

Inhaled long-acting β_2 agonists enhance glucocorticoid receptor nuclear translocation and efficacy in sputum macrophages in COPD

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Background: Combination inhaled therapy with long-acting β_2 agonists (LABAs) and corticosteroids 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 μ g, FP 500 μ g, salmeterol xinafoate (SLM) 50 μ g, and combination FP 100 μ g + SLM 50 μ g. One hour postinhalation, sputum was induced,

nuclear proteins isolated from purified macrophages, and levels of activated nuclear GR quantified by using a GR-glucocorticoid response element ELISA-based assay.

Results: Nuclear GR significantly increased after the inhalation of FP 500 μ g ($P < .01$), but not after the inhalation of FP 100 μ g or SLM 50 μ g, compared with placebo. Interestingly, SLM in combination with FP 100 μ g increased nuclear GR levels equivalent to those of FP 500 μ g alone. This was significantly greater than either FP 100 μ g ($P < .05$) or SLM 50 μ g ($P < .01$) alone. *In vitro* in a human macrophage cell line, SLM (10^{-8} mol/L) enhanced FP (10^{-9} mol/L)-induced mitogen-activated protein kinase phosphatase-1 mRNA (5.8 ± 0.6 vs $8.4 \pm 1.1 \times 10^{-6}$ copies, $P < .05$) and $2 \times$ glucocorticoid response element-luciferase reporter gene activity (250.1 ± 15.6 vs 103.1 ± 23.6 -fold induction, $P < .001$). Addition of SLM (10^{-9} mol/L) to FP (10^{-11} mol/L) significantly enhanced FP-mediated suppression of IL-1 β -induced CXCL8 ($P < .05$). **Conclusions:** Addition of SLM 50 μ g to FP 100 μ g enhanced GR nuclear translocation equivalent to that seen with a 5-fold higher dose of FP in sputum macrophages from patients with COPD. This may account for the superior clinical effects of combination LABA/corticosteroid treatment compared with either as monotherapy observed in COPD. (J Allergy Clin Immunol 2013;■■■■:■■■-■■■.)

Key words: Long-acting β_2 agonist, corticosteroid, glucocorticoid receptor, sputum, COPD, macrophage

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It is well established in asthmatic patients that the addition of long-acting β_2 agonists (LABAs) to inhaled corticosteroids (ICS) provides more effective disease control than does 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 patients with COPD.^{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 with 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

Abbreviations used

COPD: Chronic obstructive pulmonary disease
 FP: Fluticasone propionate
 GR: Glucocorticoid receptor
 GRE: Glucocorticoid response element
 ICS: Inhaled corticosteroids
 LABAs: Long-acting β_2 agonists
 MKP-1: Mitogen-activated protein kinase phosphatase-1
 SLM: Salmeterol xinafoate

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 salmeterol xinafoate (SLM)/fluticasone propionate (FP) reduces sputum differential cell counts, sputum neutrophils and eosinophils, bronchial CD45⁺, CD8⁺, and CD4⁺ cells, and cells expressing genes for TNF- α and IFN- γ in comparison to placebo.¹⁸ These anti-inflammatory effects were also accompanied by improvements in the prebronchodilator FEV₁. 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 that LABAs may have anti-inflammatory properties similar to those of ICS.^{20,21} Studies show that 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 in which they mediate corticosteroid-dependent effects.²³ Enhanced GR activation by LABAs has been demonstrated *in vitro* in human lung fibroblasts, smooth muscle cells, and^{22,24} neutrophils²⁵ and *ex vivo* in the sputum of patients with asthma.¹⁷ However, it is unknown whether LABAs cause GR translocation after ICS therapy in patients with COPD, as this disease is much less responsive than asthma to the effects of corticosteroids.²⁶ Indeed, macrophages from patients with COPD have been shown to exhibit a poor anti-inflammatory response to corticosteroids *in vitro* than did cells from healthy smokers and nonsmokers.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. Because reduced nuclear translocation of GR contributes to corticosteroid insensitivity,^{16,26} we hypothesized that the LABA, SLM, could contribute to improve corticosteroid insensitivity in cells from patients with COPD.

METHODS**Subjects**

Eight patients diagnosed with moderate COPD (Global Initiative for Chronic Obstructive Lung Disease stages 2 and 3²⁷), less than 12% reversibility in FEV₁, and a smoking history of more than 10 pack-years participated in a randomized, double-blind, placebo-controlled crossover study. All patients were aged between 40 and 80 years and were not taking either

ICS or LABAs (Table 1). At each study visit, patients' FEV₁ value 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 was treated with ICS or LABAs for at least 1 month preceding the start of the study.

Study design

Single actuations of FP 100 μ g, FP 500 μ g, SLM 50 μ g, combination therapy FP/SLM 100 μ g/50 μ g (Seretide, GlaxoSmithKline, Stevenage, United Kingdom), 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 of 7 days between treatments to prevent any crossover effects. All patients were randomized (preassigned 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 on the basis of our previous study,¹⁷ where 7 subjects received 5 treatments, which was sufficient to detect statistical differences between ICS alone versus LABA/ICS combination in asthmatic subjects.

Sputum induction and processing

Sputum induction was performed as previously described and processed within 1 hour of collection.¹⁷ Briefly, sputum was filtered (70- μ m filter) and centrifuged (1600 rpm for 5 minutes) to obtain a cell pellet. Cells were resuspended in full RPMI-1640 media (Sigma-Aldrich, Gillingham, United Kingdom), supplemented with 1% L-glutamine, 10% FCS (Invitrogen Ltd, Carlsbad, Calif), 100 U/mL penicillin, and 100 μ g/mL streptomycin (Sigma-Aldrich). Total cell count (Kimura stain) and viability (Trypan blue exclusion) were determined before cytopins were undertaken.

Ex vivo stimulation of sputum macrophages

Sputum macrophages were isolated from whole sputum by using plastic adherence at a cell density of 0.5×10^6 cells/mL for 4 hours in a 5% CO₂ humidified atmosphere at 37°C. Nonadherent 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^{-7} mol/L), FP (10^{-9} mol/L), or a combination of both for 60 minutes. The same number of macrophage cells in culture was seeded for each treatment visit and used for each of the experiments, standardized to a cell density of 0.5×10^6 cells/mL.

Isolation of blood cells

Venous blood (80 mL) was diluted 1:1 with Hanks' buffered saline solution (Invitrogen Ltd) and layered on Ficoll-Hypaque-Plus (Amersham plc, Amersham, United Kingdom). After centrifugation (30 minutes at 1100g and 18°C), PBMCs were collected, washed, and centrifuged (250g for 10 minutes). PBMCs were resuspended in culture media and counted by using Kimura dye.

Nuclear extraction

Nuclear and cytoplasmic fractions were extracted by using a Nuclear Extraction kit (Active Motif, Carlsbad, Calif). Briefly, cells were resuspended in hypotonic buffer, vortexed, and incubated on ice for 15 minutes to extract the cytoplasmic fraction. Thereafter, the remaining nuclear pellets were resuspended in complete lysis buffer for 30 minutes on ice. The suspension was centrifuged (14,000 rpm, 10 minutes, 4°C) and the nuclear fraction

TABLE I. Patient characteristics

Age (y)	65 ± 8
Gender	2 M/6 F
GOLD stage	2 stage 2, 6 stage 3
FEV ₁ (L)	1.8 ± 0.89
Postbronchodilator FEV ₁ (% predicted)	72.1 ± 11.8
Reversibility (%)	5.6 ± 4.2
FEV ₁ /FVC ratio	60.2 ± 5.2
Smoking status	2 current, 6 ex
Smoking history (pack-years)	35.3 ± 16.2
Sputum cell counts	
Macrophages (% total), median % (range)	7 (4.8-33.4)
Neutrophils (% total), median % (range)	91.9 (39.7-93.1)
Lymphocytes (% total), median % (range)	1 (0-1.7)
Eosinophils (% total), median % (range)	0.6 (0-25.7)

F, Female; FVC, forced vital capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease; M, male.

obtained. The quality and purity of the nuclear extract was determined by using Western blotting (Fig 1, A).

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, 5 µg of nuclear extract of each sample was added to a well. Each well contained multiple copies of a specific double-stranded oligonucleotide that activated GR binds to at its consensus-binding site. We confirmed by using Western blotting in U937 cells that incubation with the positive control, FP, led to an increase in nuclear GR, which correlated to an induction in GR-GRE binding, relative to the negative control of untreated cells (Fig 1, B and 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 PBS for 10 minutes at 37°C and washed 3 times with PBS. Cells were then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, Poole, United Kingdom) for 5 minutes and then incubated with primary anti-GR antibody (H-300, Santa Cruz Biotechnology, Santa Cruz, Calif) for 3 hours followed by 30 minutes with secondary antibody Alexa 488 (Invitrogen Ltd). Coverslips were washed 3 times with PBS, rinsed in distilled water, and immediately dried. Prolong Antifade 4', 6-diamidino-2-phenylindole, dihydrochloride solution (Invitrogen Ltd) was added to delineate the nuclear boundary in each cell and help define the distribution of GR protein (green) within the respective subcellular compartments (Fig 2). Coverslips were mounted onto slides and allowed to dry for 4 hours before analysis by 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 Ltd), supplemented with L-glutamine (1%, Invitrogen Ltd) and FCS (10%, Invitrogen Ltd), and differentiated as previously described.²⁸ After washing with Hanks' buffered saline solution, cells were allowed to recover in medium supplemented with 1% L-glutamine and 0.1% charcoal-stripped FCS for 48 hours before experiments.

Mitogen-activated protein kinase phosphatase-1 mRNA expression

Differentiated U937 cells were cultured in RPMI supplemented with 0.1% charcoal-stripped-FCS and L-glutamine (1%) for 24 hours before treatment.

Total RNA was isolated from cells by using an RNeasy Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 1 µg 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 using quantitative RT-PCR with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, Calif). Primers are shown in Table II. MKP-1 mRNA levels were normalized to an endogenous reference glyceraldehyde 3-phosphate dehydrogenase, 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 by 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 was determined by luciferase assay as previously described.¹⁷

CXCL8 ELISA

Serum-starved U937 cells were incubated with IL-1β for 30 minutes, 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, United Kingdom) according to the manufacturer's instructions.

Statistical analysis

In the *in vivo* study, because sputum cells are not normally distributed, a non-Gaussian distribution was adopted and data were analyzed by using nonparametric statistical analyses using the PC analysis package Graph Pad Prism (Graph Pad Prism, San Diego, Calif). Results were analyzed by using Friedman analysis of variance including factors for period, treatment, and multiple comparisons, and applying Bonferroni's posttest correction. Comparisons between treatments were made by using the Wilcoxon matched-pairs signed rank-sum test. A *P* value of less than .05 was considered statistically significant. For the difference between treatment groups in the *in vitro* data, analyses were undertaken by using the Mann-Whitney test for comparison of 2 unpaired groups with Graph Pad Prism software. A *P* value of less than .05 was considered statistically significant.

RESULTS

Patient characteristics

The mean age of the 8 patients (6 women) was 65 ± 8 years, and the mean postbronchodilator FEV₁ was 72% ± 12% of predicted (Table I). No patient showed more than 12% reversibility to albuterol, was atopic, or had a history of asthma. Median % sputum cell counts are shown in Table I 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 with the sample taken at visit 1. However, the amount of protein extracted from the isolated macrophage nuclear extracts for the GR-GRE assay was normalized 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 5 µg.

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

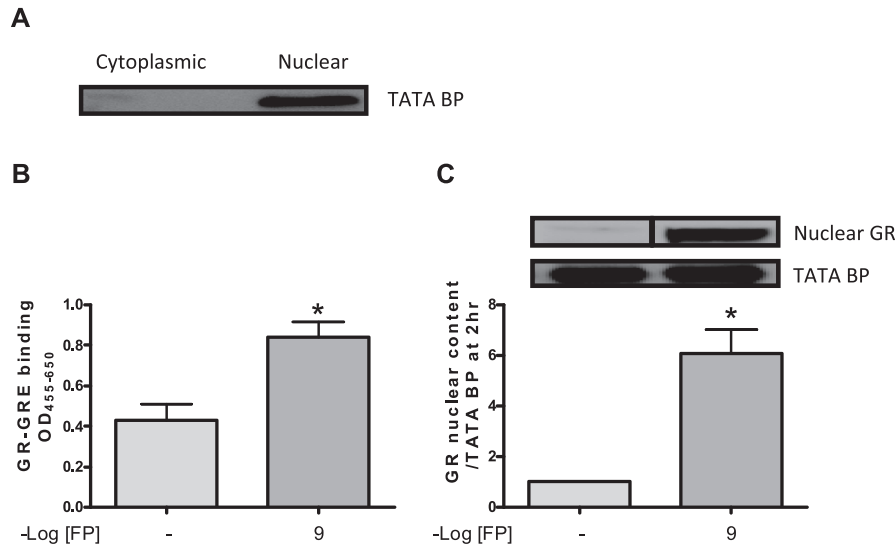


FIG 1. Validation of the quality of nuclear extract and GR-GRE binding. The quality of nuclear extracts (**A**) was determined by using Western blotting. FP-induced GR-GRE binding (**B**) and FP-induced GR nuclear localization (**C**) were assessed in U937 monocytes. Results represent the mean $\pm$ SEM of 3 independent experiments. * $P < .05$ denotes statistical significance versus untreated cells. *TATA BP*, TATA-binding protein.

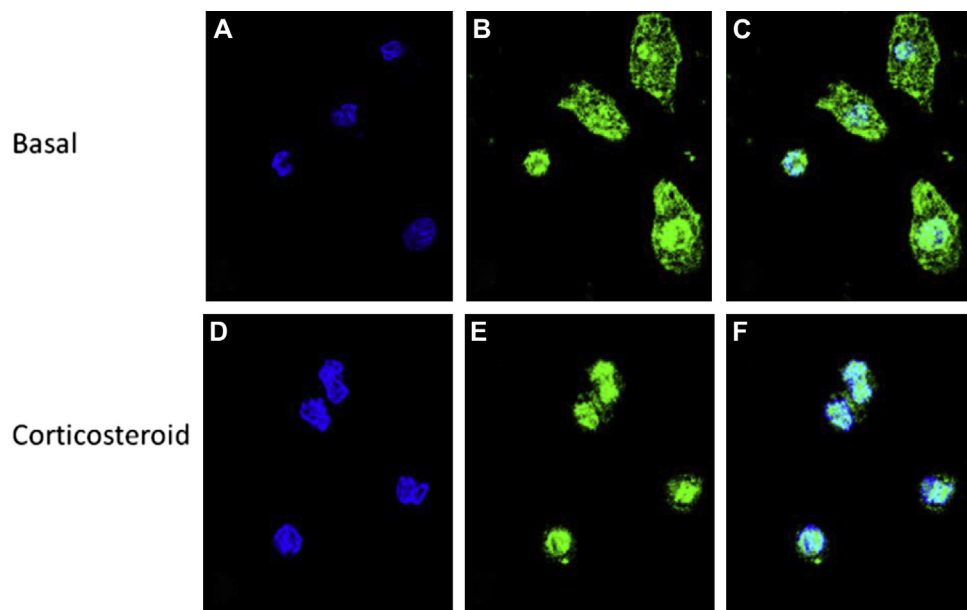


FIG 2. Confocal laser images of GR subcellular localization in sputum macrophages following corticosteroid (FP 500 μ g) treatment. **A** and **D**, 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) staining (blue channel). **B** and **E**, Cytoplasmic and nuclear GR immunostaining (green channel). **C** and **F**, Overlay of **A** and **B**.

following inhalation of FP 100 μ g or SLM 50 μ g compared with placebo (**Fig 3, A**). In contrast, FP 500 μ g significantly increased GR-GRE binding levels 2.2-fold compared with placebo (0.607 ± 0.089 vs 0.274 ± 0.038 absorbance units, $P < .01$) and induction of GR-GRE binding was significantly greater with FP 500 μ g compared with that observed with FP 100 μ g (0.607 ± 0.089 vs 0.310 ± 0.028 absorbance units, $P < .05$). Treatment with FP 100 μ g and SLM 50 μ g combination increased nuclear GR-GRE levels to levels similar to those seen with FP 500 μ g (0.598 ± 0.061 vs 0.607 ± 0.089 , respectively, $P = \text{not}$

significant). This effect observed with the combination treatment was significantly greater than that seen with placebo ($P < .01$), FP 100 μ g alone ($P < .05$), or SLM 50 μ g alone ($P < .01$). The addition of SLM 50 μ g to FP 100 μ g as combination treatment achieved an enhancement in GR activation in sputum macrophages from patients with COPD that was equivalent to that observed with a 5-fold increase in the dose of FP. That is, combination therapy allowed a 5-fold reduction in the ICS dose, yet achieved the same biological effects. Confocal microscopy documented the correlation between the GR-GRE

TABLE II. PCR primers for MKP-1 mRNA analysis

	Forward	Reverse
MKP-1	5'-GTACATCAAGTCCATCTGAC	5'-GGTCTTCTAGGAGTAGACA
GAPDH	5'-GAAGGTGAAGGTCGGAGTC	5'-GAAGATGGTGATGGGATTTTC

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; MKP-1, mitogen-activated protein kinase phosphatase-1.

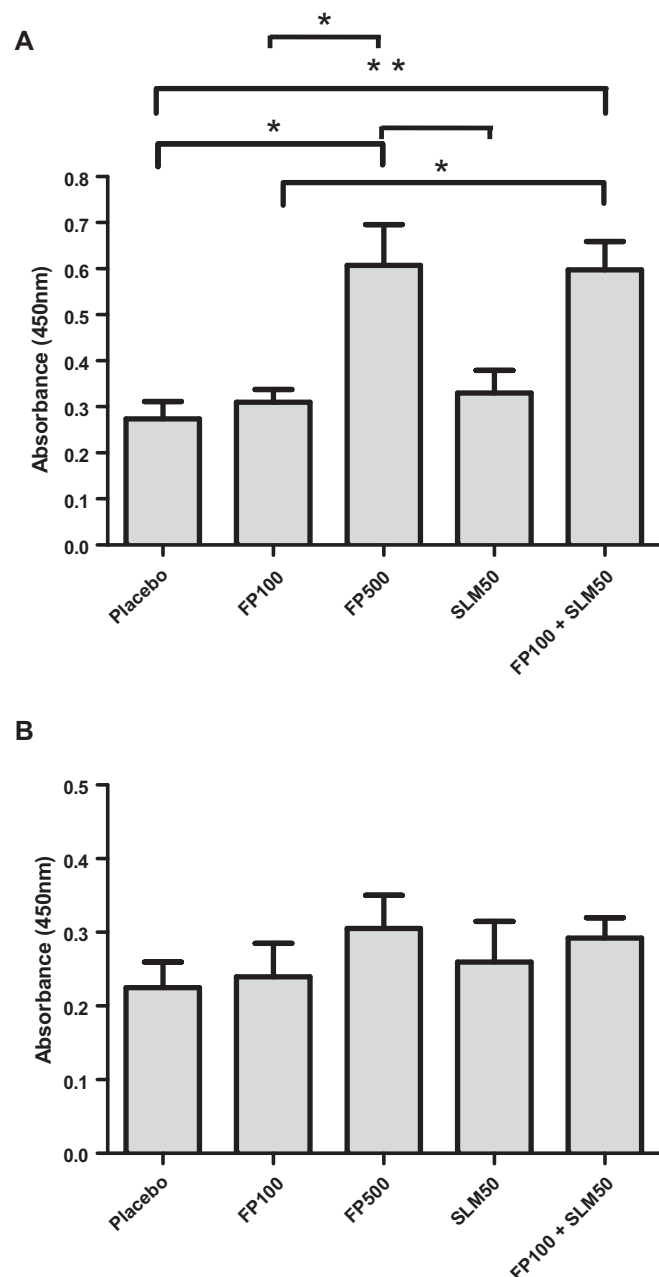


FIG 3. Effect of inhaled SLM and FP on GR-GRE binding in nuclear extracts from induced sputum macrophages (A) and PBMCs (B). Mean $\pm$ SEM absorbance values of GR-GRE binding are shown 60 minutes postinhalation of treatment, where numeric values are inhaled drug doses in micrograms. $n = 8$ subjects. * $P < .05$ and ** $P < .01$ denote statistical significance between treatments.

binding assay and the GR nuclear translocation in that treatment with corticosteroid (FP 500 μ g) led to GR nuclear translocation in sputum macrophage cells (Fig 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 the inhalation of drugs (Fig 3, B). 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 and allowed to recover for 24 hours before stimulation with SLM (10^{-7} mol/L), FP (10^{-9} mol/L), or a combination of both for 60 minutes. FP significantly enhanced nuclear GR-GRE binding compared with control unstimulated cells (0.506 ± 0.042 vs 0.252 ± 0.038 absorbance units, $P < .01$). SLM alone had no significant effect on GR-GRE binding levels compared with control (Fig 4). However, the addition of SLM to FP significantly enhanced the ability of FP alone (10^{-9} mol/L) to stimulate GR-GRE binding (0.710 ± 0.073 vs 0.506 ± 0.042 absorbance units, $P < .05$).

Induction of MKP-1 mRNA in macrophage-like U937 cells

FP (10^{-9} mol/L) alone significantly induced MKP-1 mRNA expression after 2 hours compared with the levels seen in control unstimulated cells ($5.8 \pm 0.6 \times 10^{-6}$ vs $6.4 \pm 2.5 \times 10^{-8}$ copies, $P < .001$), while SLM (10^{-8} mol/L) alone had no effect ($3.2 \pm 0.6 \times 10^{-8}$ copies, $P =$ not significant) (Fig 5, A). However, on addition to FP, SLM significantly enhanced FP-induced MKP-1 mRNA (5.8 ± 0.6 vs $8.4 \pm 1.1 \times 10^{-6}$ copies, $P < .05$).

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

SLM (10^{-8} mol/L) alone had no significant effect on a $2 \times$ GRE-luciferase reporter gene activity compared with control unstimulated cells (1.0 ± 0.6 vs 1.5 ± 0.4 -fold induction, $P =$ not significant) (Fig 5, B). In contrast, FP (10^{-9} mol/L) significantly enhanced GRE-luciferase activity compared with control (103.1 ± 23.6 -fold induction, $P < .05$). Yet, the addition of SLM to FP resulted in a highly significant increase in GRE-luciferase activity compared with that seen with FP alone (250.1 ± 15.6 vs 103.1 ± 23.6 -fold induction, $P < .001$).

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

FP (10^{-11} mol/L and 10^{-9} mol/L) significantly suppressed IL-1 β -induced CXCL8 ($28.3\% \pm 3.2\%$ and $71.2\% \pm 3.1\%$, respectively, $P < .01$), while SLM (10^{-9} mol/L) alone had no considerable effect (Fig 6). However, the addition of

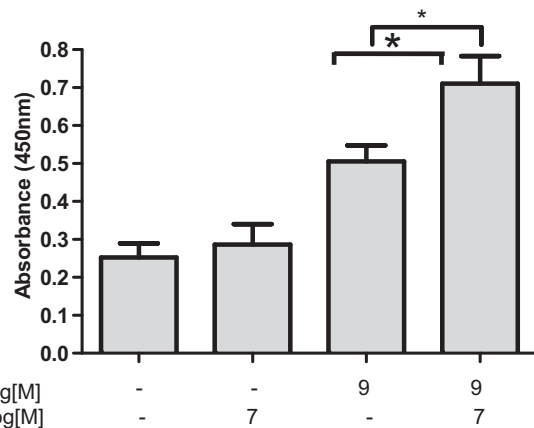


FIG 4. Effect of FP (10^{-9} mol/L) and SLM (10^{-7} mol/L) on GR-GRE DNA binding in sputum macrophages from patients with COPD *ex vivo*. Mean $\pm$ SEM absorbance values of GR-GRE binding are shown. $n = 5$ subjects. * $P < .05$ denotes statistical significance between treatments.

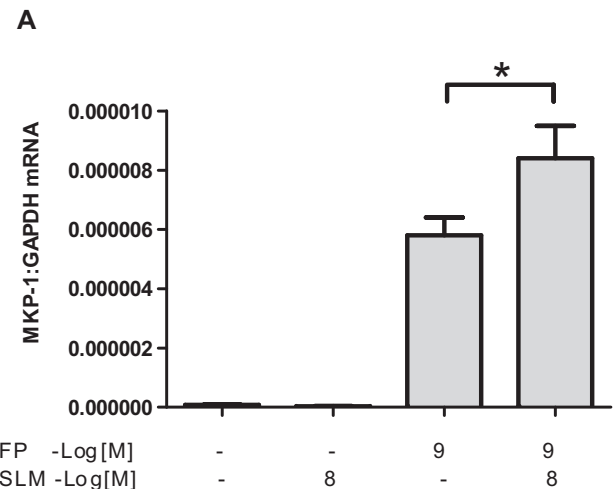
SLM (10^{-9} mol/L) to FP (10^{-11} mol/L) significantly enhanced FP-mediated suppression of IL-1 β -induced CXCL8 ($46.7\% \pm 4.8\%$ vs $28.3\% \pm 3.2\%$, $P < .05$).

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 FP 500 μ g, but not after low-dose FP 100 μ g alone or SLM 50 μ g alone. Most importantly, the addition of SLM 50 μ g to low-dose FP 100 μ g increased activated GR levels equivalent to those seen with high-dose FP 500 μ g. That is, in patients with COPD, addition of SLM to a low dose of FP achieved an enhancement of GR nuclear translocation equivalent to that observed with a 5-fold higher corticosteroid dose. Consistent with our *in vivo* patient data, we were able to demonstrate that SLM significantly enhanced the ability of FP to induce GR activation in sputum macrophages *ex vivo* and enhanced FP induction of MKP-1 mRNA and 2 $\times$ GRE reporter gene activity in a human cultured macrophage cell line *in vitro*. In addition, we were able to show a functional anti-inflammatory effect of combination therapy in that SLM significantly enhanced FP-mediated suppression of IL-1 β -induced CXCL8 in macrophage-like U937 cells.

When LABAs were introduced as an add-on therapy to ICS, 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 status³¹ 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 patients with COPD, where they are now an established treatment. The Towards a Revolution in COPD Health study demonstrated reductions in exacerbation rates and improvements in health status and lung function with SLM/FP combination compared with placebo in patients with COPD.¹⁵



B

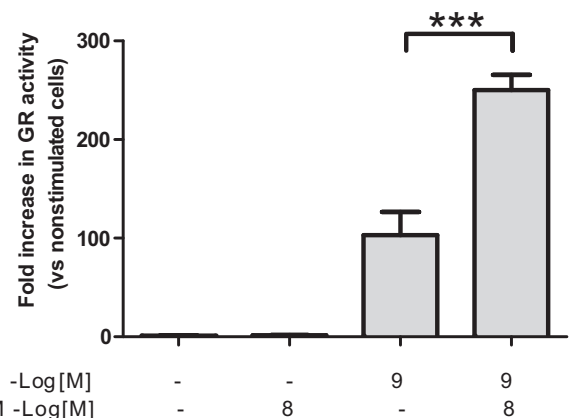


FIG 5. Effect of FP (10^{-9} mol/L) and SLM (10^{-8} mol/L) on MKP-1 mRNA induction and GRE-luciferase activity in macrophage-like PMA-treated U937 cells *in vitro*. **A**, MKP-1 mRNA expression normalized with GAPDH (Fig 5, A) and the fold-increase in GR of stimulated versus nonstimulated cells (**B**) are shown. Results represent the mean $\pm$ SEM of 3 independent experiments. * $P < .05$ and *** $P < .001$ denote statistical significance between treatments. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

The study observed a relative reduction of 17% in mortality over 3 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 with both placebo³⁴ and 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 patients with COPD.^{18,19}

Our data reveal a complementary interaction between the LABA (SLM) and the corticosteroid (FP) in that the combination therapy has a greater anti-inflammatory effect than either drug alone and was also steroid sparing, allowing a 5-fold reduction in the ICS 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

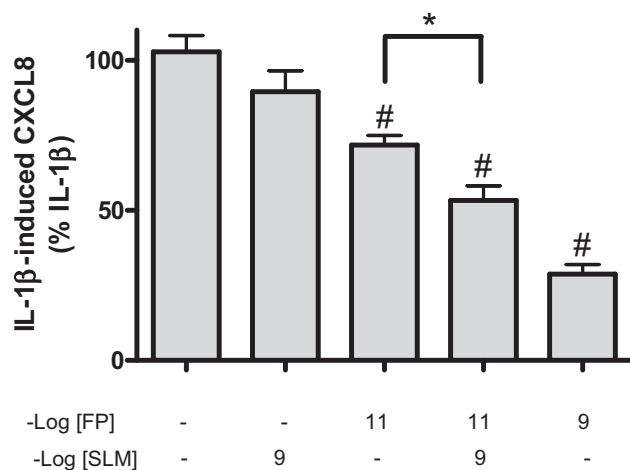


FIG 6. Effect of FP and SLM on IL-1 β -induced CXCL8 in macrophage-like U937 cells. Results represent the mean $\pm$ SEM of 8 independent experiments. * $P < .05$ denotes statistical significance versus FP (10^{-11} mol/L) and # $P < .01$ denotes statistical significance versus IL-1 β -stimulated.

for improvements in the rate of decline in lung function seen in the TORCH study.³⁵

We confirm that sputum macrophages from patients with COPD are less sensitive to corticosteroid action than are those obtained from asthmatic subjects.^{36,37} In our previous study examining the effect of corticosteroid (FP) on GR activation in sputum macrophages from subjects with mild/moderate asthma, we demonstrated that 60 minutes posttreatment, FP 100 μ g significantly enhanced GR nuclear translocation.¹⁷ Here, we show that only the higher dose of FP 500 μ g 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 (Fig 3) and this level of GR activation was maintained for at least 24 hours in culture. Because none of the patients in this study was 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 that SLM 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 have been reported in airway epithelial cells and smooth muscle cells.³⁸ Our results are consistent with literature that suggest that 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 sputum cells from asthmatic patients.¹⁷ This enhancement in GR translocation achieved by LABAs when added to corticosteroids is likely to underlie some of the clinical benefits seen with combination therapy. In addition, we demonstrated that the enhancement of GR translocation with combination therapy was associated with a functional anti-inflammatory effect in that, SLM significantly enhanced the FP-mediated suppression of IL-1 β -induced CXCL8 in U937 cells.

We were unable to assess the anti-inflammatory properties of the combination treatment in sputum macrophages *in vivo* because of 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 the inhalation of drugs will provide evidence for additional genes that are regulated in a manner similar to those described here. A role for other aspects of GR activation including GR posttranslational 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 whether 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 patients with asthma and in patients with COPD warrants further investigation, and elucidation of the precise molecular mechanisms of how LABAs (acting extracellularly via the β_2 receptor) are able to influence the 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 increases 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 patients with COPD as effectively as a 5-fold higher dose of ICS alone. This suggests that LABAs may play an important role in the benefits clinically seen with combination therapy. Our data have 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 patients with COPD, and whether this results in an alteration in sputum cytokine profiles *in vivo*.

Clinical implications: Sputum macrophages from patients with COPD are relatively corticosteroid insensitive. Inhaled SLM and FP greatly enhanced GR activation in sputum macrophages from patients with COPD.

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