

Abnormal Glucocorticoid Receptor–Activator Protein 1 Interaction in Steroid-Resistant Asthma

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Summary

Glucocorticosteroids are a very effective treatment for asthma and other chronic inflammatory diseases. However, a small proportion of patients is resistant to the therapeutic effects of glucocorticoids. Pharmacokinetic and ligand binding studies suggest that the molecular abnormality in steroid resistance lies distal to nuclear translocation. We have previously reported that there is a decreased ability of glucocorticoid receptors (GR) to bind to the DNA-binding site in peripheral blood mononuclear cells (PBMC) after dexamethasone treatment. This reduced DNA binding was due to a decrease in the number of receptors available rather than an alteration in affinity for DNA. To study this reduced DNA binding, we examined the ability of the nuclear translocated transcription factors activator protein 1 (AP-1), nuclear factor κ B (NF- κ B) and cyclic AMP response element-binding protein (CREB) to bind to their DNA-binding sites and to interact with GR in PBMC from patients with steroid-sensitive and steroid-resistant asthma. There was a significant reduction in the interaction between GR and AP-1 in these steroid-resistant patients, although interaction with other transcription factors activated in inflammation (NF- κ B and CREB) was unaffected. An increase in the basal levels of AP-1 DNA binding was also detected in the nuclei from steroid-resistant asthmatic patients. There were no differences in the amount of messenger RNA detected for the components of AP-1, c-Fos and c-Jun, nor in the sequences of these messenger RNAs. These results suggest either that the ability of the GR to bind to glucocorticoid response elements and AP-1 is altered in steroid-resistant patients or that increased levels of AP-1 prevent GR DNA binding, and that this may be the molecular basis of resistance to the antiinflammatory effect of steroids in these cells.

Glucocorticosteroids are the most effective treatment for asthma (1), and they have an important place in the control of other chronic inflammatory or immune diseases. A small proportion of asthmatic patients is resistant to the therapeutic effects of glucocorticoids, and the molecular mechanism for this steroid resistance is unclear (2). A similar defect in glucocorticoid responsiveness is also reported in patients suffering from other diseases that are treated by steroid therapy, including rheumatoid arthritis, systemic lupus erythematosus, and transplantation rejection (3, 4). Although steroid resistance is relatively rare, understanding the molecular mechanisms involved may give important insights into the mechanism of antiinflammatory actions of glucocorticoids and into chronic inflammatory processes. Furthermore, steroid resistance poses a therapeutic problem, as few alternative therapies are available. Steroid resistance has been most extensively studied in asthma, as the failure here to respond to steroids is more easily documented than in other inflammatory or immune conditions.

Steroid-resistant (SR)¹ asthma was first identified by Schwartz et al. (5), who described six asthmatic patients whose symptoms failed to improve even with high doses of oral steroids. Carmichael et al. (6) showed that SR asthmatic patients tended to be disabled for long periods of time and present with a stronger family history of asthma than those who respond to steroids. This corticosteroid resistance is not due to abnormal absorption or clearance of corticosteroids (2, 7, 8). Furthermore, these patients do not have features of Addison's disease or elevated plasma cortisol concentrations, suggesting that metabolic functions mediated by steroids are unaffected. At a cellular level, there is

¹Abbreviations used in this paper: AP-1, activator protein 1; CRE, cAMP response element; CREB, CRE-binding protein; FEV₁, forced expiratory volume in 1 s; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptors; GRE, glucocorticoid response element; mRNA, messenger RNA; NF κ B, nuclear factor κ B; RT, reverse transcription; SR, steroid resistant; SS, steroid sensitive; TRE, TPA response element.

evidence for a defective response of peripheral blood cells to corticosteroids, which correlates with clinical resistance to steroid treatment (8, 9). This functional defect has been described in blood monocytes (9) and T lymphocytes (8), and may also be observed in the skin response to topical steroids (10).

Resistance to the antiinflammatory effects of steroids in asthma does not appear to be explained by abnormal interaction between glucocorticosteroids and glucocorticoid receptors (GR). Differences in either affinity or number of GR is insufficient to account for the observed lack of steroid responses in PBMC or T lymphocytes from SR compared with steroid-sensitive (SS) asthmatic patients, and the GR-binding characteristics are similar to those reported in normal asthma (8, 9). This suggests that the major defect in steroid resistance lies beyond the point at which the GR translocates to the nucleus. Recent studies have shown that there is a reduced enhancement of GR binding to DNA after 60 min of dexamethasone treatment *in vitro* in SR patients (11) and that this reduced capacity is not due to any changes in the primary sequence of the GR in these patients (12).

The molecular basis of glucocorticoid action has been extensively studied. Glucocorticoids enter the cell and bind to an inactive cytosolic GR, which rapidly translocates to the nucleus. Within the nucleus the complex binds as a dimer to specific sites on DNA, termed glucocorticoid response elements (GREs), upstream of the promoter region in SR genes. Binding of the steroid-GR complex to the GRE enhances or represses transcription of the target gene (13). The activated translocated GR may also interact with other transcription factors, such as activator protein 1 (AP-1), nuclear factor κ B (NF- κ B), and cAMP response element-binding protein (CREB) by a direct protein-protein interaction, thus modulating an effect that they would otherwise have on gene transcription (14). An interaction between the proinflammatory transcription factor AP-1, which is a heterodimer of Fos and Jun oncoproteins, and GR has been demonstrated in cultured cells (15), in human lung (16), and in human PBMC (17), and may be an important aspect of the antiinflammatory effect of steroids (14). NF- κ B can also form protein-protein interactions with GR (16, 18), and this prevention of NF- κ B activation of gene expression by GR may underlie the mechanism of steroid action on the messenger RNA (mRNA) of many cytokines (14). An interaction between GR and CREB has also been described in various cell types (17, 19).

Glucocorticoids exert effects on expression of a wide variety of genes, including those for cytokines, receptors, and enzymes, but the absolute and relative importance of any of these to the therapeutic response in asthma is not yet clear. Many of the important antiinflammatory effects of steroids appear to be mediated via a reduction in gene transcription, and this may involve an interaction between GR and AP-1 or NF- κ B. This may be of particular relevance in chronic inflammatory diseases since cytokine- and protein kinase C-induced activation of transcription factors involved in mediating chronic inflammatory events at a transcriptional level may be inhibited by steroids (14).

We have tested the possibility that the reduced capacity of GR to bind to GRE is due to an abnormal interaction with other transcription factors. Using electrophoretic mobility shift assays, we investigated the effect of steroids on the DNA binding of transcription factors that may be activated in chronic inflammation, including AP-1, NF- κ B, and CREB.

Materials and Methods

Patients. All asthmatic subjects were defined by a history of episodic wheezing and by a >15% increase in forced expiratory volume in 1 s (FEV₁) after inhaling a bronchodilator (400 μ g albuterol). SR patients were classified as showing <5% improvement in lung function after 2 wk of treatment with 40 mg oral prednisolone per day and have previously been documented to have PBMC with reduced responsiveness to steroids *in vitro* (11). SS patients had >15% improvement in FEV₁ after oral prednisolone (40 mg daily for 2 wk). The severity of airway obstruction in both groups of patients was similar, and the bronchodilator responses to albuterol indicated that both groups had reversible airways obstruction, consistent with the diagnosis of asthma (Table 1). None of the subjects studied had taken oral steroids for at least 1 mo before study.

Isolation of PBMC. 20 ml of heparinized peripheral blood was obtained from each patient, mixed with 4 ml of 6% dextran, and allowed to separate for 2 h at 20°C. The upper layer, containing cells other than erythrocytes, was collected, and PBMC were further purified from other leukocytes by separation on Ficoll gradients. Blood was collected from all patients at the same time of day. After centrifugation at 1,200 g for 20 min at 20°C, PBMC were removed from the interface, washed twice in Hanks' modified medium, and kept at 4°C until use (9). The purity of the PBMC was 95.2 \pm 2.2% monocytes, with the remaining cells lymphocytes, as assessed by May-Grünwald-Giemsa staining.

Electrophoretic Mobility Shift Assays. Electrophoretic mobility shift assays provide a means of measuring the amount of activated tran-

Table 1. Clinical Characteristics of SS and SR Asthmatic Patients

	SS	SR	Difference
Number	6	7	NS
Age (yr)	46 \pm 5	51 \pm 6	NS
Gender	4 males	3 males	NS
Atopy	2/6	2/7	NS
Smokers	1/6	1/7	NS
FEV ₁ (% predicted)	76 \pm 4	71 \pm 3	NS
Albuterol response (% increase in FEV ₁)*	34 \pm 2	34 \pm 4	NS
Prednisolone response (% increase in FEV ₁) [‡]	36 \pm 2	3 \pm 0.8	<i>P</i> < 0.01

Mean values \pm SEM are shown.

*Albuterol response is the maximum percentage of increase in FEV₁ above baseline value after administration of 400 μ g albuterol via a metered dose inhaler.

[‡]Prednisolone was given orally for 2 wk at 40 mg/d.

scription factors within the nucleus capable of binding to their specific DNA recognition sequence. This is performed by incubating a nuclear extract with a labeled double-stranded DNA recognition sequence and detecting the bound oligonucleotide by its retardation in a nondenaturing gel. Equal numbers of cells ($0.51 \pm 0.07 \times 10^{-6}$, range $0.19\text{--}0.88 \times 10^{-6}$) were incubated with $1 \mu\text{M}$ dexamethasone, $0.1 \mu\text{M}$ PMA, or both for 15-min intervals up to 60 min. Control experiments using cells incubated in Hanks' medium alone were run in parallel. At every time point, cells were collected by microcentrifugation and were nuclear protein extracted (17). Cells were gently lysed in $100 \mu\text{l}$ of buffer A (10 mM Hepes, 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM dithiothreitol, 0.1% NP-40). After centrifugation ($<10 \text{ s}$, $14,000 \text{ g}$, 4°C), the nuclear pellet was lysed with $20 \mu\text{l}$ of buffer B (20 mM Hepes, 1.5 mM MgCl_2 , 0.42 mM NaCl, 0.5 mM dithiothreitol, 25% glycerol, 0.5 mM PMSF, 0.2 mM EDTA). The subsequent soluble fraction was mixed with $100 \mu\text{l}$ of buffer C (20 mM Hepes, 50 mM KCl, 0.5 mM dithiothreitol, 0.5 mM PMSF, 0.2 mM EDTA). Double-stranded oligonucleotides encoding the consensus target sequence for GRE (5'-TCGACTGTACAGGATGTTCTAGTACT-), AP-1 (5'-CGCTTGATGAGTCAGCCGAA-), NF- κB (5'-AGTTGAGGGGAC-TTTCCAGG-) and CREB (5'-AGAGATTGCCTGACGTCAGAGAGCTAG-) (Promega, Cambridge, UK) were end labeled using [^{32}P]ATP and T4 polynucleotide kinase. $1 \mu\text{g}$ of nuclear protein from each sample was preincubated in $25 \mu\text{l}$ incubation buffer (4% glycerol, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.5, 0.8 mg/ml sonicated salmon sperm DNA) for 20 min at 4°C before the addition of 50,000 cpm of labeled oligonucleotide. The incubation was then continued for a further 40 min at 20°C . Protein-DNA complexes were separated on 6% polyacrylamide gels using $0.25\times$ Tris-Borate-EDTA running buffer. The retarded band was detected by autoradiography and quantified by laser densitometry. All band density measurements were then expressed as a percentage of initial binding. Specificity was determined by addition of excess unlabeled double-stranded oligonucleotides. Supershift experiments were performed with $10 \mu\text{g}$ nuclear protein. Nuclear proteins were incubated with $5 \mu\text{g}$ anti-GR antibody (Cambridge Bioscience Ltd., Cambridge, UK), anti-c-Fos antibody (Serotec, Oxford, UK), anti-c-Jun antibody (Serotec), or anti-p53 antibody (Cambridge Bioscience Ltd.) for 4 h at 4°C before the addition of unlabeled oligonucleotide. After a further 40-min incubation at 20°C , complexes were resolved on 6% gels, and the retarded bands were examined. In some cases, rather than cause a further retardation (supershift) in the retarded band, the antibodies inhibited DNA binding to the protein-

DNA complexes.

Isolation of RNA and PCR Sequencing of GR, c-Fos, and c-Jun. RNA was extracted from the cytoplasm of PBMC after detergent lysis according to the method of Gough (20). Reverse transcription (RT)-generated cDNA encoding for the DNA-binding site (bp 1393-1605) and τ_1 domain (bp 520-763) of the human GR, c-Fos (bp 439-791), c-Jun (bp 5-245), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were amplified using PCR. The primers used for amplifying the cDNA were as described below and corresponded to the human GR (21), c-Fos (22), c-Jun (23), and GAPDH (24):

GR DBS forward 5'-TGCCTGGTGTGCTCTGATGAA-
reverse 5'-TCCAGGTTTCATTCCAGCCTGA-
GR τ_1 forward 5'-AATAGGTCCGACCAGTGTTC-
reverse 5'-TCTCATTTCGTCTCTTTACCTG-
c-Fos forward 5'-TAAGATGGCTGCAGCCAAATG
reverse 5'-GTCTTCAGCTCCATGCTGATG
c-Jun forward 5'-CTGCAAAGATGGAAACGACCT
reverse 5'-GGATTATCAGGCGCTCCAG
GAPDH forward 5'-ATTCCATGGCACCCTCAAGGCT-
reverse 5'-TCAGGTCCACCACTGACACGTT-

PCR products were isolated by GeneClean (Stratagene, Luton, UK) according to the manufacturer's instructions. Cycle sequencing was performed on the purified cDNA using nested primers in both forward and reverse directions using exo^{-} Pfu Cyclist (Stratagene, Cambridge, UK) using 30 cycles (denature 95°C , 30 min; anneal 50°C , 30 min; extension 72°C , 30 min) after an initial 5-min denaturing step at 95°C with ^{35}S -dATP as the label. At the end of the cycling period, aliquots were loaded onto a 7% sequencing gel, and films were exposed at -70°C .

Data Analysis. All data are expressed as mean \pm SEM. Results between treatment groups were compared by analysis of variance. At specific time, results were compared by Wilcoxon's nonparametric analysis. *P* values of <0.05 were taken as significant.

Results

Measurement of Baseline Levels of GR and AP-1. There was no difference in the amount of GR DNA binding detected in unstimulated PBMC between SS and SR groups (1.25 ± 0.2 versus 1.32 ± 0.18) (Fig. 1). The levels of AP-1 binding were significantly greater in the SR than in the SS group (1.83 ± 0.13 versus 0.77 ± 0.11 , $P < 0.01$) (Fig. 2). Confirmation that the bands detected were specific for AP-1

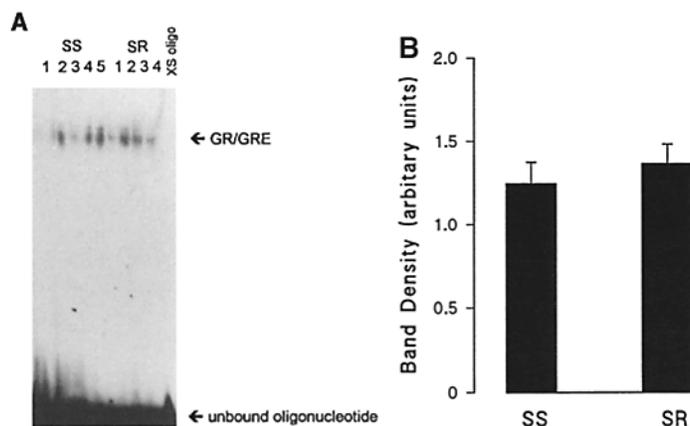


Figure 1. Comparison of basal GR DNA binding in nuclei from 10^5 PBMC from SS ($n = 5$) and SR ($n = 4$) patients at baseline. Band density is measured in arbitrary units, and SS and SR samples are run on the same gels. (A) An electrophoretic mobility shift assay from $1 \mu\text{g}$ nuclear protein from SS and SR patients. The specific GR-GRE-retarded band is indicated by an arrow. Specificity of the retarded band is shown by loss of this band after preincubation with an excess of unlabeled oligonucleotide (XS oligo). (B) The mean values (\pm SEM) of the band density of the results shown in A.

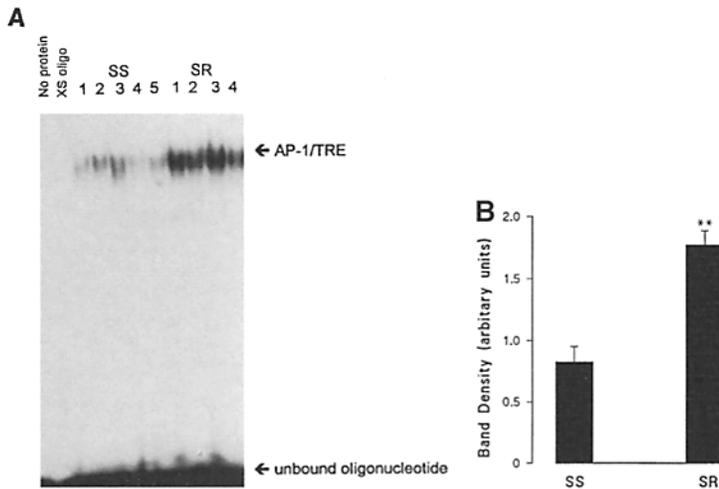


Figure 2. Comparison of basal AP-1 DNA binding in nuclei from 10^5 PBMC from SS ($n = 5$) and SR ($n = 4$) patients at baseline. Band density is measured in arbitrary units, and SS and SR samples are run on the same gels. (A) An electrophoretic mobility shift assay from $1 \mu\text{g}$ nuclear protein from SS and SR patients. The specific AP-1-TPA response element (TRE)-retarded band is indicated by an arrow. Specificity of the retarded band is shown by loss of this band after preincubation with an excess of unlabeled oligonucleotide (XS oligo). (B) Mean values (\pm SEM) of the band density of the results shown in A. ** $P < 0.01$.

or GR was obtained by the use of excess unlabeled oligonucleotide, which competed out the labeled oligo and produced a much reduced band density (Figs. 1 and 2). Further evidence that these were specific bands was obtained by supershift assays, whereby an anti-GR antibody caused a further retardation (supershift) of the specific GR-GRE complex. Antibodies to c-Fos also produced a retardation of the GR-GRE band, suggesting an interaction between GR and c-Fos (Fig. 3). Furthermore, anti-c-Fos, -c-Jun, and -GR antibodies produced a supershifted AP-1 band, which was not found after incubation with a specific anti-p53 antibody (Fig. 4).

Effect of Dexamethasone on AP-1, NF- κ B and CREB Electrophoretic Mobility Shift Assay. Dexamethasone alone caused a $52.4 \pm 11.2\%$ decrease in AP-1 binding at 60 min in SS patients, which was significantly greater than the reduction seen in SR patients ($15.5 \pm 6.4\%$ decrease, $P < 0.05$), suggesting an abnormality in the direct interaction between GR and AP-1 (Figs. 5 and 6). The effect of combined PMA and dexamethasone treatment in SS and SR patients was to block the inhibitory effect of dexamethasone on AP-1 levels and return them to baseline levels ($+18.3 \pm 9.5\%$ in SS patients and $-2.4 \pm 26.0\%$ in SR patients).

The specificity of transcription factor interactions in SS and SR patients was also examined. In contrast to the reduced fall in AP-1 activity after dexamethasone in SR patients, dexamethasone caused an equal reduction in band density of NF- κ B in both SS ($-41.6 \pm 5.6\%$) and SR ($-31.0 \pm 15.4\%$) patients (Figs. 5 and 6) and a similar reduction in CREB binding in both groups of patients ($-31.5 \pm 5.7\%$ in SS versus $-35.0 \pm 9.5\%$ in SR patients) (Figs. 5 and 6). The reduction in NF- κ B and CREB binding was found at all times studied and could be abolished, at least in part, by coincubation with $1 \mu\text{M}$ PMA.

Effect of PMA on AP-1, NF- κ B, and CREB Electrophoretic Mobility Shift Assay. AP-1 binding in cells from SS patients demonstrated a $168.3 \pm 67.2\%$ increase above baseline after PMA treatment, which was significantly greater than

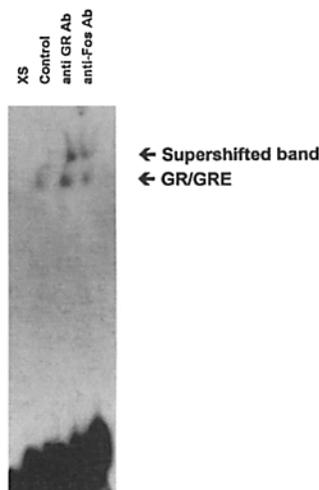


Figure 3. Supershift experiments to show interaction of GR and c-Fos proteins within the GR-GRE-binding complex from PBMC. The figure shows an electrophoretic mobility shift assay for GR-GRE binding using $1 \mu\text{g}$ nuclear proteins. The specific GR-GRE-retarded band is indicated by an arrow. Specificity of the retarded band is shown by loss of this band after preincubation with an excess of unlabeled oligonucleotide (XS). Preincubation with either anti-GR or anti-c-Fos antibody produced a further retardation (supershift) in the specific GR-GRE complex.

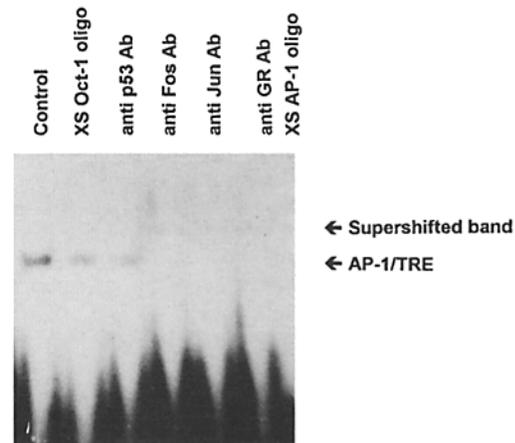


Figure 4. Supershift experiments to show interaction of c-Fos, c-Jun, and GR proteins within the AP-1-TRE-binding complex from PBMC. The figure shows an electrophoretic mobility shift assay for AP-1-TRE binding using $1 \mu\text{g}$ nuclear proteins. The specific AP-1-TRE-retarded band is indicated by an arrow. Specificity of the retarded band is shown by loss of this band after preincubation with an excess of unlabeled oligonucleotide (XS AP-1 oligo) but not with an excess of nonspecific oligo (XS Oct-1 oligo). Preincubation with either anti-Fos, anti-Jun, or anti-GR antibody produced a further retardation (supershift) in the specific GR-GRE complex. Preincubation with an anti-p53 antibody had no effect on AP-1-TRE binding.

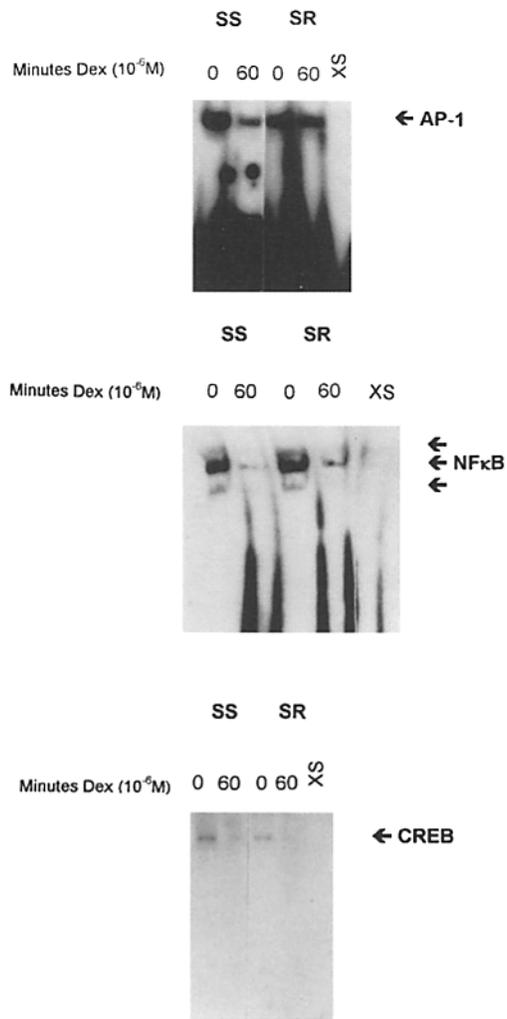


Figure 5. Action of dexamethasone (1 μ M) on AP-1, NF κ B, and CREB nuclear binding in PBMC from SS ($n = 6$) and SR ($n = 7$) patients. The upper panel shows the effect of 60-min dexamethasone treatment on binding of 1 μ g nuclear extracts from SS and SR patients to consensus double-stranded oligonucleotides encoding AP-1 as determined by electrophoretic mobility shift assays. The specific AP-1-TRE-retarded band is indicated by an arrow. Specificity of the retarded band is shown by loss of this band after preincubation with an excess of unlabeled oligonucleotide (XS). The middle panel shows the effect of 60-min dexamethasone treatment on binding of 1 μ g nuclear extracts from SS and SR patients to consensus double-stranded oligonucleotides encoding NF κ B as determined by electrophoretic mobility shift assays. The specific NF κ B-retarded bands are indicated by arrows. Specificity of the retarded bands is shown by loss of these bands after preincubation with an excess of unlabeled oligonucleotide (XS). The lower panel shows the effect of 60-min dexamethasone treatment on binding of 1 μ g nuclear extracts from SS and SR patients to consensus double-stranded oligonucleotides encoding CREB as determined by electrophoretic mobility shift assays. The specific CREB-cAMP response element (CRE)-retarded band is indicated by an arrow. Specificity of the retarded band is shown by loss of this band after preincubation with an excess of unlabeled oligonucleotide (XS).

that seen in SR patients ($9.6 \pm 18.4\%$ increase, $P < 0.05$; Fig. 7). PMA produced a similar level of increase in NF- κ B binding in both groups of subjects. SS subjects demonstrated a $159.3 \pm 54.0\%$ increase after 60-min PMA treatment, which was similar to that in SR patients ($108.0 \pm$

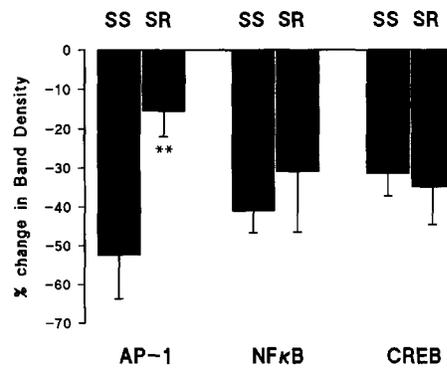


Figure 6. Action of dexamethasone (1 μ M) on nuclear transcription factor binding in PBMC from SS ($n = 6$) and SR ($n = 7$) patients. The effect of dexamethasone on binding of nuclear extracts to consensus double-stranded oligonucleotides encoding AP-1, NF- κ B, and CREB was determined using electrophoretic mobility shift assays. Mean \pm SEM values at 60 min are shown; * $P < 0.05$ (Wilcoxon's nonparametric analysis).

48.6%). Similarly, there was no significant difference in the effect of PMA on the levels of CREB binding between the two groups ($-7.8 \pm 19.5\%$ in SS versus $-32.8 \pm 16.8\%$ in SR patients) (Fig. 7). PMA produced a $43 \pm 4.4\%$ decrease in GR binding in cells from SS patients, but a much reduced inhibition of binding was found in the SR group ($-15.3 \pm 7.1\%$, $P < 0.05$) (Fig. 8).

Sequence Analysis of GR. No differences in the amounts of GR mRNA were found between the two groups of subjects by RT-PCR. cDNA sequence analysis of both the DNA-binding site (bp 1393–1605) and the τ_1 domain (bp 520–763) of the GR was performed after RT-PCR amplification of mRNA extracted from PBMC from all patients. There were no differences in the mRNA sequences of the DNA-binding domain or in the τ_1 domain of the SR patients compared with the published human cDNA sequence. There were also no differences in the amounts or sequences of the *c-fos* (bp 439–791) or *c-jun* (bp 5–245) mRNA or DNA between the SS and SR groups (Fig. 9).

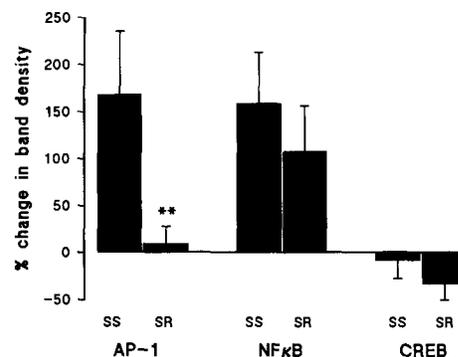


Figure 7. Action of PMA (0.1 μ M) on nuclear transcription factor binding in PBMC from SS ($n = 6$) and SR ($n = 7$) patients. The effect of PMA on binding of nuclear extracts to consensus double-stranded oligonucleotides encoding AP-1, NF- κ B, and CREB was determined using electrophoretic mobility shift assays. Mean \pm SEM values at 60 min are shown; * $P < 0.05$ (Wilcoxon's nonparametric analysis).

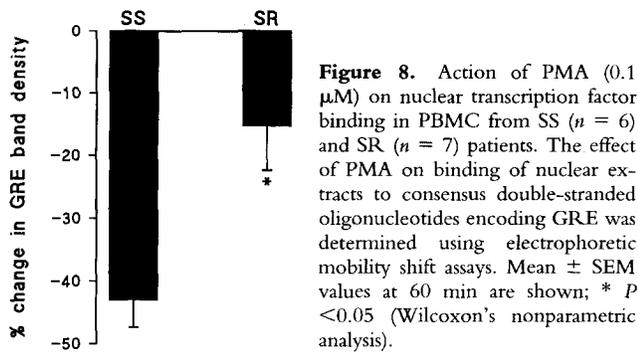


Figure 8. Action of PMA (0.1 μ M) on nuclear transcription factor binding in PBMC from SS ($n = 6$) and SR ($n = 7$) patients. The effect of PMA on binding of nuclear extracts to consensus double-stranded oligonucleotides encoding GRE was determined using electrophoretic mobility shift assays. Mean \pm SEM values at 60 min are shown; * $P < 0.05$ (Wilcoxon's nonparametric analysis).

Discussion

Previous studies of steroid resistance have failed to demonstrate any major differences in steroid binding or in nuclear localization of steroid receptors in peripheral blood monocytes or lymphocytes to account for the observed functional lack of responsiveness. A small decrease in GR affinity has been described in T lymphocytes of some patients with SR asthma (25), and a similar abnormality has been induced in healthy asthmatic patients in the presence of high concentrations of IL-2 and IL-4 (26). However, these relatively minor changes in affinity could not account for the reduced responsiveness to glucocorticoids seen in T

cells from these patients (8). We have recently reported a reduced ability of the GR to bind DNA after dexamethasone treatment in the PBMC of SR asthmatic patients compared with SS and normal subjects (11). Our results cannot be explained by a reduction in the binding between steroid and GR in the SR patients, since this has previously been reported to be unaffected in the same patients (9). We now report that there was also a significant reduction in the direct interaction between the transcription factor AP-1 and GR in SR patients, although interaction with two other transcription factors, CREB and NF κ B, was unaffected. Furthermore, there was an increase in the baseline levels of AP-1 in PBMC isolated from SR compared with SS asthmatic patients, indicating that there is increased activation of this transcription factor, even in cells that are unlikely to be affected by inflammation in the airways.

These results suggest that, in PBMC from patients with SR asthma, the decreased ability of the activated nuclear GR to bind to GRE may be due to a prior interaction of GR with other transcription factors, such as AP-1, involved in mediating chronic inflammatory responses. For example, excessive amounts of AP-1, or the formation of AP-1 with an altered affinity for GR or DNA, would reduce the number of activated GR within the nucleus available for binding to GRE. This may lead to reduced effectiveness of the transcription initiation complex in response to chronic inflammatory signals, such as cytokines. An abnormality in interaction between GR and AP-1 may explain why the steroid resistance appears to be confined to the antiinflammatory or immunomodulatory actions of steroids, rather than to their metabolic actions. Patients with steroid resistance do not suffer from steroid deficiency, have normal plasma cortisol concentrations, and show the normal adrenal suppression in response to exogenous steroids (2). This is in contrast to the high circulating levels of cortisol described in the very rare familial primary cortisol resistance, in which several abnormalities in GR function have been described, including reduced affinity and numbers of GR and reduction in nuclear localization of GR (27, 28). Various abnormalities of GR structure have been described in this condition (28).

The molecular mechanisms underlying the abnormal interaction between GR and AP-1 in SR asthma are not yet clear. A previous study has demonstrated no abnormality in GR sequence using chemical mutational analysis (12). The sequence of the DNA binding domain and the τ_1 transcription-activating domain reported here in the SR patients appear to be normal, confirming that there is unlikely to be a structural abnormality in GR in these patients. This is in contrast to the structural changes in GR reported in patients with familial glucocorticoid resistance (27). These patients differ from those studied here as they have high circulating cortisol and may have various endocrine abnormalities, none of which are present in patients with SR asthma. We have described an abnormality in the interaction between AP-1 and GR that may indicate a structural abnormality in the Fos and Jun proteins that comprise AP-1. However, sequence analysis of *c-fos* and *c-jun* showed no

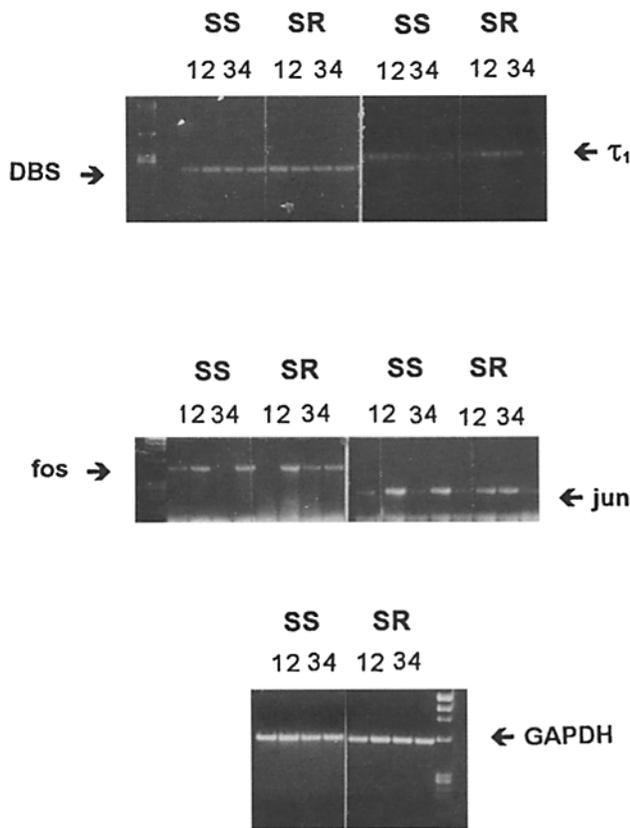


Figure 9. Basal levels of GR, *c-fos*, and *c-jun* mRNA in PBMC from SS and SR patients as measured by RT-PCR. The figure shows a representative gel of GAPDH and GR, *c-fos*, and *c-jun* RT-PCR products from PBMC isolated from SS and SR patients at $t = 0$.

abnormality compared with published sequences of these proteins. Several Fos- and Jun-related proteins may constitute AP-1, but c-Fos and c-Jun are the most common. Although no direct evidence exists, these results suggest that there may be some abnormality in the activation of these proteins, possibly due to an abnormality in the enzymes, such as Jun kinase (29), that regulate the activity of AP-1. Further studies on these enzymes are therefore indicated, particularly in view of the increased basal activation of AP-1 in PBMC that have not been exposed to inflammatory signals.

Corticosteroid resistance is not absolute but reflects a shift in the dose-response curve and tends to be studied by selecting patients at the extreme end of the spectrum of steroid sensitivity (8, 9). The sensitivity to prednisolone *in vitro* of T lymphocytes from normal subjects is highly variable, with a >10-fold variation in responsiveness (30). Presumably, this variability would only become apparent if these subjects were to develop an inflammatory or immune disease requiring glucocorticoid therapy, such as asthma or systemic lupus erythematosus.

Although steroid resistance, as strictly defined by lack of response to a large dose of oral steroids, is uncommon, elucidation of its mechanism may shed light on the mechanism of action of steroids in inflammatory diseases such as asthma. There is increasing evidence that the direct interaction between activated GR and the transcription factor

AP-1 is important in the inhibitory effects of steroids on cytokine gene transcription (14), and such interactions have been demonstrated in human lung and PBMC (16, 17). The finding that the interaction between GR and AP-1 is abnormal in patients with steroid resistance lends further support to the view that this interaction is critical in the anti-inflammatory action of steroids.

Steroid resistance poses a therapeutic problem and may result in the use of high doses of steroids, leading to serious side effects. Alternative immunomodulatory treatments, such as cyclosporin A, have been recommended (2). Cyclosporin A itself inhibits the transcription of certain cytokine genes via the inhibition of a transcription factor complex that includes AP-1 (31). The interactions of AP-1 with other transcription factors may thus be abnormal in patients with steroid resistance. This view is supported by reduced responsiveness to the immunomodulatory effects of cyclosporin A described in peripheral T lymphocytes of some SR patients (32).

Glucocorticoids inhibit the synthesis of several cytokines, including IL-1 and TNF- α (14). Although steroids may inhibit the inflammatory process at several sites, the direct interaction with transcription factors such as AP-1 and NF κ B may be of particular relevance, since this could provide a mechanism for switching off inflammation driven by cytokines known to activate these transcription factors (14).

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