Inhaled long-acting $\beta_2$-agonists enhance glucocorticoid receptor nuclear translocation and efficacy in sputum macrophages in COPD

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ABSTRACT

Background: Combination inhaled therapy with long-acting \( \beta_2 \)-agonist (LABA) and corticosteroid is beneficial in treating asthma and chronic obstructive pulmonary disease (COPD).

Objective: In asthma, LABAs enhance glucocorticoid receptor (GR) nuclear translocation in the presence of corticosteroids. Whether this biological mechanism occurs in COPD, a relatively corticosteroid-resistant disease, is uncertain.

Methods: Eight patients with mild/moderate COPD participated in a double-blind, placebo-controlled, crossover study and inhaled single-doses of; fluticasone propionate (FP)-100\( \mu \)g, FP-500\( \mu \)g, salmeterol(SLM)-50\( \mu \)g and combination FP-100\( \mu \)g+SLM-50\( \mu \)g. One hour post-inhalation sputum was induced, nuclear proteins isolated from purified macrophages and levels of activated nuclear GR quantified using a GR-GRE ELISA-based assay.

Results: Nuclear GR significantly increased after FP-500\( \mu \)g (p<0.01), but not after FP-100\( \mu \)g or SLM-50\( \mu \)g, compared to placebo. Interestingly, SLM in combination with FP-100\( \mu \)g increased nuclear GR levels equivalent to those of FP-500\( \mu \)g alone. This was significantly greater than either FP-100\( \mu \)g (p<0.05) or SLM-50\( \mu \)g (p<0.01) alone. In vitro in a human macrophage cell line, SLM (10^{-8}M) enhanced FP (10^{-9}M)-induced mitogen-activated protein kinase phosphatase-1 (MKP-1) mRNA (5.8±0.6 vs. 8.4±1.1 x 10^{-6} copies, p<0.05) and 2xGRE-luciferase reporter gene activity (250.1±15.6 vs. 103.1±23.6 fold induction, p<0.001). Addition of SLM (10^{-9}M) to FP (10^{-11}M) significantly enhanced FP-mediated suppression of IL-1\( \beta \)-induced CXCL8 (p<0.05).

Conclusion: Addition of SLM-50\( \mu \)g to FP-100\( \mu \)g, enhanced GR nuclear translocation equivalent to that seen with a five-fold higher dose of FP in sputum macrophages from COPD patients. This may account for the superior clinical effects observed in COPD of combination LABA/corticosteroid treatment compared to either as monotherapy.
Sputum macrophages from COPD patients are relatively corticosteroid insensitive. Inhaled salmeterol and fluticasone propionate greatly enhanced glucocorticoid receptor activation in sputum macrophages from COPD patients.

Combination therapy with inhaled long-acting β₂-agonist and corticosteroid improves clinical outcomes in COPD and biologically, enhanced glucocorticoid receptor activation in sputum macrophages may partly explain this phenomenon.

**KEYWORDS:** long-acting β₂-agonist, corticosteroid, transcription factor, glucocorticoid receptor, sputum, COPD, macrophage
ABBREVIATIONS

COPD Chronic obstructive pulmonary disease
FCS Fetal calf serum
FP Fluticasone propionate
FEV$_1$ Forced expiratory volume in one-second
GR Glucocorticoid receptor
GRE Glucocorticoid response element
HBSS Hanks' buffered saline solution
ICS Inhaled corticosteroids
LABAs Long-acting $\beta_2$-agonists
MKP-1 Mitogen-activated protein kinase phosphatase-1
PBMCs Peripheral blood mononuclear cells
SLM Salmeterol xinafoate
INTRODUCTION

It is well-established in asthmatic patients that the addition of long-acting $\beta_2$-agonist (LABA) to inhaled corticosteroids (ICS) provides more effective disease control than monotherapy with ICS [1-4]. Indeed, with recent concerns regarding the safety of LABA monotherapy in the treatment of asthma, current guidance supports fixed dose combinations of LABA/ICS therapy from a single inhaler [5-7]. Chronic obstructive pulmonary disease (COPD) is also characterized by chronic airways inflammation, but unlike asthma, ICS treatment on its own has relatively little effect on the accelerated decline in lung function seen in COPD patients [8,9]. However in contrast, several large clinical trials of combination LABA/ICS therapy in patients with stable COPD have shown better control of respiratory symptoms, lung function, quality of life, and exacerbations with no greater risk of side-effects, compared to the use of either ICS alone or LABA alone (10-13). Recent studies have demonstrated the effectiveness of LABA/ICS combination therapy in reducing COPD morbidity and mortality compared with ICS treatment alone (14, 15).

The scientific rationale to explain the disproportionately superior clinical efficacy achieved by the addition of LABAs to ICS in the treatment of asthma and COPD is being addressed, and elucidating the underlying molecular mechanisms may help identify novel therapeutic targets (16, 17). Combination therapy with SLM/FP reduces sputum differential cell counts, sputum neutrophils and eosinophils, bronchial CD45+, CD8+ and CD4+ cells, and cells expressing genes for tumor necrosis factor-\(\alpha\) and interferon-\(\gamma\) in comparison to placebo (18). These anti-inflammatory effects were also accompanied by improvements in the pre-bronchodilator forced expiratory volume in one-second (FEV$_1$). Treatment with SLM/FP combination has also been
shown to reduce tissue CD8+ and CD68+ cells compared with placebo, whereas no effect was observed with FP alone (19).

It has been proposed LABAs may have anti-inflammatory properties similar to ICS (20, 21). Studies show LABAs enhance corticosteroid-dependent anti-inflammatory effects, and the biological mechanism for this may be through ligand-independent priming of the glucocorticoid receptor (GR) (22). The cellular actions of corticosteroids are mediated by intracellular GRs, which after binding to corticosteroid, translocate from the cell cytoplasm into the nucleus where they mediate corticosteroid-dependent effects (23). Enhanced GR activation by LABAs has been demonstrated in vitro in human lung fibroblasts and smooth muscle cells (22, 24), in human neutrophils (25), and ex vivo in the sputum of patients with asthma (17). However, it is unknown whether LABAs cause GR translocation after ICS therapy in COPD patients, as this disease is much less responsive to the effects of corticosteroids compared to asthma (26).

Indeed, macrophages from COPD patients have been shown to exhibit a poor anti-inflammatory response to corticosteroids in vitro compared to cells from healthy smokers and non-smokers (21).

In this study, we examined the effect of SLM/FP combination treatment on GR-glucocorticoid response element (GRE) binding in the nucleus of macrophages isolated from the induced sputum of patients with COPD. Importantly, we used inhaled drug doses used in the clinic. Our aim was to determine whether GR activation in response to corticosteroid therapy was enhanced by a LABA and whether this had any functional effects. As reduced nuclear translocation of GR contributes to corticosteroid insensitivity (16, 26), we hypothesized that the
LABA, salmeterol, could contribute to improve corticosteroid insensitivity in cells from COPD patients.
METHODS

Subjects

Eight patients diagnosed with moderate COPD (GOLD stages 2 and 3 (27)), <12% reversibility in FEV₁ and a smoking history of >10 pack-years, participated in a randomized, double-blind, placebo-controlled cross-over study. All patients were aged between 40 – 80 years and were not taking either ICS or LABAs (Table 1). At each study-visit, patients’ FEV₁ was required to be within 15% of their screening FEV₁ value to control for differences in airway function and inflammation between visits. Patients were allowed to take the bronchodilators tiotropium bromide and/or albuterol during the study, but none of the subjects were treated with ICS or LABAs for at least one month preceding the start of the study.

Study design

Single actuations of fluticasone propionate (FP)-100μg, FP-500μg, salmeterol xinafoate (SLM)-50μg, combination therapy FP/SLM 100μg/50μg (Seretide™, GlaxoSmithKline, Stevenage, U.K.) or placebo were delivered via a metered dose inhaler and spacer, separately at each study visit. Sputum was induced 60 minutes after drug inhalation and peripheral blood samples were also taken at this time point. The study was approved by the Ethics Committee of the Royal Brompton & Harefield Hospitals National Health Service Trust, and all subjects gave written informed consent.

There was a minimum washout period between treatments of seven days to prevent any crossover effects. All patients were randomised (pre-assigned by picking balls out of a bag) to determine the order of treatment. In this scenario, there were 24 possible crossover orders, and with 8 subjects, there would have been possible treatment orders that were not received.
However, the sample size of 8 subjects was ethically chosen based on our previous study (17), where 7 subjects received 5 treatments, which was sufficient to detect statistical differences between ICS alone vs. LABA/ICS combination in asthmatic subjects.

**Sputum induction and processing**

Sputum induction was performed as previously described and processed within one hour of collection (17). Briefly, sputum was filtered (70μm filter) and centrifuged (1600rpm for 5 min) to obtain a cell pellet. Cells were resuspended in full RPMI-1640 media (Sigma-Aldrich), supplemented with 1% L-glutamine, 10% fetal calf serum (FCS; Invitrogen Ltd, Carlsbad, CA), 100 U/ml penicillin and 100μg/ml streptomycin (Sigma-Aldrich). Total cell count (Kimura stain) and viability (Trypan blue exclusion) were determined before cytospins were undertaken.

**Ex-vivo stimulation of sputum macrophages**

Sputum macrophages were isolated from whole sputum using plastic adherence at a cell density of 0.5x10⁶ cells/ml for 4 h in a 5% CO₂ humidified atmosphere at 37°C. Non-adherent cells were removed and macrophages resuspended in charcoal-stripped RPMI-1640 minimal media (1% L-glutamine and 0.5% FCS) overnight, before being exposed to SLM (10⁻⁷M), FP (10⁻⁹M), or a combination of both, for 60 min. The same number of macrophage cells in culture were seeded for each treatment visit and utilised for each of the experiments, standardised to a cell density of 0.5x10⁶ cells/ml.

**Isolation of blood cells**

Venous blood (80 ml) was diluted 1:1 with Hanks' buffered saline solution (HBSS, Invitrogen) and layered on Ficoll-Hypaque-Plus (Amersham plc, Amersham, UK). After centrifugation (30 min at
1,100 x g and 18°C), peripheral blood mononuclear cells (PBMCs) were collected, washed, and centrifuged (250 x g for 10 min). PBMCs were resuspended in culture media and counted using Kimura dye.

**Nuclear extraction**

Nuclear and cytoplasmic fractions were extracted using a Nuclear Extraction kit (Active Motif, Carlsbad, CA). Briefly, cells were resuspended in hypotonic buffer, vortexed and incubated on ice for 15 min to extract the cytoplasmic fraction. Thereafter, the remaining nuclear pellets were resuspended in complete lysis buffer for 30 min on ice. The suspension was centrifuged (14000rpm, 10 min, 4ºC) and the nuclear fraction obtained. The quality and purity of the nuclear extract was determined by Western Blotting (Figure 1A).

**GRE binding assay**

GR activation was determined with a GR-GRE TransAM kit (Active Motif, Frixensart, Belgium) according to the manufacturer’s instructions and nuclear GR-GRE binding was determined. Briefly, 5μg of nuclear extract of each sample was added to a well. Each well contained multiple copies of a specific double-stranded oligonucleotide which activated GR binds to at its consensus binding site. We confirmed using Western blotting in U937 cells that incubation with the positive control, fluticasone propionate, led to an increase in nuclear GR, which correlated to an induction in GR-GRE binding, relative to the negative control of untreated cells (Figure 1B & C).

**Confocal microscopy**
Sputum macrophages were seeded onto glass coverslips in 6-well plates and allowed to attach at 37°C. The cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at 37°C and washed three times with PBS. Cells were then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, Poole, UK) for 5 min and then incubated with primary anti-GR antibody (H-300, Santa Cruz Biotechnology, CA, USA) for 3 hours followed by 30 min with secondary antibody Alexa 488 (Invitrogen). Coverslips were washed three times with PBS, rinsed in distilled water and immediately dried. Prolong Antifade 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (Invitrogen) was added to delineate the nuclear boundary in each cell and help define the distribution of GR protein (green) within the respective subcellular compartments (Figure 2). Coverslips were mounted onto slides and allowed to dry for 4 hours before analysis using confocal microscopy with imaging software (Leica Confocal Software Lite, Heidelberg, Germany).

**Cell Culture**

U937 cells (ATCC, Rockville, MD) were maintained in RPMI-40 medium (Invitrogen), supplemented with L-glutamine (1%, Invitrogen) and fetal calf serum (FCS, 10%, Invitrogen), and were differentiated as previously described (28). After washing with HBSS, cells were allowed to recover in medium supplemented with 1% L-glutamine and 0.1% charcoal-stripped FCS for 48 hours before experiments.

**MKP-1 mRNA expression**

Differentiated U937 cells were cultured in RPMI supplemented with 0.1% charcoal-stripped-FCS and L-glutamine (1%) for 24 h before treatment. Total RNA was isolated from cells using an RNeasy Kit (Qiagen, Hilden, Germany). cDNA was synthesised from 1μg of total RNA with the
Qiagen Quantitect Kit (Qiagen) according to the manufacturer’s protocol. Expression of mitogen-activated protein kinase phosphatase-1 (MKP-1) mRNA was quantified by RT-qPCR with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, US). Primers are shown in Table 2. MKP-1 mRNA levels were normalized to an endogenous reference GAPDH and absolute quantification was determined by using known standards of MKP-1. MKP-1 standards were produced from PCR products containing the target sequence from U937 monocytes. The copy number of the standards was determined by measuring the concentration using a spectrophotometer.

**GR reporter gene assay**

Differentiated U937 cells were transfected with GRE-luciferase and β-galactosidase plasmids as previously described and GR transactivation determined by luciferase assay as previously described (17).

**CXCL8 enzyme-linked immunosorbent assay (ELISA)**

Serum-starved U937 cells were incubated with IL-1β for 30 min, followed by FP and SLM alone and in combination for 16 hours. Subsequently, extracellular concentrations of CXCL8 were measured with human CXCL8 Duoset ELISA kits (R&D Systems Europe, Abingdon, UK) according to the manufacturer’s instructions.

**Statistical analysis**

In the *in vivo* study, as sputum cells are not normally distributed, a non-Gaussian distribution was adopted and data were analysed using non-parametric statistical analyses using the PC.
analysis package Graph Pad Prism (Graph Pad Prism, San Diego, CA). Results were analyzed using Friedman analysis of variance (ANOVA) including factors for period, treatment, and multiple comparisons, and applying Bonferroni’s post-test correction. Comparisons between treatments were made using the Wilcoxon matched-pairs signed rank sum test. A p value of less than 0.05 was considered statistically significant. For the difference between treatment groups in the in vitro data, analyses were undertaken using the Mann-Whitney test for comparison of two unpaired groups with Graph Pad Prism software. A p value of less than 0.05 was considered statistically significant.
RESULTS

Patient characteristics

The mean age of the eight patients (6 female) was 65 ± 8 years and the mean post-
bronchodilator FEV₁ was 72 ± 12% of predicted (Table 1). No patient showed >12% reversibility
to albuterol, was atopic, or had a previous history of asthma. Median % sputum cell counts are
shown in Table 1 averaged from different study visits across all patients, and most patients had
high levels of neutrophilia. The percentage of sputum macrophages obtained varied between
visits within each patient (range -6.7% to +12.2%) compared to the sample taken at visit 1.
However, the amount of protein extracted from the isolated macrophage nuclear extracts for
the GR-GRE assay was normalised to be the same for all samples from all patients for all visits,
irrespective of the variability in the sputum macrophages yielded following sputum induction,
and this was 5µg.

Effect of LABA and corticosteroid on GR-GRE binding in sputum cells in vivo

GR-GRE binding levels in the nuclei of macrophages obtained from induced sputum were not
significantly altered at 60 minutes following inhalation of FP-100µg or SLM-50µg compared to
placebo (Figure 3A). In contrast, FP-500µg significantly increased GR-GRE binding levels 2.2-fold
compared to placebo (0.607 ± 0.089 vs. 0.274 ± 0.038 absorbance units, p<0.01) and induction
of GR-GRE binding was significantly greater with FP-500µg compared to that observed with FP-
100µg (0.607 ± 0.089 vs. 0.310 ± 0.028 absorbance units, p<0.05). Combination treatment of FP-
100µg with SLM-50µg increased nuclear GR-GRE levels to similar levels to those seen with FP-
500µg (respectively 0.598±0.061 vs. 0.607 ± 0.089, p = NS). This effect observed with the
combination treatment was significantly greater than that seen with either placebo (p<0.01), FP-
100µg alone (p<0.05) or SLM-50µg alone (p<0.01). The addition of SLM-50µg to FP-100µg as
combination treatment, achieved an enhancement of GR activation in sputum macrophages from patients with COPD that was equivalent to that observed with a five-fold increase in the dose of FP. That is, combination therapy allowed a five-fold reduction in the inhaled corticosteroid dose, yet achieved the same biological effects. Confocal microscopy documented the correlation between the glucocorticoid receptor GRE binding assay and glucocorticoid receptor nuclear translocation in that, treatment with corticosteroid (FP-500μg) led to GR nuclear translocation in sputum macrophage cells (Figure 2).

**Effect of LABA and corticosteroid on GR-GRE binding in PBMCs in vivo**

The levels of GR-GRE binding in PBMCs were measured 60 minutes after inhalation of the drugs (Figure 3B). None of the active treatments had an effect on GR-GRE binding levels and these levels did not differ significantly from those obtained with placebo.

**Effect of LABA and corticosteroid on GR-GRE binding in sputum cells ex vivo**

Sputum macrophages were isolated by adherence to plastic, allowed to recover for 24 h before stimulation with SLM (10^{-7}M), FP (10^{-9}M) or a combination of both for 60 minutes. FP significantly enhanced nuclear GR-GRE binding compared to control unstimulated cells (0.506 ± 0.042 vs. 0.252 ± 0.038 absorbance units, p<0.01). Salmeterol alone had no significant effect on GR-GRE binding levels compared to control (Figure 4). However, the addition of SLM to FP significantly enhanced the ability of FP alone (10^{-9}M) to stimulate GR-GRE binding (0.710 ± 0.073 vs. 0.506 ± 0.042 absorbance units, p<0.05).

**Induction of MKP-1 mRNA in macrophage-like U937 cells**
FP (10^{-9}M) alone significantly induced MKP-1 mRNA expression after 2 h compared to the levels seen in control unstimulated cells (5.8 ± 0.6 x 10^{-6} vs. 6.4±2.5 x 10^{-8} copies, p<0.001), whilst SLM (10^{-8}M) alone had no effect (3.2 ± 0.6 x 10^{-8} copies, p=NS) (Figure 5A). However, upon addition to FP, SLM significantly enhanced FP-induced MKP-1 mRNA (5.8 ± 0.6 vs. 8.4 ± 1.1 x 10^{-6} copies, p<0.05).

**Induction of GRE-luciferase activity by LABA and corticosteroid in macrophage-like U937 cells**

Salmeterol (10^{-8}M) alone had no significant effect on a 2xGRE-luciferase reporter gene activity compared to control unstimulated cells (1.0 ± 0.6 vs. 1.5 ± 0.4 fold induction, p=NS) (Figure 5B). In contrast, FP (10^{-9}M) significantly enhanced GRE-luciferase activity compared to control (103.1 ± 23.6 fold induction, p<0.05). Yet, the addition of SLM to FP resulted in a highly significant increase in GRE-luciferase activity compared to that seen with FP alone (250.1 ± 15.6 vs. 103.1 ± 23.6 fold induction, p<0.001).

**Effect of LABA and corticosteroid on IL-1β-induced CXCL8 in macrophage-like U937 cells**

FP (10^{-11}M) and (10^{-9}M) significantly suppressed IL-1β-induced CXCL8 (28.3 ± 3.2 and 71.2 ± 3.1 % respectively, p<0.01), whilst SLM (10^{-9}M) alone had no considerable effect (Figure 6). However, the addition of SLM (10^{-9}M) to FP (10^{-11}M) significantly enhanced FP-mediated suppression of IL-1β-induced CXCL8 (46.7 ± 4.8 vs. 28.3 ± 3.2 %, p<0.05).
DISCUSSION

Our study shows that nuclear levels of activated GR in macrophages obtained from the induced sputum of patients with COPD increased following single-dose inhalation treatment with high-dose fluticasone propionate (FP)-500 μg, but not after low-dose FP-100 μg alone or salmeterol (SLM)-50 μg alone. Most importantly, the addition of SLM-50 μg to low-dose FP-100 μg increased activated GR levels equivalent to those seen with high-dose FP-500 μg. That is, in COPD patients, addition of salmeterol to a low-dose of FP achieved an enhancement of GR nuclear translocation equivalent to that observed with a five-fold higher corticosteroid dose. Consistent with our in vivo patient data, we were able to demonstrate that salmeterol significantly enhanced the ability of FP to induce GR activation in sputum macrophages ex vivo and enhanced FP-induction of MKP-1 mRNA and 2xGRE reporter gene activity in a human cultured macrophage cell line in vitro. Additionally, we were able to show a functional anti-inflammatory effect of combination therapy in that salmeterol significantly enhanced FP-mediated suppression of IL-1β-induced CXCL8 in macrophage-like U937 cells.

When LABAs were introduced as an add-on therapy to inhaled corticosteroids, the magnitude of their clinical beneficial effect in the control of asthma, and in COPD, was not predicted. The role of LABAs was seen purely as bronchodilatation without any appreciable effect on bronchial inflammation. In asthma, LABAs combined with corticosteroids produced a better effect on symptoms (1, 3), lung function (29), exacerbation rates (30) and health status (31) than expected. Adding LABA to low-dose ICS was observed to be more effective than increasing the dose of corticosteroids (1, 29, 30, 32) and asthma guidelines were updated to reflect these findings (33).
The beneficial effects of the LABA/ICS combination have also been observed in COPD patients, where they are now an established treatment. The TORCH (TOwards a Revolution in COPD Health) study demonstrated reductions in exacerbation rates, improvements in health status and lung function with SLM/FP combination compared to placebo in COPD patients (15). The study observed a relative reduction in mortality of 17% over three years for patients receiving SLM/FP, although this just failed to reach statistical significance. A subsequent Cochrane review observed survival benefits with combination therapy compared both to placebo (34) and to ICS alone (14). In vivo studies using bronchial biopsies have generated further supporting evidence for complementary interactions between LABAs and corticosteroids on airway inflammation and pathology in COPD patients (18, 19).

Our data reveal a complementary interaction between the LABA (salmeterol) and corticosteroid (fluticasone propionate) in that the combination therapy has a greater anti-inflammatory effect than either drug alone and was also steroid sparing; allowing a five-fold reduction in the inhaled corticosteroid dose, yet achieving the same biological effects. Indeed our data support the postulate that changes in the anti-inflammatory effects with combination therapy may account for the improvements in the rate of decline in lung function seen in the TORCH study (35).

We confirm that sputum macrophages from COPD patients are less sensitive to corticosteroid action compared to those obtained from asthmatic subjects (36, 37). In our previous study examining the effect of corticosteroid (FP) on GR activation in sputum macrophages from mild/moderate asthmatic subjects we demonstrated that 60 minutes post-treatment, FP-100μg significantly enhanced GR nuclear translocation (17). Here we show that only the higher dose of FP-500μg had an effect on GR-GRE binding. These data suggest that GR-GRE binding in sputum
macrophages may be a good biomarker for corticosteroid actions. As in our previous study (17), we were able to detect a quantifiable degree of basal GR-GRE binding in sputum macrophages in the placebo-treated patients (Figure 3) and this level of GR activation was maintained for at least 24 hours in culture. Since none of the patients in this study were exposed to exogenous inhaled or oral corticosteroids this activation must reflect the action of endogenous corticosteroids.

Consistent with our in vivo patient data, our in vitro experiments, showed salmeterol enhanced the ability of low concentrations of FP to induce GRE-luciferase activity and switch on MKP-1 expression in macrophage-like U937 cells. Similar data for enhanced induction of glucocorticoid-responsive genes in the presence of a LABA such as MKP-1 and glucocorticoid inducible leucine zipper (GILZ) has been reported in airway epithelial cells and smooth muscle cells (38). Our results are consistent with literature that suggest the enhanced effects may result from the ability of LABAs to promote GR nuclear translocation in human lung fibroblasts (22), airway smooth muscle cells (24, 39, 40) and in sputum cells from asthmatic patients (17). This enhancement of GR translocation achieved by LABAs when added to corticosteroids is likely to underlie some of the clinical benefits seen with combination therapy. Additionally, we demonstrated that the enhancement of GR translocation with combination therapy was associated with a functional anti-inflammatory effect; in that, salmeterol significantly enhanced FP-mediated suppression of IL-1β-induced CXCL8 in U937 cells.

We were unable to assess the anti-inflammatory properties of the combination treatment in sputum macrophages in vivo due to limitations in cells numbers and ethical considerations regarding repeated sputum induction in patients. Future experiments examining gene
expression profiles in sputum macrophages 4 hours after inhaled drugs will provide evidence for additional genes that are regulated in a similar manner to those described here. A role for other aspects of GR activation including GR post-translational modifications and the activity of GR chaperone proteins is difficult to rule out as potential modulators of GR-GRE binding. An experimental clinical patient study specifically designed to address these points would be needed. In contrast to our observations in sputum macrophages, the levels of activated GR in the nuclei of PBMCs did not change significantly after any treatment. This is likely to be due to peripheral blood levels of the study medications being too low after a single-dose inhalation to exert a measurable effect on the biological machinery of GR translocation. It would be interesting to see if a measurable effect is achieved after a more prolonged course of treatment.

The marked effect of LABAs on GR translocation in vitro and in vivo, in asthmatics and in COPD patients warrants further investigation, and elucidation of the precise molecular mechanisms of how LABAs (which acting extracelluarily via the β2 receptor) are able to influence translocation of cytoplasmic GRs. Altered phosphorylation, ‘priming’ of the GR increasing its tendency to translocate without the need for its natural ligand has been proposed (41, 42). Recently we have shown that LABA increase GR nuclear translocation through the activation of a phosphatase PPA2, which dephosphorylates hyperphosphorylated GR (43).

In conclusion, we report here for the first-time that a LABA in combination with a low-dose of ICS enhances GR-GRE binding in vivo in COPD patients as effectively as a five-fold higher dose of ICS alone. This suggests LABAs may play an important role in the benefits clinically seen with combination therapy. Our data has clinical relevance to the use of combination inhalers in view of the reduced sensitivity to ICS in COPD. Further studies are needed to address whether these
differences are maintained following chronic treatment of LABAs with ICS in COPD patients, and whether this results in an alteration in sputum cytokine profiles in vivo.

REFERENCES


### Patient characteristics

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### Sputum Cell Counts

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<tr>
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<td>Lymphocytes (% total)</td>
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<td>Eosinophils (% total)</td>
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### TABLE 2.

**PCR primers for MKP-1 mRNA analysis**

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<td>5'- GAAGGTGAAGGTCGGAGTC</td>
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**FIGURE LEGENDS**

**Figure 1. Validation of the quality of nuclear extract and GR-GRE binding.**

The quality of nuclear extracts (panel A) was determined by Western Blotting. Fluticasone propionate (FP)-induced GR-GRE binding (panel B) and FP-induced GR nuclear localisation (panel C) were assessed in U937 monocytes. Results represent the mean ± SEM of 3 independent experiments. *p<0.05 denotes statistical significance vs. untreated cells.

**Figure 2. Confocal laser images of GR subcellular localisation in sputum macrophages following corticosteroid (FP 500µg) treatment.** (panels A, D) 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) staining (blue channel); (panels B, E) cytoplasmic and nuclear GR immunostaining (green channel); (panels C, F) overlay of panels A and B.

**Figure 3. Effect of inhaled salmeterol (SLM) and fluticasone propionate (FP) on GR-GRE binding in nuclear extracts from induced sputum macrophages (panel A) and PBMCs (panel B).** Mean ± SEM absorbance values of GR-GRE binding are shown 60 minutes post-inhalation of treatment, where numeric values are inhaled drug doses in micrograms. n = 8 subjects. *p<0.05 and **p<0.01 denote statistical significance between treatments.

**Figure 4. Effect of fluticasone propionate (FP, 10⁻⁹M) and salmeterol (SLM, 10⁻⁷M) on GR-GRE DNA binding in sputum macrophages from COPD patients ex vivo.** Mean ± SEM absorbance values of GR-GRE binding are shown. n = 5 subjects. *p<0.05 denotes statistical significance between treatments.
Figure 5. Effect of fluticasone (FP, $10^{-9}$M) and salmeterol (SLM, $10^{-8}$M) on MKP-1 mRNA induction and GRE-luciferase activity in macrophage-like PMA-treated U937 cells in vitro. (A)

MKP-1 mRNA expression normalized with GAPDH (panel A) and the fold-increase in GR of stimulated vs. non stimulated cells (panel B) are shown. Results represent the mean ± SEM of three independent experiments. * $p<0.05$ and *** $p<0.001$ denote statistical significance between treatments.

Figure 6. Effect of fluticasone propionate (FP) and salmeterol (SLM) on IL-1β-induced CXCL8 in macrophage-like U937 cells. Results represent the mean ± SEM of 8 independent experiments.

* $p<0.05$ denotes statistical significance vs. FP ($10^{-11}$M) and # $p<0.01$ denotes statistical significance vs. IL-1β stimulated.