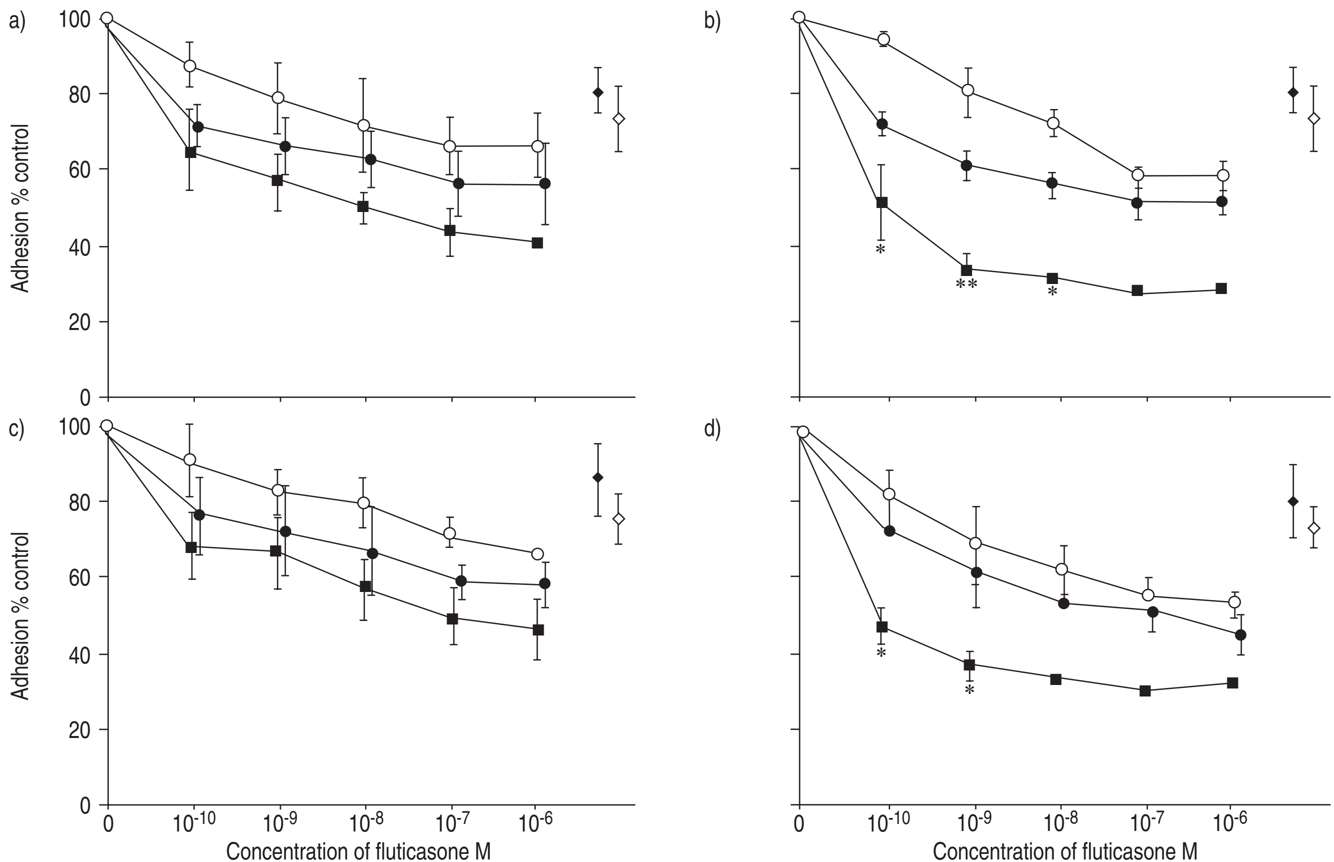

Fig. 2. – Salmeterol augmented the inhibitory effect of fluticasone propionate (FP) on interleukin (IL)-5- or eotaxin-stimulated eosinophil adhesion to plated bovine serum albumin. Eosinophils were pre-incubated with indicated concentrations of FP for 12 h (fig. 2a, c), 24 h (fig. 2b, d) and with SM for 30 min (●: 10^{-10} M SM; ■: 10^{-7} M SM; ○: no SM pre-incubation) and then stimulated by either 10 ng·mL^{-1} IL-5 (fig. 2a, b) or 100 ng·mL^{-1} eotaxin (fig. 2c, d). Eosinophil adhesion was measured by residual eosinophil peroxidase activity (n=5). IL-5 caused 23.7±4.5% and 24.8±1.2% adhesion when eosinophils were incubated without FP for 12 h and 24 h, respectively. Eotaxin caused 17.1±3.8% and 16.8±1.8% adhesion when eosinophils were incubated without FP for 12 h and 24 h, respectively. Results are presented as the mean±SEM. ◆: The effect of 10^{-10} SM alone; ◇: the effect of 10^{-7} SM alone. *: $p<0.05$ and **: $p<0.01$ compared with the sum of inhibition by each drug alone, and denotes the synergistic effect of FP+SM.

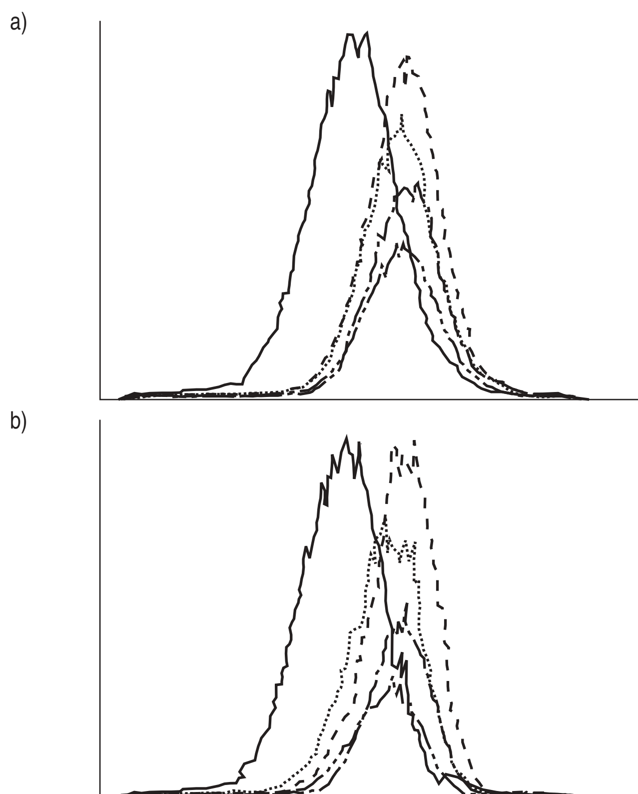

Fig. 3. – Effect of salmeterol (SM) and fluticasone propionate (FP) on CD11b expression. Eosinophils were pre-incubated with or without 10^{-7} M FP for 24 h and 10^{-7} M SM for 30 min, followed by addition of interleukin-5 (a) or eotaxin (b) and incubation for 30 min. CD11b surface expression was measured by flow cytometry as described in the Material and Methods section. Histograms show typical results of CD11b expression. Solid lines: nonstimulated controls; - - -: stimulated controls; ·········: 10^{-7} M FP-treated cells; – - – : 10^{-7} M SM-treated cells; – - - –: combination of 10^{-7} M FP and 10^{-7} M SM-treated cells.

Effects of fluticasone propionate on cytosolic phospholipase A_2 protein expression and phosphorylation

To determine the mechanism by which SM and FP caused additive/synergistic augmentation of the blockade of β_2-integrin adhesion, the effect of SM and FP on Serine[505] phosphorylation of $cPLA_2$ was examined by Western blot analysis. Incubation of eosinophils with FP (10^{-9} M) for 24 h or SM (10^{-7} M) for 30 min did not inhibit $cPLA_2$ phosphorylation or total protein expression. Combined treatment with the combination of 10^{-9} M FP + and 10^{-7} M SM, which caused >70% blockade of eosinophil adhesion (fig. 2) also did not inhibit $cPLA_2$ phosphorylation (fig. 4).

Effects of fluticasone propionate on cytosolic phospholipase A_2 translocation

To determine further the putative mechanism of FP and SM in blocking β_2-integrin adhesion, the intracellular distribution of $cPLA_2$ was examined by immunofluorescent staining (fig. 5). Negative controls displayed minimal fluorescence background (fig. 5k). $cPLA_2$ was predominantly localised to the cytoplasm in unstimulated cells (fig. 5l). After stimulation with either 2 μM A23187 (as a positive control), 10 ng·mL^{-1} IL-5 or 100 ng·mL^{-1} eotaxin for 30 min, the immunofluorescent staining for $cPLA_2$ was localised to the perinuclear region of the cells (fig. 5a, f, m). Pretreatment with 10^{-7} M FP for 24 h completely inhibited $cPLA_2$ translocation to the nucleus caused by activation with both IL-5 and eotaxin (fig. 5b, g). Pretreatment with either 10^{-7} M SM for 30 min or 10^{-9} M FP for 24 h did not substantially block $cPLA_2$ translocation (fig. 5c, d, h, i). However, the combination of 10^{-9} M FP +10^{-7} M SM inhibited completely the translocation of $cPLA_2$ to the nuclear envelope (fig. 5e, j).

Discussion

The objective of this investigation was to determine the independent effects of β_2-adrenoceptor stimulation by SM and corticosteroid treatment with FP on β_2-integrin (CD11b/CD18)-mediated eosinophil adhesion. Further studies were generated to determine if SM augmented the anti-adhesive effects of FP, and additional studies were performed to determine the mechanism by which additive or synergistic augmentation of the blockade of eosinophil adhesion caused by IL-5 or eotaxin might occur.

The current data indicate that both SM and FP inhibited IL-5- or eotaxin-induced eosinophil adhesion in a concentration- and a time-dependent manner, although neither compound was exceptionally effective alone in concentrations likely to be achieved in human airways during inhalation (GlaxoSmithKline, Uxbridge, UK). However, inhibition of IL-5- or eotaxin-induced eosinophil adhesion caused by 10^{-9} M FP was substantially enhanced by 10^{-7} M SM (fig. 2b, d), which caused a >2 log shift in the efficacy of FP at lower concentrations. The combination of SM and FP was synergistic (FP $\leqslant 10^{-9}$ M at 24 h with 10^{-7} M SM for eotaxin and FP $\leqslant 10^{-8}$ M at 24 h with 10^{-7} M SM for IL-5) or additive (with all other combinations tested) in the blockade of the β_2-integrin adhesion of human eosinophils (fig. 2).

To determine the mechanism of additive and/or synergistic augmentation of adhesion between FP and SM, studies were performed to examine the effect of these compounds individually and in combination on 1) β_2-integrin upregulation on the eosinophil surface; 2) $cPLA_2$-phosphorylation and 3) translocation of phosphorylated $cPLA_2$ to the perinuclear membrane [23]. Upregulation of β_2-integrin, $cPLA_2$ phosphorylation and translocation of $cPLA_2$ all are required for IL-5-stimulated eosinophil adhesion [20]. Blockade of any step of these processes also causes blockade of adhesion [20, 26, 27]. It was found that concentration of SM + FP that caused substantial blockade of eosinophil adhesion at 24 h had no effect either on Mac-1 upregulation (fig. 3) or $cPLA_2$ phosphorylation (fig. 4) caused by IL-5 or eotaxin. By contrast, very small concentrations of SM+FP caused substantial blockade of $cPLA_2$ translocation (fig. 5). The current authors have previously shown that FP blocks eosinophil secretion of LTC_4 by preventing translocation of $cPLA_2$ to the perinuclear membrane [28]. The mechanism by which this occurs has not been further defined; however, inhibition required 48 h incubation, suggesting that FP-induced transcription is essential for the blockade of $cPLA_2$ migration. This study found substantial augmentation of FP-induced blockade of adhesion at 24 h after treatment with SM. SM also greatly augmented the effect of FP in blocking translocation (fig 5). These data suggest that β_2-adrenoceptor stimulation, which has been shown to augment translocation of the cytosolic corticosteroid receptor to the nucleus, likely results in temporal and quantitative augmentation of transcriptional events that block integrin adhesion. The precise mechanism by which $cPLA_2$ translocation is blocked, however, still remains to be elucidated.

In the experiments, the present authors ensured that eosinophil viability was maintained during culture. ZHANG

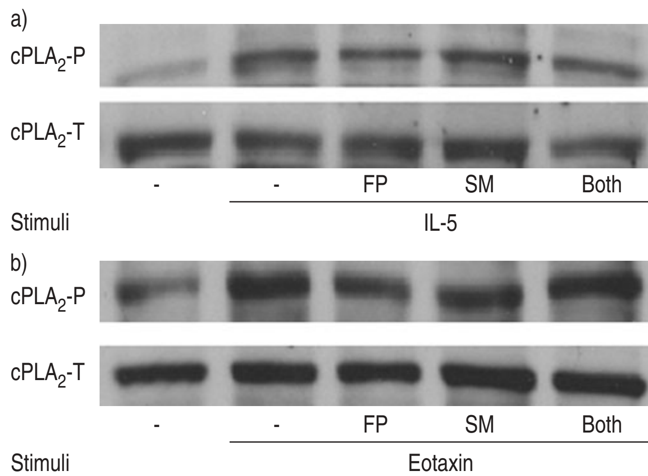

Fig. 4. – Effect of salmeterol (SM) and fluticasone propionate (FP) on cytosolic phospholipase A_2 ($cPLA_2$) expression or phosphorylation. Eosinophils were pre-incubated with 10^{-9} M FP for 24 h or 10^{-7} M SM for 30 min and then stimulated by a) interleukin (IL)-5 or b) eotaxin for 30 min. Phosphorylation of $cPLA_2$ ($cPLA_2$-P) and total protein expression ($cPLA_2$-T) was measured by Western blot using Ser^{505} phosphorylation-specific and nonspecific $cPLA_2$ antibody respectively.

et al. [29] reported that FP induces eosinophil apoptosis and addition of IL-5 attenuates the toxic effect of FP on cell viability [29, 30]. Accordingly, 10 pg·mL^{-1} IL-5 was added to the culture medium for all experiments in the current study. Viability of eosinophils after 24 h incubation with FP was >98%. However, IL-5 in concentrations used to maintain viability had no effect on eosinophil adhesion in these studies.

The current results contrast with some studies that found no effect of glucocorticoids on eosinophil adhesion to endothelial cells [18, 19]. The mechanism underlying these differences is not certain but may be related to a difference in technique for measurement of adhesion. The current study used single purified ligands in concentrations established previously [20, 25] rather than cultured endothelial cells, which express multiple counter ligands such as ICAM-1, vascular cell adhesion molecule-1 and endothelial leukocyte adhesion molecule-1 and have potential to secrete pro-inflammatory mediators. FP also has significantly greater potency than dexamethasone. Finally, in preliminary experiments for this study, the authors found little efficacy of FP in concentrations comparable to those achieved in airways after inhalation. The addition of a moderate concentration SM to FP was essential to achieve substantial blockade of inhibition, and this blockade was achieved within a relatively short time course (12–24 h) in comparison with prior studies using corticosteroids alone [28].

It is important to consider some important limitations of the current findings. While the concentrations of FP and SM used were $\leqslant$ those achieved in human airways (data provided by GlaxoSmithKline, Uxbridge, UK), these studies were nonetheless conducted *in vitro*. IL-5 was introduced to insure eosinophil survival, thus insuring that blockade of adhesion was not related to cell death. Freshly isolated human eosinophils were also used in these studies. Nonetheless, these data cannot be directly extrapolated to the human asthmatic state or to other cell types that may be involved in asthmatic airway inflammation. Accordingly, these data do not affirm or disprove a role for the eosinophil in human asthma. Rather, the current study shows, in an inflammatory cell that is highly prevalent in human asthma, that SM + FP act additively and synergistically to prevent translocation of $cPLA_2$, which is an essential enzyme in all inflammatory cell adhesion and first step synthesis of prostaglandins and leukotrienes. Accordingly, the data suggest one mechanism by which the addition of a β_2-adrenoceptor agonist may

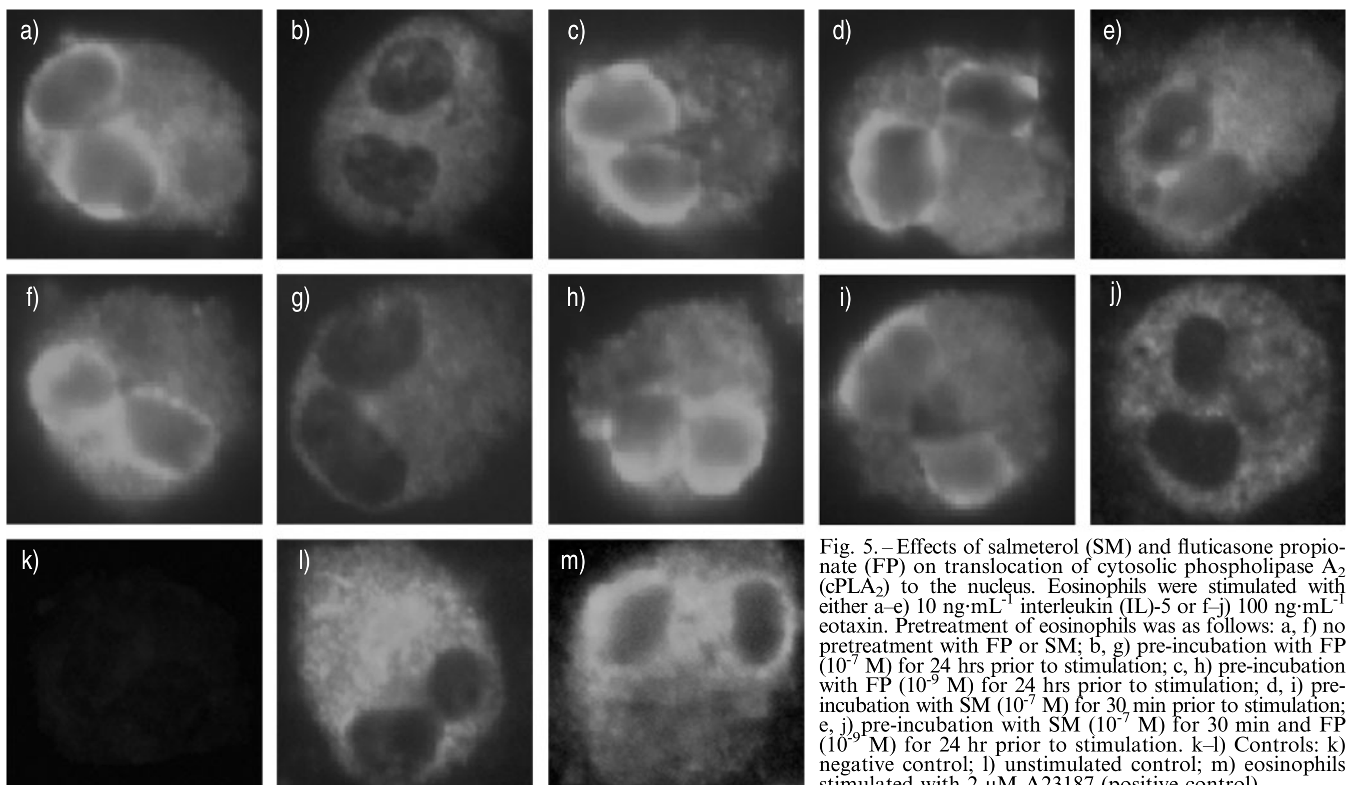

Fig. 5. – Effects of salmeterol (SM) and fluticasone propionate (FP) on translocation of cytosolic phospholipase A_2 ($cPLA_2$) to the nucleus. Eosinophils were stimulated with either a–e) 10 ng·mL^{-1} interleukin (IL)-5 or f–j) 100 ng·mL^{-1} eotaxin. Pretreatment of eosinophils was as follows: a, f) no pretreatment with FP or SM; b, g) pre-incubation with FP (10^{-7} M) for 24 hrs prior to stimulation; c, h) pre-incubation with FP (10^{-9} M) for 24 hrs prior to stimulation; d, i) pre-incubation with SM (10^{-7} M) for 30 min prior to stimulation; e, j) pre-incubation with SM (10^{-7} M) for 30 min and FP (10^{-9} M) for 24 hr prior to stimulation. k–l) Controls: k) negative control; l) unstimulated control; m) eosinophils stimulated with 2 µM A23187 (positive control).

augment at the cellular level the anti-inflammatory effects of corticosteroids.

To conclude, neither salmeterol nor fluticasone propionate alone cause substantial blockade of β_2-integrin adhesion caused by interleukin-5 or eotaxin. However, in combination, there is a significant additive effect, which, at low concentrations, is synergistic in blocking integrin adhesion. Augmented blockade of *in vitro* adhesion is caused neither by blockade of Mac-1 upregulation nor by blockade of cytosolic phospholipase A_2 phosphorylation but rather by augmentation of corticosteroid-induced inhibition of cytosolic phospholipase A_2 translocation to the perinuclear envelope.

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