

In vivo modulation of glucocorticoid receptor mRNA by inhaled fluticasone propionate in bronchial mucosa and blood lymphocytes in subjects with mild asthma

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Background: In vivo regulation of the glucocorticoid receptor (GR) by glucocorticoids provides a means of modulating sensitivity of targeted cells.

Objective: We sought to determine the in vivo modulation of GR mRNA expression by fluticasone propionate (FP) in subjects with mild asthma.

Methods: Ten atopic asthmatic subjects were treated with FP 250 µg twice daily for 4 weeks. Before and after treatment, the patients underwent fiberoptic bronchoscopy with endobronchial biopsy and sampling of venous blood for measurements of GR mRNA levels. A solution hybridization assay was used for quantitative analysis of GR mRNA. In addition, a 24-hour urinary cortisol excretion and an adrenocorticotrophic hormone test before and after treatment with FP were performed.

Results: A high interindividual variation in GR mRNA expression was seen. However, we detected a significant reduction of the GR mRNA levels in the endobronchial biopsy specimens after FP treatment (36.6 ± 23.1 and 25.0 ± 10.9 amol GR mRNA/µg RNA, respectively; $P < .01$). In the peripheral blood lymphocytes an even more striking downregulation of the GR by its cognate ligand was documented (30.3 ± 26.5 and 8.8 ± 5 amol GR mRNA/µg RNA, respectively; $P < .001$), possibly reflecting differences in glucocorticoid sensitivity between tissues. A small but significant reduction of the 24-hour urinary cortisol excretion was observed (233 ± 109 and 157 ± 66 nmol/L, respectively; $P < .01$), whereas the feedback regulation of glucocorticoid synthesis by means of the hypothalamic-pituitary-adrenal axis as assessed by the adrenocorticotrophic hor-

Abbreviations used

ACTH:	Adrenocorticotrophic hormone
BAL:	Bronchoalveolar lavage
CCSP:	Clara cell secretory protein
FP:	Fluticasone propionate
GR:	Glucocorticoid receptor

mone test remained normal after treatment with FP.

Conclusion: The results in this study confirm the potency of the inhaled corticosteroid FP and provide evidence for a considerable tissue-specific interindividual variation in the expression of the GR. (*J Allergy Clin Immunol* 1999;103:595-600.)

Key words: Glucocorticoid receptor, asthma, bronchoalveolar lavage, fluticasone, Clara cell secretory protein

Asthma is currently recognized as an inflammatory disease, and hence administration of glucocorticoids to asthmatic patients is strongly encouraged in most asthma treatment guidelines.¹ The physiologic effects of glucocorticoids are mediated by intracellular glucocorticoid receptors (GRs), which function as ligand-activated transcription factors transducing the hormone signal to a transcriptional response at the level of specific target genes.² This occurs by means of binding of activated GRs to palindromic *cis*-acting DNA sequences known as glucocorticoid receptor-responsive elements (GREs) and results in enhanced or repressed transcription in responsive genes.^{3,4} In addition, the GR can modulate gene transcription through protein-protein interaction with transcription factors such as AP-1 and NF-κB, thereby enabling cross-talk between glucocorticoids and other polypeptides and cytokines.⁵ In particular, the mutual interference between hormone-activated GR and NF-κB is of relevance to the immunosuppressive effects of glucocorticoids.^{6,7}

Ubiquitous expression of GR in the adult human lung has recently been demonstrated,⁸ with the highest concentration of GR mRNA noted in the alveolar walls and vascular endothelium and smooth muscle of bronchial and pulmonary vessels and lesser amounts present in the airway epithelial cells and smooth muscle cells. The pleiotropy of hormone responses is maintained by one

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single GR gene,⁹ and the receptor protein is believed to be identical in all cells.^{10,11} In human subjects and in rats, but seemingly not in mice,¹² alternative splicing gives rise to one GR- α and one GR- β isoform of the receptor.¹⁰ Considerable interest has been focused on the nonligand-binding GR- β isoform on the basis of the assumption that it could function as a competitive inhibitor of transactivation of GR target genes.¹³ However, the physiologic significance of the β -isoform appears questionable.¹⁴

Given the large variety of biologic functions carried by a single GR protein and the variability in steroid sensitivity in different target tissues, it is thus evident that other factors can modulate the function of the receptor. In this way autologous regulation of GR by its cognate ligand, leading to a change in the number of available receptors in target cells, provides a mechanism to alter cell sensitivity to glucocorticoid hormones.^{15,16} Inherent to this concept, some studies performed in patients with hematologic malignancies have demonstrated a correlation between the therapeutic response to glucocorticoids and the number of GRs in cells,¹⁷ although this issue still remains controversial. Ligand-induced downregulation of steroid hormone receptors might also be of importance to limit the magnitude and duration of a specific biologic response in a tissue. Agonist-mediated downregulation of GRs has been shown to be time-dependent and tissue-specific and may possibly also be influenced by developmental and disease status.¹⁸ In addition, this regulation can be mediated at several molecular levels: the transcriptional level, the posttranscriptional level, and the posttranslational level.¹⁹

Because of the potential role of the bronchial epithelium in asthma^{20,22} and because the bronchial epithelium may be the target tissue of inhaled glucocorticoids, we decided to examine the autoregulation of GR mRNA *in vivo*. The levels of GR mRNA in endobronchial biopsy specimens, as well as in blood lymphocytes, obtained from subjects with mild asthma before and after 1 month of treatment with inhaled fluticasone propionate (FP) were evaluated. In parallel, we examined the feedback regulation of glucocorticoid synthesis through the hypothalamic-pituitary-adrenal axis,²³ as well as the urinary secretion of cortisol in these patients.

METHODS

Study population

Ten atopic asthmatic patients (5 women and 5 men) with a mean age of 29 years (range, 24 to 37 years) were recruited to participate in the study (Table I). All of the patients gave written informed consent to participate in the study, and the study was approved by the Ethics Committee of the Huddinge University Hospital. One of the patients decided not to undergo a second bronchoscopy, which precluded analysis of corresponding data, but apart from that, the study was conducted as planned. All patients were nonsmokers, and at the entry of the study, they were clinically fit as assessed by medical examination and routine serum biochemistry; lung function was normal as assessed by FEV₁ (100% of predicted value), and the mean sensitivity to histamine as documented as the histamine dose to cause a 20% fall in FEV₁ (PD₂₀) was 880 μ g. Asthma symptoms

were controlled by inhaled β -agonists on demand, and none of the included patients had taken oral or inhaled corticosteroids within 3 months before entry into the study.

Study design

After a screening visit, 24-hour urinary excretion for cortisol measurements was obtained, an adrenocorticotrophic hormone (ACTH) test was performed, and baseline histamine sensitivity was assessed. Two weeks later, 5 mL of venous blood was obtained from each patient for preparation of total RNA from lymphocytes, and the first bronchoscopy was performed. Before bronchoscopy, the patients inhaled 5 mg salbutamol (Ventoline; GlaxoWellcome, Greenford, UK) by means of a nebulizer (Ailos; Ailos Systems, Karlstad, Sweden). All patients were premedicated with morphine scopolamine hydrobromide (Morfin-scopolamin; Kabi-Pharmacia, Uppsala, Sweden). By using a flexible fiberoptic bronchoscope (BF type 1T20; Olympus Optical, Tokyo, Japan), bronchoalveolar lavage (BAL) was performed in the right middle lobe by instillation of 4 portions of 50 mL lukewarm, sterile phosphate buffer, pH 7.4, which was gently suctioned back. After this, endobronchial biopsy specimens for preparation of total RNA were taken from the carina between the right upper lobe bronchus and the intermediate bronchus. During bronchoscopy, intermittent doses of 0.5 mL of lidocaine (40 mg/mL) were sprayed on the vocal cords and in the bronchial tree with a dose standardized to 8.0 mL of lidocaine. The day after the first bronchoscopy, the patients were instructed to start inhaling 250 μ g of FP (Flutide, Diskhaler, GlaxoWellcome) twice daily at 8 AM and 8 PM. The dosage range and scheduling regime (FP 250 μ g twice daily) used in this study was chosen to reflect its common use in clinical practice in Sweden. One month later, 24-hour urinary excretion was obtained for cortisol measurements. The next day, 5 mL of venous blood was obtained for preparation of total RNA from lymphocytes, and a second bronchoscopy (at 10 AM, approximately 2 hours after the last inhalation of FP; thus FP treatment was terminated at the same day as the second bronchoscopy was performed) was performed, including endobronchial biopsy and BAL as described for bronchoscopy number one. All bronchoscopies were performed out of season. Finally, at a minimum of 2 weeks after the second bronchoscopy, a second ACTH test was performed.

BAL fluid analysis

The BAL fluid obtained from each patient was kept on ice, and a small aliquot was used to prepare smears for differential counts by cytocentrifugation at 500 rpm for 3 minutes (Cytospin 2Shandon; Southern Products Ltd, Runcorn, UK). Smears were stained with May-Grünwald-Giemsa, and 500 cells were counted. The rest of the BAL fluid was centrifuged at 400g for 10 minutes at 4°C, and the supernatant fraction was stored at -70°C until further analyzed.

Urinary cortisol

Twenty-four hour urinary cortisol was determined by a solid phase ¹²⁵I-RIA (Coat-A-Count CORTISOL; Diagnostic Products Corp, Calif). In each case urine was collected for 24 hours, the total volume was recorded, and an aliquot was extracted and analyzed according to the manufacturer's protocol.

ACTH test

Synthetic ACTH (tetracoside, Synacthen, Ciba-Geigy; 0.25 mg) was given intravenously, and blood samples for determination of plasma cortisol concentrations were taken before and 30, 60, 120, and 180 minutes after its administration. Patients were at rest during this procedure, which was performed between 8 AM and 10 AM. The samples were frozen for later determination of the plasma cor-

TABLE I. Demographic data

Sex ratio (M:F)	5:5
Age, years (range)	29 (24-37)
FEV _{1.0} (% predicted)	98 (92-115)
Histamine sensitivity (PD ₂₀)	880 µg (337-2120)
Atopy (positive skin test response)	Birch (4/10), Timothy grass (2/10), cat (8/10)

tisol by a solid phase ¹²⁵I-RIA (Coat-A-Count CORTISOL; Diagnostic Products Corp).

Solution hybridization assay

After homogenization, bronchial biopsy sample RNA was extracted with a modified guanidine thiocyanate water-saturated phenol/chloroform extraction method according to Chomczynski and Sacchi.²⁴ Lymphocytes were prepared from whole blood by Ficoll separation, and RNA was extracted from the homogenized cells as described for bronchial biopsy specimens. The quantity and purity of the extracted RNA was determined spectrophotometrically by measuring the absorbance at 260/280 nm; the absorbance 260/280 ratio was greater than 1.8 in all samples analyzed. For construction of the human GR probe, 2 complementary oligonucleotides corresponding to the nucleotides 159-209 in the human GR cDNA were synthesized, annealed, and ligated into the Pst 1/Hind III sites in pGEM TM1. The sequence of the insert in pGEM TM1 was confirmed by DNA sequencing with the Sanger dideoxy chain-termination method. Northern blot analysis showed that the GR probe hybridized to RNA species of the correct (7.0 and 6.1 kb) size. The plasmid designated pHGR was then used for in vitro synthesis of cRNA radiolabeled with ³⁵S-UTP (uridine triphosphate) by using SP6 RNA polymerase. The opposite mRNA strand was synthesized by using T7 polymerase. For solution hybridization, RNA samples were hybridized to 20,000 cpm of ³⁵S cRNA probe in 40 µL of 0.5 mol/L NaCl, 20 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EDTA, 0.1% SDS, 1 mmol/L dithiothreitol, and 25% formamide at 68°C for 12 to 16 hours. The samples were then treated with RNase A, 2 µg RNase T1 (Boehringer-Mannheim GFR), and 100 µg herring sperm DNA. Radioactivity protected against RNase digestion was precipitated by the addition of 100 µL 6M trichloroacetic acid and collected on a filter (Whatman GF/C). The hybridization signal of a sample was compared with a standard curve constructed from incubations with known amounts of the synthetic oligonucleotide mRNA. A standard curve (3 to 300 × 10⁻¹⁸ mol mRNA/incubation) was included in each assay, and each RNA sample was analyzed in duplicate or triplicate. For each specific probe, the corresponding amount of sample mRNA was calculated from the linear part of the standard curve. The between-assay variation and the variation between different identical samples was less than 10%, as estimated by analyzing the same sample in triplicate from 3 different experiments.

SDS-PAGE and Western blot immunoassay

PAGE was performed in the presence of SDS, essentially as described by Laemmli,²⁵ on a vertical slab gel unit (model SE 400; Hoefer Scientific Instruments, San Francisco, Calif) under nonreducing conditions. The gels were 10%, and proteins from SDS-PAGE were blotted onto nitrocellulose filters and detected immunologically as described earlier.²⁶ Antibody-antigen complexes were visualized by reaction with ¹²⁵I-protein A and phosphorimager plates (Fujix BAS-2000 Bio-Imager; Fuji, Tokyo, Japan). The antibodies have been described earlier.²⁶ To permit a quantitative analysis of human Clara cell secretory protein (CCSP) in BAL fluid, Western

TABLE II. Expression and in vivo regulation of GR mRNA by FP in endobronchial biopsy specimens and peripheral blood lymphocytes from asthmatic subjects

Cell/tissue sample material	Before FP treatment	After 1 month of FP treatment
Endobronchial biopsy specimens	36.6 ± 23.1 amol GR mRNA/µg RNA	25.0 ± 10.9 amol GR mRNA/µg RNA
Peripheral blood lymphocytes	30.3 ± 26.5 amol GR mRNA/µg RNA	8.8 ± 5 amol GR mRNA/µg RNA

Endobronchial biopsy specimens were obtained through bronchoscopy, and lymphocytes were obtained from peripheral blood from asthmatic patients. The levels of GR mRNA in the RNA samples prepared from the tissues and cells, respectively, were determined by solution hybridization as described in the Methods section. Results are presented as means ± SD. Each sample was analyzed in duplicate or triplicate.

standard curves were constructed. Thus we included known amounts of purified recombinant human CCSP²⁷ in each Western immunoassay. For each specific BAL fluid sample, the corresponding amount of BAL sample CCSP was constructed from the linear part of the standard curve.

Statistical methods

For comparison of levels of GR mRNA in endobronchial biopsy specimens and lymphocytes, as well as CCSP levels in BAL fluid before and after FP treatment, the nonparametric Wilcoxon's signed-rank test was used. The same method was applied for comparison of the urinary cortisol excretion values before and after FP treatment. Significance was set at *P* values less than .05.

RESULTS

GR expression and regulation

The solution hybridization assay was used to measure GR mRNA levels because of the high sensitivity of the method (less than 0.3 pg of RNA), which is of importance in studies of low abundant mRNA species such as the GR. In addition, the method provides absolute values for mRNA measurements. As shown in Table II, there was a small but significant decrease in the steady state levels of GR mRNA in the bronchial biopsy samples as a result of FP treatment (36.6 ± 23.1 [mean ± SD] and 25.0 ± 10.9 amol GR mRNA/µg RNA, respectively; *P* < .01). This downregulation of GR mRNA after FP treatment was even more pronounced in the peripheral blood lymphocytes (30.3 ± 26.5 and 8.8 ± 5 amol GR mRNA/µg RNA, respectively; *P* < .01).

ACTH test and 24-hour urinary cortisol

All of the patients included in the study had a normal ACTH test response at the entry of the study after a standard dose (250 µg) of ACTH, as evidenced by a rise of serum cortisol above 500 nmol/L 60 minutes after administration of ACTH. Treatment with inhaled FP 250 µg twice daily did not affect this response to ACTH, which is in keeping with a maintained hypothalamic-pituitary-adrenal axis (data not shown). In contrast, a small but significant (233 ± 109 and 157 ± 66 nmol/L,

respectively; $P < .01$) reduction of the urinary 24-hour cortisol excretion was seen after 1 month of treatment with inhaled FP 250 μg twice daily.

CCSP expression and regulation

CCSP is a 16 kd, globular, secretory protein localized to the airway epithelium and the genital organs.^{28,29} To investigate a putative regulation of CCSP by glucocorticoids in adult human lungs, we measured the levels of CCSP in BAL fluid before and after 1 month of FP 250 μg twice daily. We were able to detect CCSP in BAL fluid in all patients who participated in the study. However, treatment with FP 250 μg twice daily did not significantly alter the levels of CCSP in BAL fluid (136.5 ± 60.4 ng CCSP/ μg total protein before treatment vs 144.8 ± 92.7 ng CCSP/ μg total protein after treatment, corresponding to 8.20 ± 5.43 ng CCSP/ μL BAL fluid before treatment vs 9.19 ng CCSP/ μL BAL fluid after treatment).

BAL cell differentials

Before FP treatment, some of the patients appeared to have had an increased number of inflammatory cells in their respective BAL fluid as judged from the BAL cell differentials (expressed as mean of total BAL cells and range): macrophages, 83.1% (range, 66% to 100%); lymphocytes, 13.2% (range, 1% to 26%); neutrophils, 3.3% (range, 1% to 11%); and eosinophils, 0.4% (range, 0% to 3%). However, the group mean for the BAL cell differentials was within the normal values as compared with the BAL cell differentials for healthy volunteers in our laboratory. Similarly, although there were changes in individual BAL cell differentials after FP treatment, no significant changes were seen for the whole group of patients after FP treatment: macrophages, 84.7% (range, 79% to 92%); lymphocytes, 9.3% (range, 3% to 16%); neutrophils, 5.4% (range, 0% to 12%); and eosinophils, 0.6% (range, 0% to 2%).

Lung function measurements

Given the normal lung function of the asthmatic subjects on inclusion in the study, lung function measurements were not a main investigative purpose of this study. It was felt, however, that morning and evening peak expiratory flow recordings throughout the study would be informative to rule out unexpected events. In summary, the peak expiratory flow values did not differ significantly during the treatment period (data not shown). In addition, none of the patients experienced any adverse events related to the treatment during the study.

DISCUSSION

In this study we demonstrate an *in vivo* downregulation of GR mRNA in the bronchial mucosal samples, as well as in the peripheral blood lymphocytes, in atopic subjects with mild asthma after treatment with FP (250 μg twice daily) for 1 month. The rationale for 1 month of FP treatment was the observation that 2 weeks of FP treatment intranasally was sufficient to cause a signifi-

cant downregulation of GR mRNA in nasal mucosal biopsy specimens.³⁰ Experiments in the same study making use of nasal brush lavage samples gave inconclusive results, suggesting that biopsy specimens would be more suitable for quantification of GR mRNA levels in the lung. Furthermore, *in vitro* experiments in cell lines have demonstrated a maximum downregulation of GR after 2 weeks of glucocorticoid treatment.³¹ During the FP treatment period, the 24-hour urinary cortisol excretion was shown to be significantly reduced, whereas the integrity of the hypothalamic-pituitary-adrenal axis was maintained.

The biologic response elicited by GRs depends on the actual number of GRs within a target cell, and therefore factors that regulate the expression of GR may affect cellular glucocorticoid sensitivity.³² It is generally accepted that downregulation of GRs is a very complex process involving transcriptional, posttranscriptional, and post-translational mechanisms.³³ Irrespective of the mechanism, a 50% to 70% agonist-mediated downregulation of GR, both at the protein and at the mRNA level, has been documented in a number of cell lines and tissues, whereas adrenalectomy consistently causes an upregulation of GR number in the same order of magnitude.³⁴ The ligand-induced negative autoregulation of the GR mRNA thus provides a means of modulating sensitivity of targeted cells and tissues to glucocorticoids and may in this respect be regarded as an important homeostatic event.

The pharmacologic properties of inhaled glucocorticoids that determine the local effects are water solubility/lipid solubility, receptor-binding affinity, and receptor-binding half-life. Of the inhaled glucocorticoids, FP is known to have a very high plasma protein binding. It must be remembered that it is only the free, unbound drug that is able to interact with the GR. FP exhibits a high degree of lung tissue binding (4.89 ng/mg)³⁵ and a high³⁶ and sustained GR binding. In keeping with this, a significant downregulation of the GR mRNA levels in the bronchial tissue was seen in this study. In all likelihood the 4 weeks of continuous FP treatment is responsible for the observed downregulation of the receptor. For obvious reasons, this study could not address the issue of whether glucocorticoids administered by inhalation twice daily affect the bronchial mucosa in a chronic way. It should also be pointed out that the changes in mRNA levels observed in this study do not necessarily reflect changes in protein level. However, our results show that inhaled glucocorticoids affect the expression of the GR gene. The present study, which was performed in individuals with normal lung function at the entrance of the study, cannot therefore address the issue of a decrease in pulmonary effects on receptor downregulation.

In this study inhaled FP in a moderate dose (250 μg twice daily) caused a 70% decrease in the GR mRNA expression in the peripheral blood lymphocytes. Even though subjects inhaling 1 mg of FP before open lung surgery were found to have a ratio of FP in the lung compared with plasma ranging from 78:1 to 152:1,³⁷ a systemic effect by FP is likely to be the cause of the

observed decrease in GR mRNA in the peripheral blood lymphocytes. In support of this notion, a small but significant reduction of the 24-hour urinary cortisol excretion was seen in this study. The functional significance of GR autoregulation in terms of glucocorticoid responsiveness is difficult to evaluate in intact animals and in humans and seems to involve a more complex pattern of regulatory and modulatory factors than previously thought. For inhaled glucocorticoids like FP, with a high degree of first metabolism, the main determinant of systemic effects is the fraction of the inhaled drug absorbed through the lung. The large volume of distribution and a long elimination half-life of FP is likely to cause the observed downregulation of GR mRNA in peripheral lymphocytes.

Because of the suggested anti-inflammatory role of the high abundant CCSP, we measured the levels of CCSP in BAL fluid in the study group. The levels of CCSP remained unchanged during treatment with FP, arguing against a regulation of CCSP levels by inhaled glucocorticoids at the indicated dosages. Several years ago, it was noted that glucocorticoids increase the expression of CCSP 2- to 3-fold in rabbit lung³⁸ and 2-fold in rat and mouse lung.^{39,40} This glucocorticoid effect is believed to be mediated by an enhanced transcription, although an increased stability of the CCSP mRNA offers an alternative explanation.⁴¹ During the late fetal period in rats, glucocorticoids are known to increase the levels of CCSP and steady-state CCSP mRNA.⁴² We thus hypothesized that FP treatment would increase the levels of CCSP in BAL fluid. However, the levels of CCSP in BAL fluid, although readily detected in all patients examined, were unaffected by FP treatment. Cigarette smoking is known to significantly decrease the levels of CCSP in BAL fluid,⁴³ and this was recently shown to be correlated to a decrease in the numbers of Clara cells within the lung.⁴⁴ It may therefore be the case that changes in the ultrastructure of the lung are necessary for alterations in the levels of CCSP in the BAL fluid.

In summary, the results in this study show the GR message repression in vivo in 2 different tissue/cell compartments in patients with mild asthma mediated by a pharmacologic ligand, inhaled FP. In all likelihood, the relatively high lung tissue binding, high receptor occupancy, large volume of distribution, and long biologic half-life of FP explain the obtained results. In the context of the heterogeneity in glucocorticoid sensitivity in asthmatic subjects, we suggest that pharmacologically acquired downregulation of GR may be of physiologic importance.

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