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Anti-inflammatory activity of β_2-agonists in primary lung epithelial cells is independent of glucocorticoid receptor

J. Lovén, N. Svitacheva, A. Jerre, A. Miller-Larsson and S.H. Korn

ABSTRACT: In patients with asthma and chronic obstructive pulmonary disease, the addition of long-acting β_2-agonists (LABA) to glucocorticosteroids (GCS) results in better control than increasing the dose of GCS alone. In smooth muscle cells and fibroblasts, one apparent underlying mechanism involves the ability of LABAs to activate the glucocorticoid receptor (GR).

The present study investigates the effects of formoterol (FORM), salmeterol (SALM) and budesonide (BUD) on GR activation in bronchial epithelial cells *via* tumour necrosis factor-α-stimulated granulocyte-macrophage colony-stimulating factor (GM-CSF) release, GR nuclear translocation and GR-regulated reporter gene activity.

Both BUD and FORM inhibited GM-CSF release by $\leqslant$50%. The combination of these two drugs, in clinically relevant concentrations, inhibited GM-CSF release by 85% down to unstimulated levels. A similar inhibition was obtained when combining BUD and SALM. The ability of FORM to inhibit GM-CSF synthesis was not altered by small interfering RNA-mediated depletion of GR and FORM nor SALM-induced GR translocation into the cell nucleus. In addition, FORM did not activate GR-regulated reporter gene activity (SALM was not tested), in contrast to the clear effect of BUD.

It was concluded that in bronchial epithelial cells, inhibition of granulocyte-macrophage colony-stimulating factor synthesis by formoterol and salmeterol does not act *via* previously demonstrated glucocorticoid receptor-related mechanisms, suggesting an alternative pathway in these cells.

KEYWORDS: Anti-inflammatory, glucocorticoid receptor, granulocyte-macrophage colony-stimulating factor, long-acting β_2-agonists, small interfering RNA, Western blot

Glucocorticoids (GCS) and long-acting β_2-agonists (LABAs) are the two most effective drugs in the treatment of asthma; they result in better asthma control than higher doses of GCS alone, especially when used in combination [1–3]. The addition of formoterol (FORM) to budesonide (BUD) leads to improved disease control in asthma [1, 3] and chronic obstructive pulmonary disease [4, 5]. Clearly the bronchodilator activity of FORM and other LABAs contributes to this benefit, but recent evidence has demonstrated that β_2-agonists can contribute to the control of asthma *via* other mechanisms. For example, β_2-agonists (or other cyclic adenosine monophosphate (cAMP)-elevating agents) exert anti-inflammatory activity [6–9] and reduce airway smooth muscle proliferation [10].

β_2-Agonists have the potential to activate the glucocorticoid receptor (GR) in the absence of a GR ligand, one of the mechanisms proposed for the anti-inflammatory activity of β_2-agonists [10–12]. Primary human lung fibroblasts, airway and vascular smooth muscle cells, macrophages and bronchial epithelial cells were exposed to salmeterol (SALM) or FORM; this led to GR translocation into the nucleus and binding of the GR to glucocorticoid responsive elements (GREs). The events were blocked by propranolol, a β-receptor antagonist, indicating that the process was receptor-dependent. In a related study, performed in rat hepatoma cells, induction of cAMP by forskolin increased GR protein and mRNA levels within 4 h, as demonstrated by ^{3}H-dexamethasone binding assays and RNA blot hybridisation, due to prolongation of the GR message half-life [13]. However, increased binding of ^{3}H-dexamethasone may also reflect an altered phosphorylation state of the GR, induced by cAMP-activated protein kinase A (PKA). It is

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known that seven different phosphorylation sites on the GR regulate the switch between the inactive, nonhormone-binding form of the GR and the active GCS-binding receptor [14, 15]. In addition, a change in the ''active'' phosphorylation state can further enhance the activation state of the GR, resulting in a more potent receptor with, for example, greater transactivation capacities [14]. This is further supported in a study by DOUCAS *et al.* [16], in which PKA is shown to interact with the GR, thereby altering its activity (possibly by phosphorylation of the GR) and subsequently binding to nuclear factor (NF)-κB.

Airway epithelial cells amplify inflammation in asthma by generating a wide variety of mediators, including granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-8 [17]. Airway epithelial cells are also the first cells to be exposed to therapeutic GCS and β_2-agonists, and the combined action of medications from these two classes reduces the secretion of pro-inflammatory mediators [9, 18, 19]. In the present study, the mechanism behind the previously described anti-inflammatory activities of FORM [9] was investigated, testing whether the concept that FORM activates the GR is also applicable in human bronchial epithelial cells. FORM and SALM were studied in the absence and presence of BUD, in clinically relevant concentrations at a clinically relevant ratio. The chief findings were that BUD and FORM, applied in combination, provided a superior inhibition of tumour necrosis factor (TNF)-α-triggered GM-CSF release by normal human bronchial epithelial cells, compared with either drug alone. A similar inhibition of GM-CSF was obtained with low (10^{-12} M BUD/10^{-10} M FORM) and high (both 10^{-6} M) concentration combinations. The observed effects could not be attributed to activation of the GR by FORM, as demonstrated by, for example, small interfering (si)RNA and GR nuclear translocation. Similar results were obtained with SALM. In contrast to earlier publications [11, 12] concerning various cell types, the evidence presented in the present paper indicates that another mechanism is responsible for the anti-inflammatory activities of FORM in normal human bronchial epithelial cells.

MATERIALS AND METHODS

Cell culture

Primary normal human bronchial epithelial (NHBE) cells (Clonetics NHBE 7310; Clonetics, San Diego, CA, USA) were obtained from Biowhittaker (Västra Frölunda, Sweden) and cultured in bronchial epithelial cell growth medium (BEGM) according to the manufacturer's instruction (Clonetics). During the experiments, cells were grown in incomplete BEGM medium, lacking hydrocortisone, retinoic acid and epinephrine, hereafter referred to as -BEGM medium. At ~60% confluency, experiments were commenced by starving the cells in -BEGM medium for 24 h; thereafter, TNF-α, FORM, SALM and/or BUD were then added for various lengths of time and at various concentrations. The experiments were terminated at 80–90% confluency, either by washing the cells once with ice-cold PBS (Western blot) or by sampling the cell medium and measuring the DNA content in the corresponding wells (ELISA). Experiments were repeated at different passage numbers (p3–p6).

The human bronchial epithelial cell line ChaGo-K1 was obtained from the American Type Culture Collection (Rockville, MD, USA). ChaGo-K1 cells were propagated in RPMI 1640 medium (Invitrogen, Paisley, UK) with Glutamax™-1 (L-analyl-L-glutamine; Invitrogen), supplemented with 10% foetal calf serum (FCS; Gibco, Auckland, New Zealand), 1% nonessential amino acids (Invitrogen), 1% sodium pyruvate (Invitrogen), and 1% 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Invitrogen). ChaGo-K1 cells stably transfected with pSV-β-galactosidase reporter vectors (Promega, Madison, WI, USA) were used to monitor GRE and 12-*O*-tetradecanoylphorbol-13-acetate response element (TRE)-mediated effects: ChaGo-GRE with an engineered pMAMneo vector that includes the regulatory sequence 4×GRE TATA fused to plasmid DNA encoding the reporter gene lacZ, and ChaGo-TRE with an engineered pMAMneo vector that includes the regulatory sequence 5×TRE TATA fused to plasmid DNA encoding the reporter gene lacZ. At ~60%, confluency cells were exposed to 4-β-phorbol-12-myristate-13-acetate (PMA), FORM and/or BUD for 24 h at various concentrations. Experiments were terminated at 80–90% confluency by adding a colour substrate to stop β-galactosidase activity (reporter gene assay).

siRNA transfection

All siRNA duplexes used in the current study were pre-synthesised and ordered through Dharmacon Research, Inc. (Lafayette, CO, USA). Human bronchial epithelial cells (ChaGo-K1 and NHBE cells) were grown in six- or 24-well plates, respectively, and human GR siRNA duplexes were transfected into the cells using Oligofectamine™ (Invitrogen), according to the manufacturer's instructions with the following slight alterations. Prior to transfection, cells were washed once with 1 mL FCS-free culture medium (ChaGo cells) or 1 mL bovine pituitary extract (BPE)-free culture medium (NHBE cells) and subsequently re-fed with 600 μL fresh FCS/BPE-free culture medium before 400 μL of the appropriate transfection mix was added to each well. Cells were then incubated for 4 h in a CO_2 incubator (5% CO_2, 95% humidity, 37°C). Following incubation, 1 mL of fresh culture medium containing double FCS/BPE was added to each well and cells were then re-incubated. At 24 h post-transfection, cells were washed twice with PBS (without calcium, magnesium and sodium bicarbonate) and experiments were initiated by starving the cells with 1 mL -BEGM medium. At 48 h post-transfection, total proteins were either harvested for Western blot analysis, to determine the knockdown of GR, or propagated further to perform functional experiments (reporter assays and ELISA).

Western blot analysis of GR translocation

Nuclear and cytosolic extracts from stimulated or unstimulated cells were prepared at the indicated time-points according to EICKELBERG *et al.* [11]. Cells were scraped from 60-mm dishes into Eppendorf tubes in 200 μL of low salt buffer (20 mM hydroxyethyl piperazine ethane sulphonic acid (HEPES), pH 7.9; 10 mM KCl; 0.1 mM Na_3VO_4; 1 mM EDTA; 0.2% Nonidet P-40; 10% glycerol supplemented with a set of proteinase inhibitors (Complete™; Roche Diagnostics, Mannheim, Germany)). After 10 min of incubation on ice, the samples were centrifuged at 13,000×*g* for 2 min at 4°C, and the supernatants were taken as cytosolic extracts. Pellets containing nuclei were resuspended in high salt buffer (20 mM HEPES, pH 7.9; 420 mM NaCl; 10 mM KCl; 0.1 mM $NaVO_4$; 1 mM EDTA;

20% glycerol, supplemented with Complete™), and nuclear proteins were extracted by shaking on ice for 30 min. Samples were then centrifuged at 13,000 × *g* for 10 min at 4°C, and the supernatants were taken as nuclear extracts. To determine the knockdown of the GR after transfection of cells with siRNA, total cell proteins were isolated [20]. Cells were scraped from six-well plates into Eppendorf tubes in 100 μL lysis buffer pH 6.5 (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, 10 mM Na_3VO_4, 0.1% Nonidet P-40, 0.5 mM dithiothreitol and 100 μM NaF, supplemented with Complete™) and incubated for 10 min on ice. Lysates were frozen and, before use, thawed and cleared by centrifugation at 16,000 × *g* at 4°C for 10 min. In order to load equal protein amounts for gel analysis, protein concentrations were determined using the Bradford method (Dc Protein Assay; Bio-Rad, Hercules, CA, USA). Cytoplasmic proteins (80 μg), nuclear proteins (20 μg) and total cell proteins (30 μg) were mixed with 4× Laemmli sample buffer. Samples were boiled for 8 min and loaded onto a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose (Amersham Pharmacia Biotech, Amersham, UK), and membranes were subsequently blocked overnight in 5% milk/0.05% Tween-20 in PBS at 4°C. Membranes were washed twice for 5 min in PBS/0.05% Tween and incubated with the primary antibody against GR for 1.5 h in PBS/0.5% Tween/5% milk on a shaking platform at room temperature. Membranes were washed three times for 15 min in PBS/0.5% Tween and incubated with a peroxidase-conjugated secondary antibody in PBS/0.5% Tween/5% milk for 1 h at room temperature. After three 10-min washes with PBS/0.5% Tween and one final wash in PBS, conjugated peroxidase was detected by chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech). As a control for the amount of proteins loaded, membranes were evaluated for actin, a cytoskeletal protein not influenced by the drugs. To quantify the nuclear import of GR, the density of the lanes was measured and analysed *via* ImageQuant (Amersham/GE Healthcare, Uppsala, Sweden) and was presented graphically.

Reporter gene assays

Reporter gene analysis was based on β-galactosidase activity in lysates prepared from two stable transfectants of ChaGo-K1 cells grown in 96-well plates. BUD exposure to ChaGo-GRE cells induced the regulatory GRE promoter sequence, resulting in activation of the reporter enzyme molecule β-galactosidase. Exposure of ChaGo-TRE cells to 10 ng·mL^{-1} PMA stimulated, *via* the transcription factor activator protein (AP)-1, the TRE promoter sequence, which subsequently resulted in β-galactosidase activity. Twenty-four hours after stimulation, cells were treated for 1 h at 37°C with the appropriate substrate: ChaGo-GRE with o-nitrophenyl-β-D-galactoparanoside and ChaGo-TRE with 4-methylumbelliferyl-β-D-galactoparanoside. During this period, β-galactosidase hydrolysed the colourless substrates to either o-nitrophenol (yellow in colour) or 7-hydroxy-4-methylcoumarin (pink in colour). After termination of the hydrolysis reactions, absorbance was measured at 420 nm for ChaGo-GRE lysates, while fluorescence levels (excitation 360/40; emission 460/40) were determined for ChaGo-TRE lysates.

DNA determination and ELISA

Amounts of DNA were determined *via* incorporation of Hoechst H33342 (Sigma Aldrich, Stockholm, Sweden), measuring fluorescence at excitation (λ_{ex}) and emission (λ_{em}) wavelengths λ_{ex} 360/λ_{em} 460, according to a modified method from BLAHETA *et al.* [21]. After centrifuging the plates and removing the cell medium, the 24-well plates were kept on ice, as follows. A measurement of 600 μL PBS (or standard curve samples) were added to the wells, followed by 400 μL of 10 μg·mL^{-1} H33342. The plates were covered with foil and incubated for 30 min at room temperature. The fluorescence was measured and DNA amounts were calculated in μg·mL^{-1}. GM-CSF levels were determined for each experimental condition using ELISA kits from R&D Systems (Abingdon, UK), according to the manufacturer's instructions. The levels of GM-CSF were expressed in μg·mL^{-1} culture medium, and related to the DNA content per well. All samples were analysed in duplicate.

Data analysis

Experiments were repeated at least three times. All reporter gene activity and cytokine data are presented as arithmetic mean ± SEM. Vehicle-treated cells are used as controls. For statistical analysis, the unequal variance unpaired t-test was used, and data were considered significant at $p \leqslant 0.05$.

The upregulation of GRE activity and the inhibition of TRE activity were calculated as follows:

$$\%\ \text{upregulation} = ((\text{Abs drugs}/\text{Abs control}) - 1) \times 100 \quad (1)$$

$$\%\ \text{inhibition} = (1 - (\lambda_{em}\ \text{drugs}/\lambda_{em}\ \text{PMA control})) \times 100 \quad (2)$$

where Abs is absorbance.

RESULTS

Inhibition of TNF-α-induced GM-CSF release from NHBE cells by budesonide and formoterol

The effect of BUD and/or FORM (10^{-12}–10^{-6} M for 18 h) on GM-CSF release by NHBE cells was investigated after stimulation of cells with TNF-α (10 ng·mL^{-1}). On average, TNF-α induced GM-CSF levels six-fold, from ~200 pg·mL^{-1} to 1,100 pg·mL^{-1}. BUD or FORM reduced the release of GM-CSF by ⩽50% in a concentration-dependent fashion (fig. 1a). A similar concentration-dependent inhibition was seen for SALM (data not shown). The effect of FORM was suboptimal at 10^{-11} M and optimal at ⩾10^{-10} M. The efficacy of BUD was suboptimal at 10^{-9} M and maximal at ⩾10^{-8} M. When cells were treated with BUD and FORM combined at various concentrations and ratios, a further decrease in GM-CSF by 85% was observed, even to below basal levels (fig. 1b). Thus, in contrast to the partial inhibition obtained by the agents alone, combining BUD with FORM completely blocked the triggering of GM-CSF release, even at lower concentration combinations.

The BUD/FORM ratio of 35:1 that is used commonly in the clinic was also tested with BUD at 10^{-8} M and FORM at 2.8×10^{-10} M or a 10-fold lower concentration of the fixed combination (BUD 10^{-9} M, FORM 2.8×10^{-11} M) for 24 h (fig. 1c). As for other concentrations, BUD and FORM in combination resulted in a more potent inhibition of TNF-α-induced GM-CSF release compared with the effect of the monocomponents. A similar effect was observed when BUD was combined with SALM at 10^{-9} and 10^{-8} M (fig. 1c).

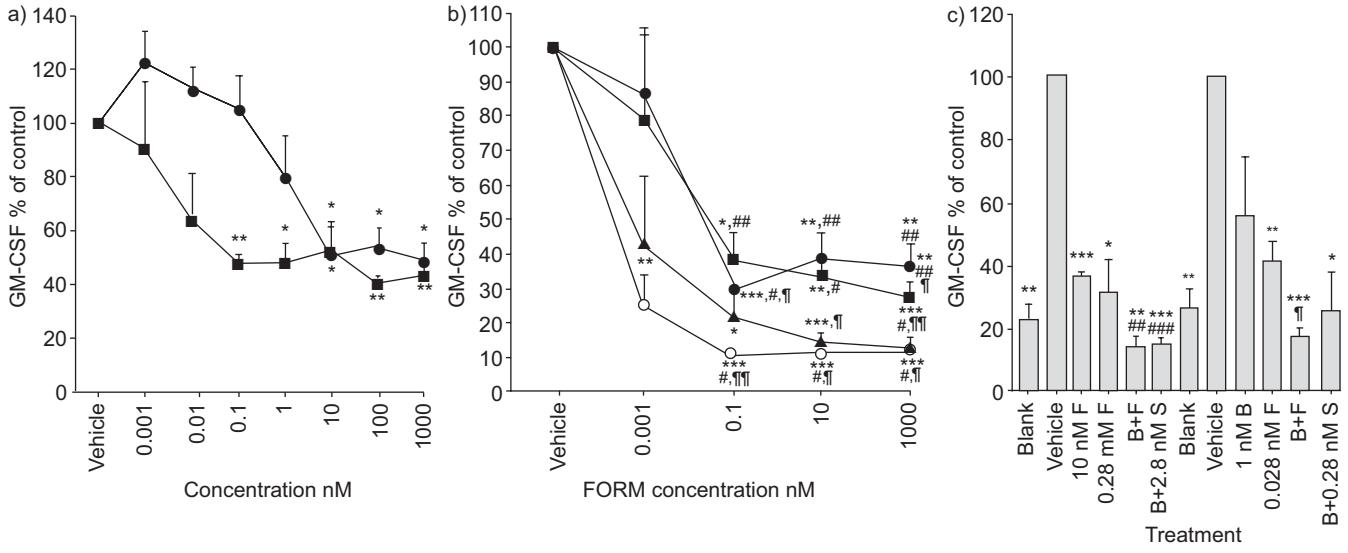

FIGURE 1. Suppression of granulocyte-macrophage colony-stimulating factor (GM-CSF) release by the budesonide (BUDB)/formoterol (FORMF) or BUD/salmeterol (SALMS) combination. Normal human bronchial epithelial cells were stimulated for 18 h with tumour necrosis factor-α (10 ng·mL^{-1}) plus BUD and/or FORM at the indicated concentrations (10^{-12}–10^{-6} M). Effects of a) monocomponents (●: BUD; ■: FORM) and b) combinations (●: BUD 0.001 nM; ■: BUD 0.1 nM; ▲: BUD 10 nM; ○: BUD 1000 nM) are shown. Each data point represents the mean±SEM of three independent experiments performed in duplicate. c) Cells were exposed to BUD/FORM combinations for 24 h in the clinically relevant ratio (35:1); SALM in combination with BUD was also tested. *: $p<0.05$ *versus* control (vehicle-treated cells); **: $p<0.01$ *versus* control (vehicle-treated cells); ***: $p<0.001$ *versus* control (vehicle-treated cells); #: $p<0.05$ *versus* the corresponding BUD concentration; ##: $p<0.01$ *versus* the corresponding BUD concentration; ###: $p<0.05$ *versus* the corresponding BUD concentration; ¶: $p<0.05$ *versus* the corresponding FORM concentration; ¶¶: $p<0.01$ *versus* the corresponding FORM concentration.

Test of possible formoterol effects on activation of GR

In order to explore whether FORM was able to activate the GR and whether the anti-inflammatory efficacy of FORM was regulated *via* the GR, Western blot assays were performed (to investigate GR translocation from cytoplasmic to nuclear compartments), GRE/TRE-regulated reporter gene assays were performed (to investigate GR activation of transcription), and GR-depletion experiments were completed *via* siRNA.

BUD induced the concentration- and time-dependent translocation of GR into the nucleus within 15 min, where it remained for at least 4 h. At 6 h, the nuclear signal had returned to

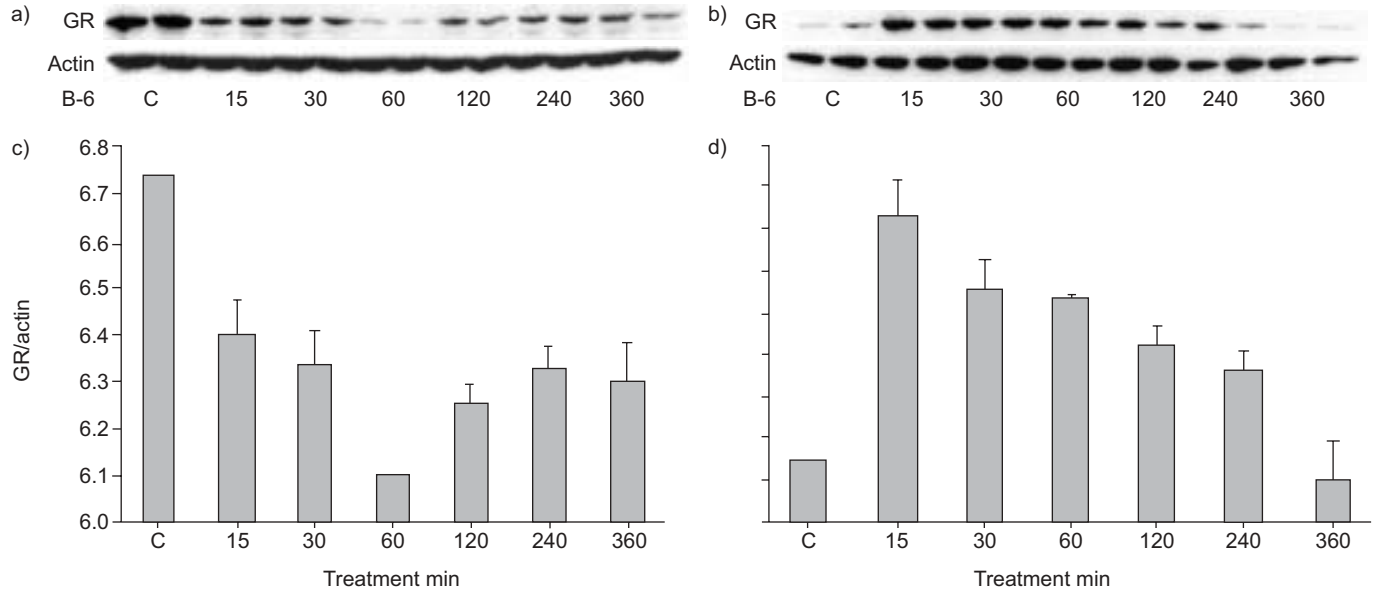

FIGURE 2. A time-dependent glucocorticoid receptor (GR) nuclear translocation by 10^{-6} M budesonide (B) is shown. a) Cytoplasmic and b) nuclear proteins were detected by Western blot analysis for GR and actin. The blots are representative of three independent experiments with similar outcomes. c) Cytoplasmic and d) nuclear GR translocation of the corresponding blot, measured by optical densitometry. B was able to translocate the GR into the nucleus, with a peak at 60 min. C: control.

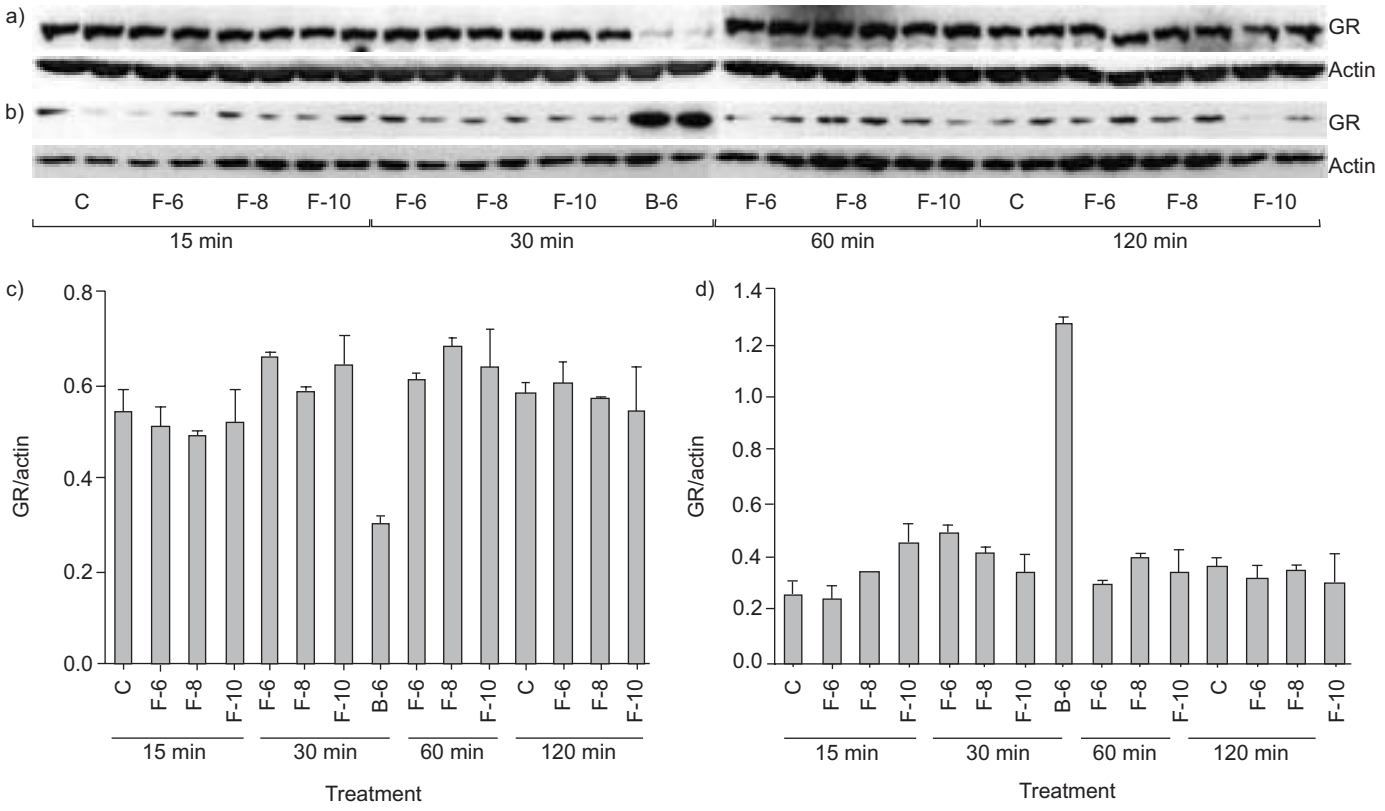

FIGURE 3. Lack of glucocorticoid receptor (GR) nuclear translocation by the long-acting β_2-agonist formoterol (F) in contrast to a time- and concentration-dependent GR translocation by budesonide (B) is shown. a) Cytoplasmic and b) nuclear proteins were detected by Western blot analysis for GR and actin. Each blot is a representative of two independent experiments with similar outcomes. c) Cytoplasmic and d) nuclear GR translocation of the blots presented, as measured by optical densitometry. F showed no GR nuclear translocation at neither timepoint nor concentration tested. This is in contrast to a clear translocation by B in the same experiment. C: control; F-6: 10^{-6} M F; F-8: 10^{-8} M F; F-10: 10^{-10} M F; B-6: 10^{-6} M B.

normal (fig. 2). Neither FORM (fig. 3) nor SALM (fig. 4) induced translocation at any of the time-points or concentrations examined. In addition, FORM did not alter GR translocation induced by BUD (fig. 5).

In order to investigate possible additive effects of BUD and FORM, GRE and TRE reporter systems were employed. The GRE reporter system is regulated by the anti-inflammatory GR and the TRE reporter system is activated by the fos-jun heterodimer AP-1. In the GRE-dependent system, FORM had no independent activity (except at the highest concentration) and did not influence activation by BUD in either a positive or negative direction (fig. 6). Various concentration combinations were tested (data not shown); all resulted in the same lack of effect of FORM on BUD's ability to increase GRE activity.

When the compounds were tested in a TRE-dependent reporter system, both BUD and FORM exhibited modest, concentration-dependent inhibition of gene expression (data not shown). When they were added together in various combinations, the inhibitory effect was greater than for either component alone, confirming the results seen in NHBE cells. These results prove that the lack of effect of FORM on GRE activity is not due to toxicity but is indeed due to the inability of FORM to (further) activate the GR.

To test whether the suppression of cytokine synthesis by either BUD or FORM is dependent on the function of the GR, NHBE cells were transfected with siRNA against the GR, resulting in an almost total reduction of the GR expression in the cells within 48 h (fig. 7a represents a Western blot taken 72 h post-transfection). This reduction was due to the siRNA-mediated knockdown of the GR, and not due to nonspecific toxicity, as cells treated with scramble and lamin siRNAs show a clear GR band. In addition, by visual inspection, the general morphology of the cells did not change due to GR siRNA treatment.

In control-transfected cells, both BUD and FORM were able to inhibit TNF-α-induced GM-CSF levels, resulting in additive inhibition when cells were exposed to both drugs. Reduction of GR levels by specific siRNA inhibited only the effects of BUD on TNF-α-induced GM-CSF levels, whereas the effects of FORM were unchanged (fig. 7).

DISCUSSION

The rationale for β_2-agonist use in asthma is based principally on their bronchodilation properties; however, other effects on bronchial epithelial and inflammatory cells may contribute to better asthma control at lower GCS concentrations [22–24]. For example, β_2-agonists inhibit histamine release from mast cells, reduce plasma exudation by preventing endothelial cells separation, inhibit cytokine release from bronchial epithelial

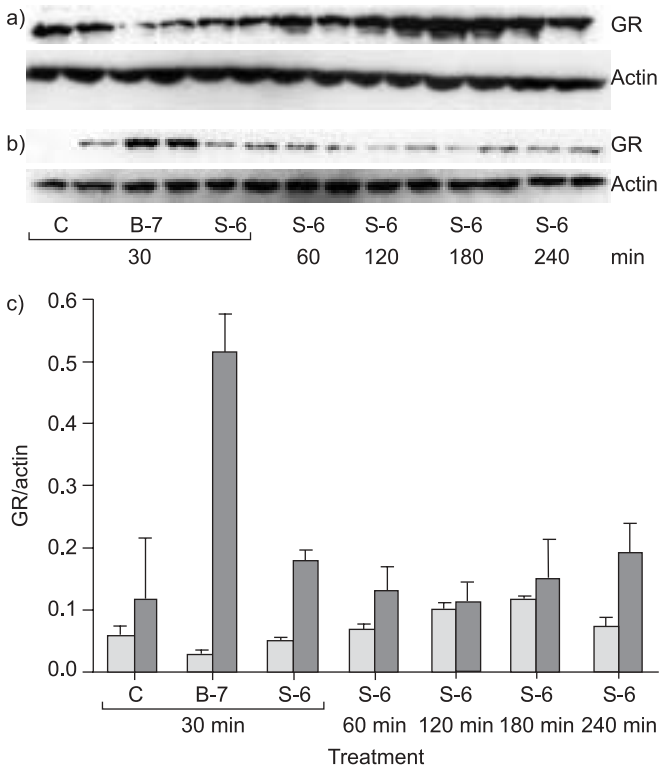

FIGURE 4. The lack of glucocorticoid receptor (GR) nuclear translocation by the long-acting β_2-agonist salmeterol (S) in contrast to a time- and concentration-dependent GR translocation by budesonide (B) is shown. a) Cytoplasmic and b) nuclear proteins were detected by Western blot analysis for GR and actin. The blot is a representative of two independent experiments with similar outcomes. c) Cytoplasmic (□) and nuclear (■) GR translocation as measured by optical densitometry. S showed no GR nuclear translocation at the timepoints tested in contrast to a clear translocation by B in the same experiment. C: control; B-7: 10^{-7} M B; S-6: 10^{-6} M S.

cell and fibroblasts, and block activation of sensory nerves [8, 9, 22]. One of the potential underlying mechanisms is the activation of the GR by β_2-agonists, as previously described in human lung fibroblasts and airway/vascular smooth muscle cells [10, 11]. In addition, a recent paper by USMANI *et al.* [12] demonstrated GR translocation by SALM in sputum-derived epithelial cells and macrophages from asthmatic patients.

The present study focused on TNF-α-stimulated GM-CSF production because this cytokine is secreted in increased amounts by bronchial epithelial cells of patients with asthma and contributes to the pathogenesis of airway inflammation by supporting the influx of activated eosinophils [25, 26]. GM-CSF is able to promote the differentiation, activation and survival of eosinophils and other inflammatory cells, a process triggered by various stimuli, including TNF-α, environmental particles, oxidants *etc.*, that result in the activation of pro-inflammatory transcription factors, such as NF-κB and AP-1, and subsequent transcription of GM-CSF by the airway epithelium [18, 27, 28]. In the present paper, BUD, FORM and SALM concentration-dependently decreased GM-CSF levels by ~50%. In combination, their efficacy was at least additive, resulting in a decrease of GM-CSF to basal levels. When BUD and FORM were administered in the ratio that is most common for Symbicort® (AstraZeneca, Lund, Sweden) in clinics (35:1), it was possible to observe a shift of the dose–response curve, such that BUD/FORM combinations of 10^{-9} M/2.8×10^{-11} M reduced stimulated GM-CSF levels to a similar degree, as did high concentration combinations of 10^{-6} M/10^{-6} M. This fits very well into the described clinical observations that addition of FORM to BUD gives equal or better asthma control than a four-fold higher concentration of BUD alone [1].

The present authors next investigated whether this anti-inflammatory function of the LABAs in NHBE cells was regulated through a ligand-independent activation of the GR. Nuclear translocation and activation of the GR by β_2-agonists has been described in human lung fibroblasts, airway/vascular smooth muscle cells and sputum-derived epithelial cells and macrophages [10–12]. However, in NHBE cells in the present study, neither FORM nor SALM translocated GR into the nucleus, at any of the time-points or concentrations tested. This was reflected in a lack of GRE activity in the reporter system by FORM. As expected, BUD induced both GR nuclear translocation and GRE activity. FORM did not enhance the nuclear translocation of the GR induced by BUD, which was again reflected in the GRE reporter results. These results are in conflict with the results of USMANI *et al.* [12], in which GR nuclear translocation occurred (although very weakly, by only 8%) with SALM in sputum epithelial cells, and GR nuclear translocation induced by fluticasone propionate (FP) was enhanced by SALM (by 12% as compared with FP alone). In the same study [12], SALM alone had no effect on GR translocation in bronchial epithelial cell line BEAS2B; in addition, GRE reporter in these cells was not affected, although SALM enhanced those effects induced by FP. Similarly, at an earlier date, both SALM and FORM have been shown to enhance dexamethasone-induced GR binding to GRE and dexamethasone-induced production of secretory leukocyte protease inhibitor (a GRE-dependent process), but neither SALM nor FORM were found to enhance GR translocation [29].

In the present study, FORM and BUD were able to reduce AP-1-regulated TRE activity independently by 30–40%. Additive TRE inhibition was obtained when combining FORM and BUD, which was compatible with the results for GM-CSF production. Additional experiments, in which the GR was knocked out largely by using GR-specific siRNA, demonstrated that the inhibiting effects of FORM on GM-CSF were sustained after removal of the GR. This is in agreement with results obtained previously, in which the GR antagonist RU486 did not change the reducing capacity of FORM on GM-CSF [9]. However, since, on some occasions, RU486 has been shown to behave as an agonist, confirming the results with the novel technique using siRNA clarifies the mechanism. Taken together, these results confirm the lack of involvement of the GR in the anti-inflammatory activities of FORM in bronchial epithelial cells. In their recent paper, NIE *et al.* [30] described an alternative mechanism for the anti-inflammatory activity of β_2-agonists. In human airway smooth muscle cells, SALM was shown to inhibit histone H4 acetylation and binding of NF-κB (p65) to DNA, resulting in a diminished TNF-α-induced eotaxin release. The inhibition was not due to a reduced p65 nuclear translocation, suggesting instead a nuclear interaction at the CREB binding protein (CBP)/p300. Binding of GR and/

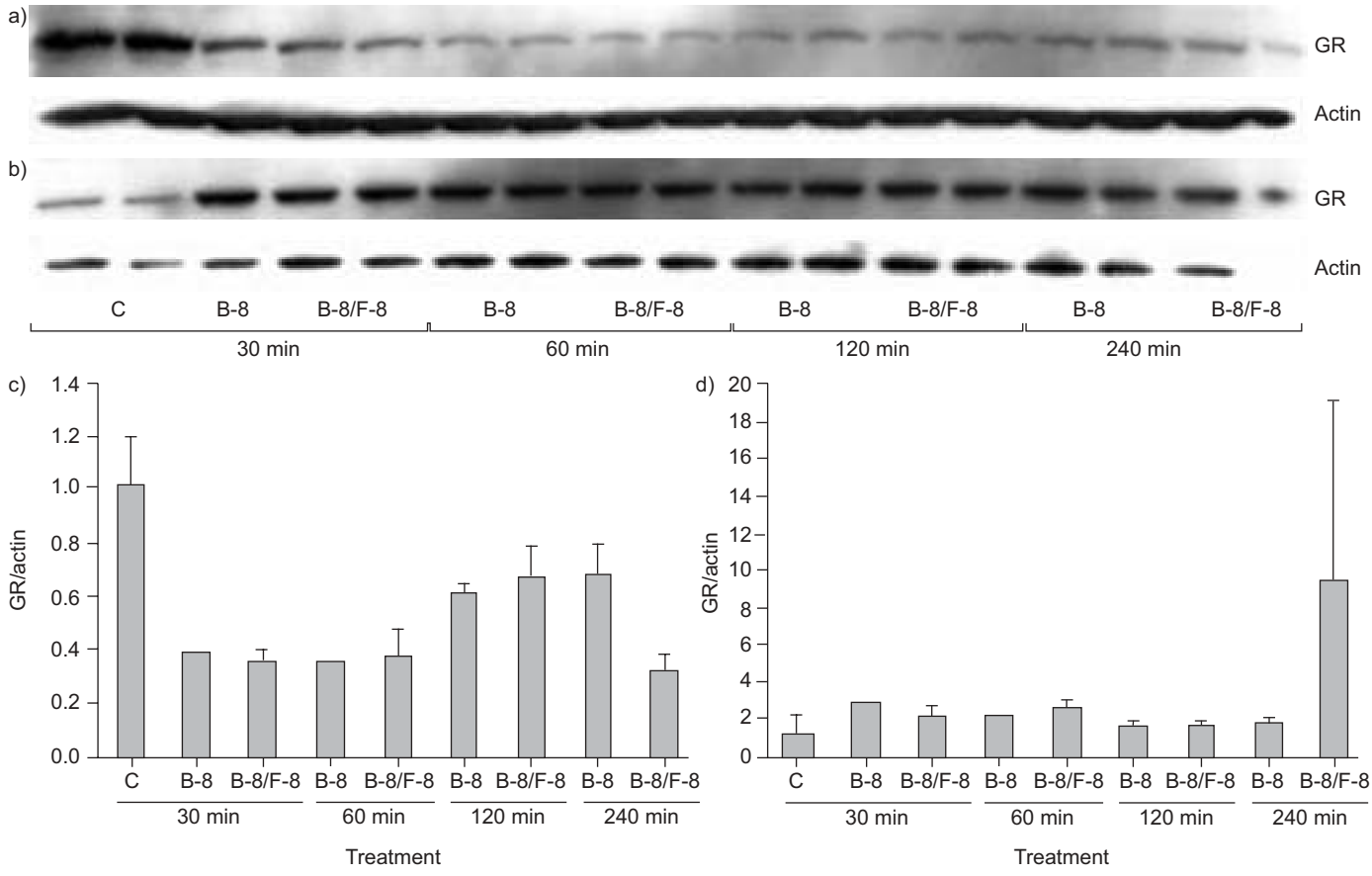

FIGURE 5. Inability of formoterol (F) to affect glucocorticoid receptor (GR) translocation induced by budesonide (B). Western blot from normal human bronchial epithelial cells exposed to B and F. a) Cytoplasmic and b) nuclear proteins were detected with GR and actin antibodies. Each blot is representative of two independent experiments with similar outcomes. c) Cytoplasmic and d) nuclear GR translocation of the blots presented, as measured by optical densitometry. C: control; B-8: 10^{-8} MB; F-8 10^{-8} M F.

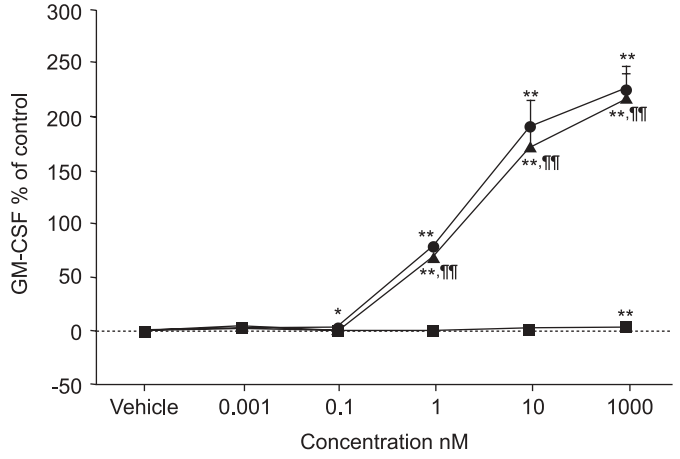

FIGURE 6. The failure of formoterol (FORM) to affect budesonide (BUD)-mediated glucocorticoid receptor (GR) activity in a reporter system is shown graphically through the effects of BUD and/or FORM on glucocorticoid response element (GRE) reporter gene activity in ChaGo-GRE cells. ●: BUD; ■: FORM; ▲: BUD+1 nM FORM. Each data point represents the mean ± SEM of three independent experiments performed in duplicate. GM-CSF: granulocyte-macrophage colony-stimulating factor. *: $p<0.05$ *versus* control (vehicle-treated cells); **: $p<0.01$ *versus* control (vehicle-treated cells); ¶: $p<0.05$ *versus* the corresponding FORM concentration; ¶¶: $p<0.01$ *versus* the corresponding FORM concentration.

or CREB to CBP/p300 could disturb the interaction with or recruitment by NF-κB, blocking its histone acetyltransferase (HAT) activity, making the promoter unavailable for NF-κB-binding. This would fit well with the findings of ITO *et al*. [31], which demonstrate a better anti-inflammatory efficacy when the GR is in a deacetylated form. Thus, if HAT activity is blocked, neither histone H4 nor GR will be acetylated, resulting in a diminished inflammation. This mechanism might apply in the present study; this is supported by the observation that dexamethasone did not block p65 translocation into the nucleus in HeLa cells and addition of TNF-α to BUD-stimulated cells did not change GR nuclear translocation (data not shown), indicating that all inhibiting interactions occur in the nucleus.

From the present experiments, it can be concluded that the anti-inflammatory effects of FORM seen in human bronchial epithelial cells cannot be attributed to the activation of the GR. Various alternative mechanisms that could explain the anti-inflammatory effect of β_2-agonists and the improved anti-inflammatory activity of the β_2-agonist/GCS combination observed at lower BUD concentrations have been described. For example, β_2-agonists can increase the mRNA half-life of the GR, resulting in increased GR levels, and thus an increased susceptibility for GCS [13]. There does not seem to be any evidence for the occurrence of this mechanism in the NHBE

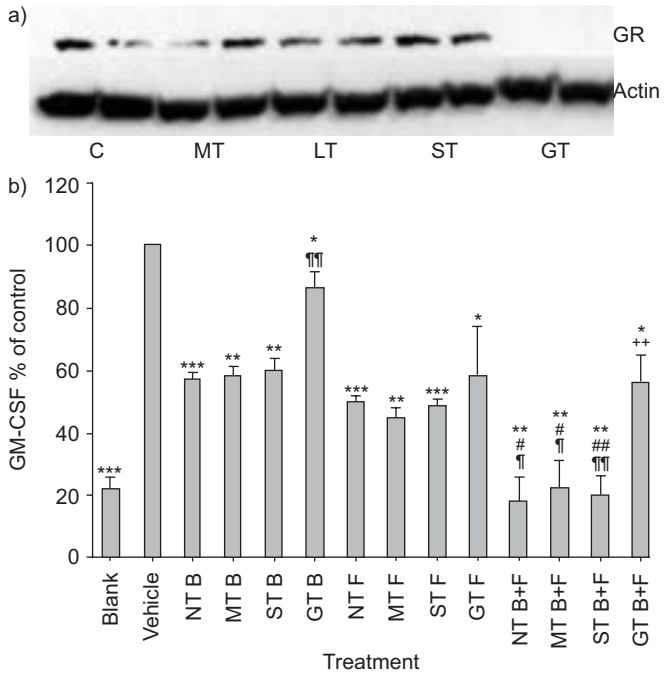

FIGURE 7. Effects of budesonide (BUDB) and/or formoterol (FORMF) on granulocyte-macrophage colony-stimulating factor (GM-CSF) production in normal human bronchial epithelial cells (b) following small interfering (si)RNA-mediated reduction of the glucocorticoid receptor (GR; a). NT: nontransfected; GR1: siRNA duplex 1; MT: mock-transfected, *i.e.* transfected without siRNA; ST: scramble-transfected, *i.e.* transfected with unspecific siRNA; LT: lamin transfected; GT: GR1 transfected. Each data point represents the mean±SEM of three independent experiments performed in duplicate.*: $p<0.05$ *versus* control (vehicle-treated cells); **: $p<0.01$ *versus* control (vehicle-treated cells); ***: $p<0.001$ *versus* control (vehicle-treated cells); #: $p<0.05$ *versus* nontransfected cells treated with FORM; ##: $p<0.01$ *versus* nontransfected cells treated with FORM; ¶: $p<0.05$ *versus* nontransfected cells treated with BUD; ¶¶: $p<0.01$ *versus* nontransfected cells treated with BUD; ++: $p<0.01$ *versus* nontransfected cells treated with FORM+BUD.

cells, since no change in GR amounts was observed by Western blot analysis within the 6- h timescale measured. However, since no mRNA was measured and no time-points beyond 6 h were investigated, this mechanism cannot be completely ruled out. In addition, alteration of the phosphorylation state of the GR by PKA, activated by β_2-agonists, results in a more susceptible GR, with an improved GCS/GR or GR/NF-κB binding [13, 16]. Due to the fact that FORM did not alter GR translocation or GRE-activation induced by BUD, it was doubtful whether FORM was altering the phosphorylation state of the ligand-activated GR. Alternatively, β_2-agonists are able to activate the transcription factor cAMP response element binding protein, which can interact, similarly to the GR, with pro-inflammatory transcription factors NF-κB and AP-1, or can interfere with their signal transduction pathways, and thereby reduce inflammation [22]. The fact that it has been possible to block the inhibitory effects of FORM on TNF-α-induced GM-CSF with a PKA inhibitor provides support both for the existence of the signal transduction pathway and the anti-inflammatory efficacy of cAMP. This signal transduction pathway could explain the results of the present study, and might be a possible way for β_2-agonists to convey anti-inflammatory activities in bronchial epithelial cells.

Taking the present and earlier published results collectively, it appears that the effects of β_2-agonists on glucocorticoid receptor signalling involves different mechanisms in different cell types. Though the mechanism of action in the normal bronchial epithelial cells has not yet been defined, the present work nevertheless suggests that in asthma therapy, the combination of budesonide and formoterol will limit inflammation-induced production of granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells to a greater extent, and at lower concentrations, than either component alone.

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