

Effects of Salmeterol Xinafoate and Fluticasone Propionate on Immunological Activation of Human Cultured Mast Cells

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ABSTRACT

Background: The clinical efficacy of combination therapy comprising a long acting β_2 -agonist (LABA) and corticosteroid is widely recognized for the treatment of adult asthma. Here we examine the effect of salmeterol xinafoate (SX) and fluticasone propionate (FP) alone and in combination on the immunological activation of human cultured mast cells (HCMC) *in vitro*.

Methods: HCMC were passively sensitized with IgE antibody and then activated by challenging with anti-IgE antibody. The effect of drugs on the activation of mast cells was examined by measuring the amount of released chemical mediators (histamine, leukotrienes (LT) and prostaglandin D₂ (PGD₂)) and granulocyte macrophage colony stimulating factor (GM-CSF).

Results: The release of each chemical mediator was inhibited by 10^{-9} – 10^{-8} M SX but not by 10^{-10} – 10^{-7} M FP. The production of GM-CSF was inhibited by a concentration of 10^{-8} M in both drugs and the inhibition was augmented by combined treatment with 10^{-11} M of each drug.

Conclusions: The immunological release of chemical mediators (histamine, LT, PGD₂) from HCMC was inhibited by SX but not by FP. SX and FP inhibited the production of GM-CSF by HCMC and both drug showed synergistic inhibition in the production of GM-CSF.

KEY WORDS

chemical mediator, fluticasone, GM-CSF, mast cell, salmeterol

INTRODUCTION

Over the last decade there has been a dramatic improvement in the treatment of allergy, largely due to the early use of potent anti-allergic or anti-inflammatory drugs.¹⁻³ For example, bronchial asthma can be effectively managed using an inhaled corticosteroid with or without β_2 -agonists. However in patients with severe bronchial asthma, theophylline and an anti-cholinergic bronchodilator are also used.⁴⁻⁶ Whereas short acting β_2 -agonists are very effective in the relief of symptoms due to bronchodilator effects, occasionally some patients experience

tremor and palpitations.^{7,8} These adverse effects can usually be avoided by reducing the dose and/or frequency of the β_2 -agonist and by combination therapy with other drugs, including anti-cholinergic bronchodilator or glucocorticoids.^{9,10}

Recently, long acting β_2 -agonists (LABAs) have been introduced for the treatment of bronchial asthma.^{11,12} LABA, salmeterol and formoterol have prolonged smooth muscle effects and a high affinity for the β_2 -receptor compared to salbutamol.¹³ The pharmacological activity of LABAs is not restricted to airway smooth muscle because these agents also inhibit mast cell mediator release and pro-inflammatory

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Table 1 Effects of SX and Isoproterenol on IgE-mediated Histamine, LTs and PGD₂ Release

Concentration (M)	Histamine (%)	LTs (pg/mL)	PGD ₂ (pg/mL)
Spon	5.02+++	106.78+++	322.12+++
Control	58.93	36311.45	17719.47
10 ⁻¹²	55.28	41797.65	20348.69
10 ⁻¹¹	52.33	35360.27	17692.86
10 ⁻¹⁰	32.52*	15915.31*	13072.12
10 ⁻⁹	20.76***	8818.88**	8100.66
10 ⁻⁸	18.70***	6777.65**	7036.74*
ISO	9.60+++	1056.62+++	2346.27+

Each figure represents the mean of three to five experiments. For clarity, standard error is not shown, but it was less than 18.2% of the mean value at each point. HCMC were passively sensitized by incubation with 1 µg/ml human myeloma IgE overnight. HCMC were treated with each drug for 10 minutes and then stimulated with anti-IgE antibody for 30 minutes.

*, **, ***: $p < 0.05$, 0.01 and 0.001 (vs control; Dunnett's multiple range test)

+, +++, $p < 0.05$ and 0.001 (vs control; Student's *t*-test)

cytokine production following segmental allergen challenge of atopic asthma patients.¹⁴ In addition, LABA was shown to produce a clinically relevant reduction in inhaled steroid dose, in addition to strong bronchodilation. Furthermore, there is growing evidence to suggest that LABA has complementary actions to glucocorticoids. For example, LABA increases glucocorticoid induced-eosinophil apoptosis, inhibition of cytokine/chemokine release and respiratory mucosal cytoprotection.¹⁵⁻¹⁸

This study was conducted to investigate the complementary action of LABAs and glucocorticoids on the immunological activation of human cultured mast cells (HCMC). We chose salmeterol xinafoate (SX) as the LABA and fluticasone propionate (FP) as the glucocorticoid. The activation of HCMC was examined by measuring the released chemical mediators (histamine, leukotriene (LT) and prostaglandin D₂ (PGD₂)) and the production of granulocyte-macrophage colony stimulating factor (GM-CSF) after stimulation with anti-IgE antibody.

METHODS

DRUGS

SX and FP were kindly donated by GlaxoSmithKline K.K. (Tokyo, Japan). These drugs were dissolved in dimethyl sulfoxide and stored. Isoproterenol was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

MAST CELL CULTURE

HCMC were obtained by culturing progenitor cells in human umbilical cord blood donated from healthy mothers after obtaining informed consent at Ogaki Municipal Hospital, Gifu, according to the method described by Saito *et al.*^{19,20} with some modifications. Briefly, freshly obtained heparin-treated cord blood was layered on a lymphocyte separating medium (ICN Biomedicals, Inc., Aurora, OH, USA) and centrifuged. The mononuclear cell layer was collected and

CD34 positive cells were purified using a CD34 isolation kit and Magnetic Cell Sorting System (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Purified CD 34 positive cells were cultured in an α -minimum essential medium (Sigma-Aldrich) containing 15% fetal bovine serum (Thermo Trace Ltd., Melbourne, Australia), 80 ng/ml human recombinant stem cell factor (Kirin Brewery Co., Ltd., Maebashi, Japan) and 50 ng/ml human recombinant interleukin-6 (Kirin Brewery, Maebashi, Japan). Cells were harvested weekly and resuspended in a fresh medium. The purity of mast cells was determined by toluidine blue-staining. Beyond 12 weeks, almost 100% of the cultured cells were mast cells.

CHEMICAL MEDIATOR RELEASE AND CYTOKINE PRODUCTION

HCMC were sensitized with 1 µg/ml human myeloma IgE (Chemicon Int. Inc., Temecula, CA, USA) at 37°C overnight. In the experiment on IgE-mediated chemical mediator (histamine, LTs and PGD₂) release, cells were resuspended at a concentration of 1×10^5 cells/ml in Tyrode's solution (126 mM NaCl, 4 mM KCl, 0.64 mM NaH₂PO₄, 1 mM CaCl₂, 0.6 mM MgCl₂, 0.1% glucose, 0.03% bovine serum albumin (BSA), 10 mM HEPES, pH 7.4). The cell suspension was pretreated with each drug for 10 minutes and then challenged with 4 µg/ml anti-human IgE antibody (DAKO, Glostrup, Denmark) for 30 minutes at 37°C. To examine the effect of the agents on cytokine (GM-CSF) production, cells were resuspended at a concentration of 1×10^6 cells/ml in fresh culture medium, pretreated with each drug for 10 min and then challenged with 1.5 µg/ml anti-human IgE for 6 hours at 37°C. After stimulation with anti-IgE, reaction mixtures were centrifuged and the supernatants were separated and stored until measurement.

Table 2 Effects of FP and Isoproterenol on IgE-mediated Histamine, LTs and PGD₂ Release

Concentration (M)	Histamine (%)	LTs (pg/mL)	PGD ₂ (pg/mL)
Spon	2.55+++	1605.23+++	4.27+++
Control	49.48	15599.24	10339.74
10 ⁻¹⁰	58.34	19058.51	11538.19
10 ⁻⁹	63.50	19129.28	13199.20
10 ⁻⁸	63.99	15098.61	12993.80
10 ⁻⁷	62.03	13228.60	12923.93
ISO	7.52+++	3271.16+++	1132.64+++

Each figure represents the mean of three to five experiments. For clarity, standard error is not shown, but it was less than 21.5% of the mean value at each point. HCMC were passively sensitized by incubation with 1 µg/ml human myeloma IgE overnight. HCMC were treated with each drug for 10 minutes and then stimulated with anti-IgE antibody for 30 minutes.

+++; $p < 0.001$ (vs control; Student's *t*-test).

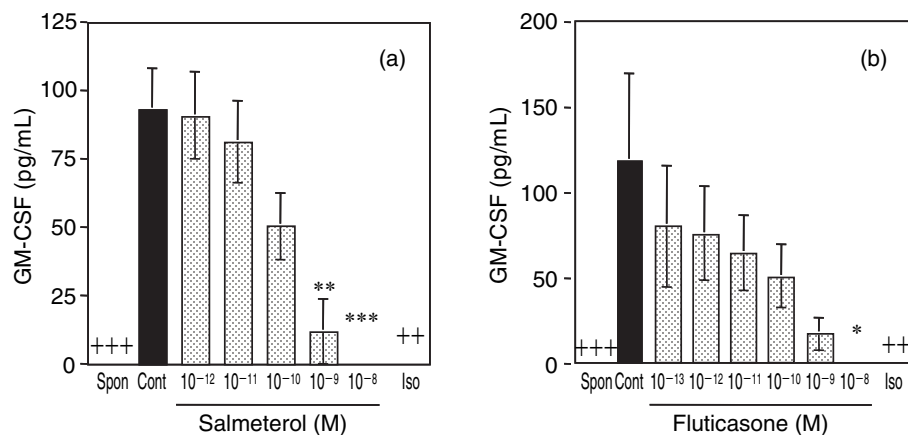


Fig. 1 Effects of salmeterol, fluticasone and isoproterenol on IgE-mediated GM-CSF production from HCMC. HCMC were passively sensitized with 1 µg/mL human myeloma IgE overnight. HCMC were treated with each drug for 10 minutes and then stimulated with anti-IgE for 30 minutes or 6 hours. Each result represents the mean of the S.E.M. of three to four independent experiments. Cont: control, Iso: isoproterenol, Spon: spontaneous. *, **, ***: $p < 0.05, 0.01, 0.001$ (vs Cont; Dunnett's multiple range test). +, ++, +++: $p < 0.001$ (vs Cont; Student's *t*-test).

MEASUREMENT OF HISTAMINE, LTS, PGD₂ AND GM-CSF

Histamine content was quantified by the method of post-column derivatization as previously reported.²¹ Histamine release is expressed as the percentage of total histamine content determined by cell lysis with perchloric acid. For the analysis of LTs, we used a commercial ELISA kit provided by Bülmann Laboratories AG (Allschwil, Switzerland). The ELISA kit was based on the monoclonal antibody that recognizes sulfidoleukotrienes (LTC₄, LTD₄ and LTE₄). PGD₂ was measured by PGD₂-MOX EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA). This kit is based on the conversion of PGD₂ to a stable methoxime derivative by treatment with methoxamine hydrochloride. GM-CSF content in the supernatants was measured with an ELISA kit (R&D Systems, Minneapolis,

MN, USA), according to the manufacture's protocol.

STATISTICS

All data are represented as the mean $\pm$ S.E.M of three or four independent experiments and analyzed using Dunnett's multiple range test and Student's *t*-test.

RESULTS

EFFECTS OF SX AND FP ON THE RELEASE OF HISTAMINE, LTS AND PGD₂ FROM HCMC

Firstly, we examined the effect of SX and FP on the release of chemical mediators from HCMC. After stimulation with anti-human IgE antibody, the level of histamine, LTs and PGD₂ in the supernatant was significantly elevated. As shown in Table 1, SX (10⁻¹¹ – 10⁻⁸ M) showed a tendency to inhibit or inhibited the release of histamine, LT and PGD₂ in a concentration-

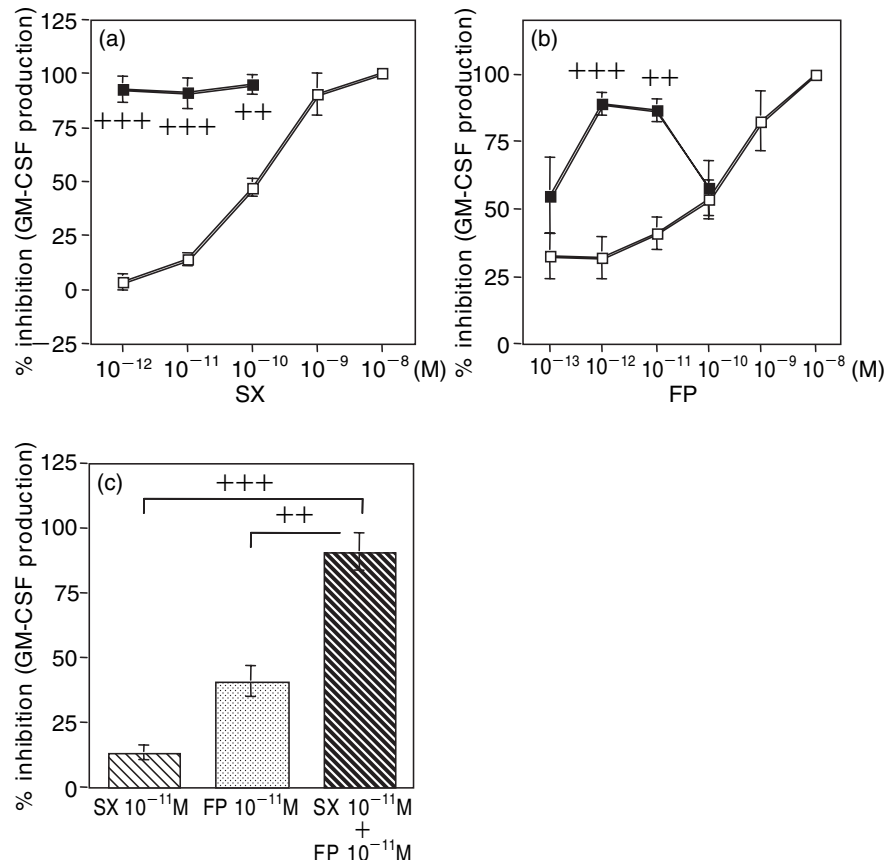


Fig. 2 Combined effects of salmeterol and fluticasone on IgE-mediated GM-CSF production from HCMC. HCMC were passively sensitized with 1 μ g/mL human myeloma IgE overnight. HCMC were treated with each drug for 10 minutes and then stimulated with anti-IgE for 6 hours. Each result represents the mean with the S.E.M. of three or four independent experiments. (a) Inhibitory effect of salmeterol alone ($\square$) or salmeterol with 1×10^{-11} M fluticasone ($\blacksquare$). (b) Inhibitory effect of fluticasone alone ($\square$) or fluticasone with 1×10^{-11} M salmeterol ($\blacksquare$). (c) Comparison of the inhibitory effects of fluticasone and salmeterol alone or in combination. SM: 1×10^{-11} M salmeterol, FP: 1×10^{-11} M fluticasone. ++, +++: $p < 0.01$ and 0.001 (vs alone; Student's *t*-test).

dependent manner. SX showed no inhibitory effect at 1×10^{-11} M, but significantly inhibited the release of chemical mediators at a concentration of 1×10^{-8} M. Pretreatment with FP did not affect the release of the chemical mediators (Table 2). Isoproterenol at a concentration of 10^{-8} M, which was used as a positive control, clearly inhibited the release of histamine, LT and PGD₂.

EFFECT OF SX AND FP ON THE PRODUCTION OF GM-CSF BY HCMC

Treatment of HCMC with either SX or FP inhibited the production of GM-CSF in a concentration-dependent manner (*i.e.* no effect on GM-CSF production at 1×10^{-12} M, but complete inhibition at 1×10^{-8} M). Isoproterenol at a concentration of 1×10^{-7} M completely inhibited the production of GM-CSF (Fig. 1).

We also examined the effect of combined treatment of HCMC with SX and FP. The inhibition of GM-CSF production by SX at concentrations between 1×10^{-12} and 1×10^{-10} M was clearly augmented by the addition of 1×10^{-11} M FP (Fig. 2(a)). Furthermore, the inhibition by FP at concentrations between 1×10^{-13} M and 1×10^{-11} M was clearly potentiated by the addition of 1×10^{-11} M SX, although no synergistic effect was observed at a concentration of 1×10^{-10} M FP (Fig. 2(b)). The inhibition of GM-CSF production by SX (1×10^{-11} M) and FP (1×10^{-11} M) alone and in combination is depicted in Figure 2(c). Although the inhibition of GM-CSF production by SX and FP was 14 and 41%, respectively, it reached 91% when the drugs were used in combination. When the combined effect on the release of histamine was examined under the same conditions, no synergistic effect was observed.

DISCUSSION

In this study, we investigated the effects of SX and FP alone or in combination on IgE-mediated mast cell activation, including chemical mediator release and cytokine production by human mast cells derived from cord blood progenitor cells.

We initially examined the effect of these drugs on chemical mediator release from mast cells. Our results indicate that SX inhibits the release of histamine, LTs and PGD₂ in a concentration-dependent manner. The same effect is also observed for isoproterenol. Our previous data indicated that β_2 agonists are strong inhibitors of mediator release from mast cells when compared to the effect of mast cell stabilizers, such as disodium cromoglycate and azelastine.²² Furthermore, Chong *et al.* also demonstrated that SX inhibits chemical mediator release from human lung mast cells.²³ They reported that β_2 agonists elevate intracellular cAMP levels through β_2 adrenoceptors, resulting in the activation of a downstream effector, protein kinase A, that plays a key role in the inhibitory effects of the secretion of chemical mediators in addition to the relaxation of smooth muscle cells. Our previous studies using rat mesenteric mast cells also indicate the same mechanism.²⁴ Further experiments designed to measure the level of cAMP in mast cells will be necessary to explain an anti-inflammatory activity of SX in the acute phase reaction.

In contrast to β_2 agonists, FP did not show any inhibitory effect on mediator release from mast cells. Our preliminary experiments using HCMC and human lung tissues also indicated that prednisolone and dexamethasone failed to inhibit allergic chemical mediator release (data not shown). Similar data has been reported by other investigators.²⁵⁻²⁷ These experiments suggest that corticosteroids do not directly affect chemical mediator release from human mast cells. However some investigators reported that there is an inhibitory action of glucocorticoids on the maturation of human mast cells, which results in a decrease in tissue mast cells by reducing the production of the c-kit ligand, a stem cell factor.^{28,29} These data suggest that the main mechanism of glucocorticoid induced inhibition of allergic mediator release may be related to a mechanism other than mast cell activation.

In contrast to allergic chemical mediators, SX and FP clearly inhibit the production of GM-CSF by HCMC even at low concentrations. Whereas numerous studies have demonstrated the inhibitory action of glucocorticoids on the production of cytokines,³⁰⁻³⁴ only a few studies have been carried out to examine the effect of β_2 -agonists on the production of cytokines by HCMC.³⁵ The present study has clearly demonstrated the efficacy of SX on the production of GM-CSF by HCMC activated through immunological stimuli. Combination therapy using SX and FP has re-

cently been recognized as a potent treatment for bronchial asthma. Therefore we also examined the effect of SX and FP in combination on the production of GM-CSF by HCMC. The present results clearly demonstrate that the inhibition of GM-CSF production from human mast cells is synergistically potentiated by simultaneous treatment with low concentrations of SX and FP. The anti-inflammatory activity of β_2 agonists detected in basic studies has not been clinically evaluated. Thus it is not known whether β_2 agonist enhance the anti-inflammatory activity of corticosteroids in clinical practice. Indeed, the potentiation of the anti-inflammatory activity of corticosteroids by β_2 agonists may have important implications concerning the clinical efficacy of the combined use of these two drugs.

Regarding the effect of other cytokine production, we did not try to measure any other cytokine without GM-CSF in this experiment. However some investigators reported a synergistic action of SX and FP on the inhibition of IL-5 production and IL-10 secretion by CD4⁺ T cells.³⁶⁻³⁸ Further experiments designed to measure IL-5 or other cytokine production by HCMC is necessary in near future.

Similar synergistic effects were reported for SX and FP on the *in vitro* production of TNF- α -induced IL-8 and eotaxin by human airway smooth muscle cells and human lung fibroblasts.³⁹⁻⁴¹ Moreover, other combinations of LABA and ICS, such as formoterol and budesonide, also depress cytokine production in human bronchial epithelial cells.⁴² These reports together with the results presented in this paper support the clinical efficacy of combination therapy for LABA and ICS in the treatment of bronchial asthma. The addition of salmeterol to ICS gives improved lung function and symptom control in asthmatic patients and is more effective than doubling the dose of ICS.^{43,44} We predict that combination therapy with SX and FP will soon become the major treatment for asthma in Japan. The underlying mechanism for the combined use of the two drugs was not examined in the present study. Some studies have indicated that corticosteroids increase the expression of β_2 adrenoceptors and that β_2 agonists activate the glucocorticoid receptor.^{45,46} These mechanisms may explain the efficacy of combination therapy. We plan to investigate the molecular mechanism of combined therapy including c-AMP levels, mRNA expression of functional molecules and alternation of nucleic factors in near future. This is the first report to demonstrate the combination effect of SX and FP on HCMC. Our results support the clinical efficacy of combination therapy comprising LABA and ICS for the treatment of asthma.

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