Effects of inhaled β agonist and corticosteroid treatment on nuclear transcription factors in bronchial mucosa in asthma

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Abstract

***Background*—Inhaled corticosteroids and β agonists are the most commonly used treatments in asthma and are often used together. Recent evidence suggests that many of the anti-inflammatory actions of corticosteroids are mediated by cross-talk between the activated glucocorticoid receptor (GR) and other transcription factors such as the pro-inflammatory nuclear factor kappa B (NFκB). Beta agonists can activate the transcription factor cAMP response element binding protein (CREB). A mutual inhibition between GR and CREB occurs in vitro which raises the possibility of a negative interaction between corticosteroid and β agonist drugs. A study was undertaken to determine whether these interactions occur during treatment with β_2 agonists and corticosteroids in asthma.**

***Methods*—Seven subjects who were participating in a randomised, placebo controlled, crossover study of six weeks treatment with inhaled budesonide (400 μg twice daily), terbutaline (1 mg four times daily), and combined treatment were recruited. Biopsy samples of the bronchial mucosa were obtained after each treatment and analysed for the DNA binding activity of GR, CREB, and NFκB.**

***Results*—Budesonide increased GR activity ($p<0.05$) and decreased NFκB activity ($p<0.05$). No treatment combination altered CREB activity and terbutaline had no significant effects on any transcription factor.**

***Conclusions*—Inhaled corticosteroids have significant effects on GR and NFκB activity in bronchial mucosa. A negative interaction between inhaled corticosteroids and β agonists was not found.**

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Corticosteroids have potent anti-inflammatory effects which are beneficial in the treatment of asthma. The mechanisms of their anti-inflammatory action have been extensively investigated in recent years and are believed to be mediated by altering gene expression. This may occur directly by the binding of the activated glucocorticoid receptor (GR) to glucocorticoid response elements (GREs) in the promoter region of target genes or indirectly via the ability of the GR to interact with other nuclear transcription factors thereby enhancing or suppressing their effects.[1] This cross-talk between transcription factors can occur at the level of DNA binding where GREs occur close to binding sites for other transcription factors.[2 3] Transcriptional cross-talk may also occur due to direct protein-protein interactions between transcription factors or by binding of co-factors such as the cAMP response element binding protein (CREB) binding protein (CBP) and P300 which may be necessary for gene transcription.[4]

The GR has been shown to interact with the pro-inflammatory transcription factor nuclear factor kappa B (NFκB) by binding to the p65 subunit of the NFκB heterodimer.[5] This suppresses the DNA binding activity of NFκB[5 6] and prevents transcription of inflammatory genes such as those for the cytokines interleukins 6 and 8.[5 7] NFκB regulates genes for many of the cytokines, inflammatory enzymes, and adhesion molecules which are activated in asthma, and thus inhibition of NFκB binding may be an important mechanism of the anti-inflammatory effects of corticosteroids.[1]

Conversely, other transcription factors may inhibit the activity of corticosteroids through similar mechanisms.[5 8 9] An interaction between the GR and CREB has been demonstrated in rat lung in vitro. Incubation with corticosteroids enhances GR binding to DNA whereas incubation with β agonists increases CREB binding to DNA. Simultaneous incubation with both drugs causes a reciprocal reduction of the increase in binding of both GRs and CREB by 40–50%.[10] As a consequence, β agonists may inhibit the anti-inflammatory activity of corticosteroids.

The relevance of these in vitro findings to the treatment of asthma is unclear. Many patients use both inhaled corticosteroids and β agonists for the treatment of asthma, raising the possibility that there may be a negative interaction in vivo between the two drugs. This has not been extensively investigated in the clinical setting although two recent studies have found that the beneficial effects of inhaled budesonide on the early asthmatic response to allergen are reduced by concomitant treatment with inhaled β agonists.[11 12]

This study was part of a larger investigation of the effects of inhaled corticosteroids and β_2 agonists in asthma[13] and was designed to examine the effects of each drug singly and in

combination on transcription factor binding in bronchial mucosa.

Methods

SUBJECTS

The inclusion criteria for the study have been reported previously.[13] Briefly, non-smoking subjects with mild to moderate atopic asthma and evidence of bronchial hyperresponsiveness (PC_{20} methacholine ≤ 8 mg/ml[14]) were recruited. For this subgroup study all subjects who had successfully completed the run in to the principal study were invited to participate before starting on the first of the blinded treatments. Other than restricting participation to adults (aged ≥ 18 years), subjects were unselected. Recruitment commenced when ethical approval for this subgroup study had been obtained and finished when the target number of subjects (6) had been exceeded.

STUDY DESIGN

The study design has been reported in detail elsewhere.[13] Following a four week run in period subjects were assigned to a double blind, random sequence of the following four treatments: (1) budesonide 400 μg twice daily + terbutaline 1 mg four times daily; (2) budesonide 400 μg twice daily + placebo four times daily; (3) placebo twice daily + terbutaline 1 mg four times daily; (4) placebo twice daily + placebo four times daily. Drugs were given by Turbuhaler (Astra Draco, Lund, Sweden). Each treatment was given for six weeks with a four week washout between each treatment period. During the run in and washout periods subjects used identical inhalers containing placebo (single blind). Inhaled ipratropium bromide (Atrovent Forte, Boehringer Ingelheim, Germany) was used for symptom relief throughout the study and subjects were instructed not to take any other asthma treatment (including nasal corticosteroids) except in an emergency.

BRONCHOSCOPY PROCEDURE

The primary end point of this study was the analysis of GR, CREB, and NFκB binding to DNA in bronchial mucosa samples obtained by fibreoptic bronchoscopy at the end of each treatment period. After measurement of the pulmonary function end points required for the principal study,[13] subjects continued treatment for a further 3–7 days before the bronchoscopic procedure.

Subjects were instructed to fast and not to take their study inhalers or ipratropium inhaler for six hours before bronchoscopy. On arrival asthma stability was assessed and forced expiratory volume in one second (FEV_1) was measured (Vitalograph, UK) to ensure that it was safe to proceed with bronchoscopy (FEV_1 >50% predicted). Premedication was given with intravenous atropine 0.6 mg and intravenous pethidine 25–50 mg. Subjects then inhaled nebulised lignocaine (1%) for 10 minutes. Lignocaine jelly (2%) was applied to the nostril prior to inserting the bronchoscope and further lignocaine solution (1%) was sprayed into the airway as required during the procedure. Additional sedation (intravenous alfentanyl 0.5–1 mg) was given as required. Supplemental oxygen 2 l/min was given throughout and pulse oximetry was monitored continuously.

Several biopsy specimens (5–8) were obtained at the level of second order carinae from the right middle and lower lobe bronchi. These were snap frozen in liquid nitrogen within 15–20 minutes.

All subjects received nebulised salbutamol 2.5 mg on completion of the bronchoscopy. Spirometric tests were repeated 10–15 minutes later. After a period of observation subjects were allowed to leave.

TRANSCRIPTION FACTOR ANALYSIS

Biopsy samples were kept at –70°C until the study was complete. They were then transferred on dry ice to the National Heart and Lung Institute in London. Nuclear and cytosolic proteins were extracted from biopsy tissue and transcription factor binding was measured by electrophoretic mobility shift assay as previously described.[15]

In brief, cells were lysed with 200 μl buffer A (20 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% Nonidet P40) and incubated at 4°C for 15 minutes. After microcentrifugation for 10 seconds and collection of the cytosolic fraction, the nuclear pellet was lysed with 20 μl of buffer B (20 mM HEPES, 1.5 mM $MgCl_2$, 0.42 mM NaCl, 0.5 mM DTT, 25% glycerol, 0.5 mM PMSF, 0.2 mM EDTA). The subsequent soluble fraction was mixed with 100 μl of buffer C (20 mM HEPES, 50 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA). 2 μg nuclear protein from each sample was preincubated at 4°C for 30 minutes in binding buffer (10 mM Tris HCl, pH 7.5, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 4% glycerol, 0.1 μg/μl salmon sperm DNA). Double stranded oligonucleotides encoding the specific sequences of GRE (5'-TCGACT GTACAGGATGTTCTAGCTACT-), NFκB (5'-AGTTGAGGGGACTTTCCCAGGC-), CRE (5'-AGAGATTGCCTGACGTCAGA GAGCTAG-) and Oct-1 (TGTCGAATG CAAATCACTAGAA) (Promega, Cambridge, UK) were end-labelled with (γ-^{32}P)-ATP and T4 polynucleotide kinase. Each sample was incubated with 50 000 cpm of labelled oligonucleotide for 40 minutes at 4°C. The protein-DNA complexes were separated on a 6% polyacrylamide gel using Tris-Borate-EDTA running buffer. Gels were autoradiographed for 1–3 days using Kodak OMAT XS film at –70°C with an intensifying screen and analysed by laser densitometry using a PDI image analysis system which covers a 3 OD unit range (Protein Databases Inc, New York, USA). Specificity of binding was determined by adding excess unlabelled oligonucleotide. Binding of the transcription factors GR, NFκB, and CREB was expressed as a percentage of the binding of the transcription factor Oct-1 (fig 1).

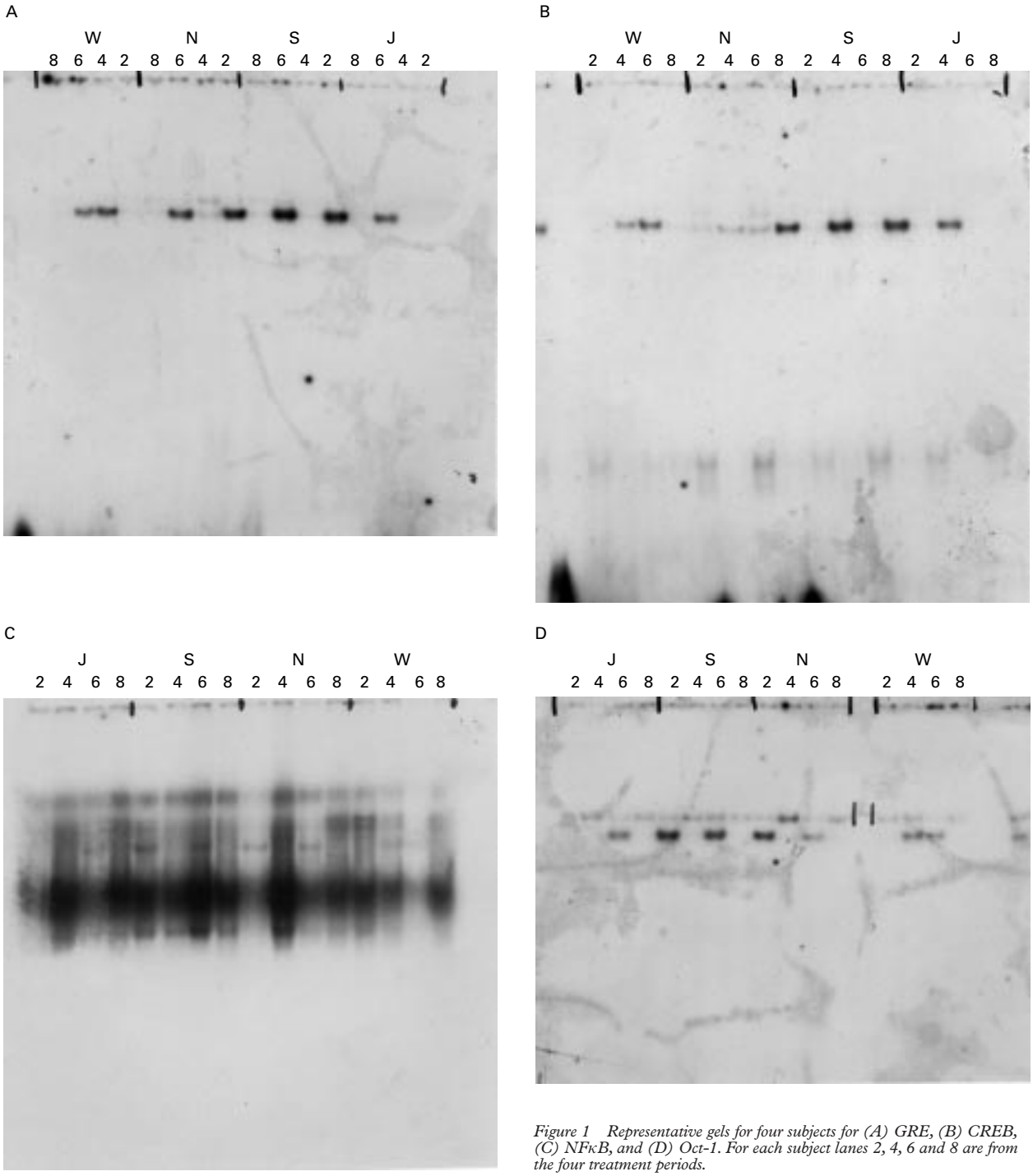

Figure 1 Representative gels for four subjects for (A) GRE, (B) CREB, (C) NFκB, and (D) Oct-1. For each subject lanes 2, 4, 6 and 8 are from the four treatment periods.

STATISTICAL ANALYSIS

The results for the transcription factor binding were analysed using a 2^2 factorial two way analysis of variance procedure for repeated measures (MANOVA procedure of SPSS). In vitro studies show a reciprocal inhibition of GR and CREB binding by terbutaline and budesonide, respectively, of 40–50%.[10] A sample size of six subjects was calculated to provide >80% power to detect a difference of this magnitude at $p<0.05$ in a paired analysis.

ETHICAL CONSIDERATIONS

The bronchoscopic procedures and the pre-bronchoscopy assessment were designed to ensure patient safety.[16] Subjects had access to the investigators at all times, and clear instructions for the emergency treatment of an asthma exacerbation in the form of an individualised action plan were given to all subjects. The study was approved by the Otago ethics committee. Written informed consent was obtained from each subject before entering the study and before each bronchoscopic procedure.

Results

SUBJECTS

All seven subjects (one male, age 18–45) completed the study. The mean (SD) duration of

Table 1 Transcription factor binding to DNA during each treatment period (as % of Oct-1 binding)

	GR binding				*CREB binding*				*NFκB binding*			
Subject	*P*	*T*	*B*	*BT*	*P*	*T*	*B*	*BT*	*P*	*T*	*B*	*BT*
1	0.76	0.76	0.93	1.52	0.40	0.43	0.55	0.64	8.11	7.06	4.63	8.29
2	1.00	0.65	1.18	2.91	0.56	0.40	0.62	0.40	14.25	11.29	2.39	4.66
3	0.92	0.75	0.76	0.88	0.51	0.29	0.48	0.48	1.39	8.03	5.83	3.26
4	0.97	0.72	1.02	1.60	0.85	1.47	1.10	0.71	20.33	7.12	5.88	14.33
5	0.56	0.34	0.88	0.66	0.31	0.41	0.51	0.51	9.33	10.10	4.25	4.10
6	1.06	1.38	1.85	1.38	1.74	2.14	0.74	1.25	13.10	14.88	18.10	0.99
7	0.74	0.55	0.6	0.84	0.54	1.11	0.28	0.68	1.79	18.50	5.52	13.66
Mean	0.86	0.74	1.03	1.40	0.70	0.89	0.61	0.67	9.76	11.00	6.66	7.04
SD	0.16	0.30	0.38	0.70	0.45	0.65	0.24	0.26	6.32	3.99	4.81	4.84

GR = glucocorticoid receptor; CREB = cAMP response element binding protein; NFκB = nuclear factor kappa B; P = placebo; T = terbutaline; B = budesonide; BT = budesonide + terbutaline.

their asthma was 21 (9.5) years. On entry to the study the mean (SD) FEV_1 was 100 (19)% predicted. Geometric mean PC_{20} methacholine was 1.37 mg/ml (range 0.09–5.32). Only two of the subjects were using regular inhaled corticosteroid treatment before entering the study. Two subjects had exacerbations of asthma during one of the treatment periods requiring oral prednisone (both were receiving budesonide + placebo). Without unblinding either the patients or the investigators, these treatments were repeated without further incident at the end of the scheduled treatment sequence.

TRANSCRIPTION FACTORS

The results of the transcription factor binding analysis are shown in table 1.

GR binding

Budesonide significantly increased GR binding by a mean of 52% (95% CI 6 to 99) (p = 0.033). There was a trend to higher GR binding when budesonide was combined with terbutaline but this did not reach statistical significance (p = 0.19). Terbutaline did not affect GR binding.

CREB binding

CREB binding was similar during all treatment periods. Although there were trends to higher levels of CREB binding (mean increase 19% (95% CI –18 to 55)) during terbutaline treatment and lower levels of CREB binding (mean decrease 20% (95% CI –27 to 66)) during budesonide treatment, neither of these was significant. There was no significant interaction between budesonide and terbutaline on CREB binding.

NFκB binding

Budesonide significantly reduced NFκB binding by a mean of 34% (95% CI 5 to 63) (p = 0.028). Terbutaline did not significantly alter NFκB binding and there was no significant interaction between budesonide and terbutaline.

Discussion

The results of this study show that inhaled budesonide causes a small but significant increase in GR binding to DNA in the bronchial mucosa in asthmatic subjects. This increase is in keeping with the results of in vitro studies and with those predicted by the molecular actions of corticosteroids, but the magnitude is reduced.[8 10] The failure to demonstrate a greater increase in GR:GRE binding may be due to differences in the concentrations of corticosteroid achieved in the airway in this study and the concentrations used in vitro. Alternatively, the reduced effect could be due to downregulation of the glucocorticoid receptor which has been shown in a number of in vitro and in vivo models.[17–21]

We have also shown for the first time that inhaled corticosteroids reduce binding activity of the pro-inflammatory transcription factor NFκB in vivo. This confirms the results of in vitro studies which have shown that corticosteroids reduce the level of NFκB binding to DNA in human lung tissue and peripheral blood mononuclear cells,[5 6] and supports the view that this may be an important mode of action of inhaled corticosteroids.[1] The exact mechanism of this inhibition is not certain but may be due to a direct protein-protein interaction between GR and the p65 subunit of NFκB. Such a mechanism appears to be responsible for the inhibition of NFκB activation of the IL-8 promoter by dexamethasone.[7] Alternatively, the reduction in NFκB DNA binding may reflect an induction of the inhibitor of NFκB, IκBα, by budesonide.[22] Induction of IκBα synthesis by corticosteroids is associated with inhibition of NFκB activity in some cultured mononuclear cells,[23 24] but studies using other cells have found that the induction of IκBα is neither sufficient nor necessary for the inhibition of NFκB transcriptional activity by corticosteroids.[25 26]

In this study we did not find any significant effect of β agonist or corticosteroid treatment on the DNA binding activity of CREB. This is in contrast to the results of in vitro studies of lung tissue in which CREB binding was increased by incubation with salbutamol or fenoterol and reduced by incubation with dexamethasone.[10] Although non-significant trends to an increase in CREB binding during terbutaline and a decrease in CREB binding during budesonide were observed, these changes were small in comparison with the results of in vitro studies. Thus, although this study may have had insufficient power to demonstrate a significant effect, it is clear that the size of the effect is likely to be small. There are several possible reasons for the difference between our results and those obtained in vitro. Firstly, the dose of terbutaline used in this

study may have been too low to raise intracellular levels of cAMP and thereby activate CREB. Although a standard therapeutic dose was used, it may be that at these doses terbutaline causes smooth muscle relaxation without significant activation of the cAMP pathway. Secondly, the timing of taking the bronchial biopsy specimen with regard to the last dose of the inhaled drugs may have missed their effects on CREB binding. Most of the bronchoscopies were performed in the morning and the interval between the last dose of the study medication and obtaining the biopsy specimen would have been approximately 12 hours. Although β agonists produce a sustained increase in CREB activation in vitro, this may be shorter in vivo. Thirdly, the biopsy specimens were of bronchial mucosa rather than lung parenchyma which was used in the in vitro experiments.[10] It is possible that these tissues differ in their response to increases in intracellular cAMP. Finally, the level of CREB binding in vivo may be subject to homeostatic influences which are not reproduced in tissue culture. During six weeks of maintenance treatment with inhaled β agonists there are likely to be counter-regulatory mechanisms which adjust to the effects of sustained activation of β_2 receptors. Downregulation of the β_2 receptor or of its ability to activate CREB, or modulation of the co-factor CBP by other transcription factors are possible mechanisms by which the effects of β agonists on gene transcription might be reduced.

This study has failed to confirm the negative interaction between β agonists and corticosteroids found in in vitro studies. In rat lung tissue, 24 hours incubation with the β agonists fenoterol and salbutamol increases CREB binding to DNA but decreases GR binding. Conversely, incubation with dexamethasone increases GR binding but decreases CREB binding. Incubation with both agents produces a mutual inhibition of their effects.[10] However, no such interaction was observed during asthma treatment in this study. Similarly, there was no apparent interaction between the effects of budesonide and terbutaline on NFκB binding. This may have been because terbutaline did not produce a sustained activation of CREB, and thus the inhibition of GR binding by CREB would not be expected to have occurred.

In conclusion, the results of the present study suggest that the anti-asthmatic effects of inhaled corticosteroids may be mediated by interactions between the GR and pro-inflammatory transcription factors such as NFκB. We have been unable to demonstrate any effects of either corticosteroids or β agonists on CREB activation. Importantly, there was no evidence that inhaled β agonists inhibit the effects of corticosteroids on transcription factors in vivo.

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