p38 Mitogen-activated protein kinase–induced glucocorticoid receptor phosphorylation reduces its activity: Role in steroid-insensitive asthma

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Background: Although glucocorticoids are the most effective treatment for chronic inflammatory diseases, such as asthma, some patients show a poor response. IL-2 combined with IL-4 can alter glucocorticoid receptor (GR) ligand-binding affinity and modulate glucocorticoid function.
Objective: We sought to confirm the altered ligand-binding affinity in a distinct group of steroid-dependent asthmatic subjects and examine the mechanism by which IL-2 and IL-4 modify the ligand-binding affinity of the GR.
Methods: We examined PBMCs from healthy subjects, subjects with mild asthma, and steroid-dependent subjects with severe asthma using dexamethasone-binding assays and Western blot analysis of GR and phosphorylated activated transcription factor 2 expression. GR phosphorylation was measured after orthophosphate labeling and immunoprecipitation and cytokine production by means of ELISA.
Results: GR ligand-binding affinity was reduced in the nucleus but not in the cytoplasm of steroid-dependent asthmatic subjects compared with that seen in healthy subjects (dissociation constant, 39.8 ± 4.6 vs 6.79 ± 0.8 nmol/L). This difference in ligand-binding affinity could be mimicked by IL-2 and IL-4 cotreatment and was blocked by the p38 mitogen-activated kinase (MAPK) inhibitor SB203580. Activation of p38 MAPK by IL-2 and IL-4, as shown by means of phosphorylation of activated transcription factor 2, resulted in GR phosphorylation and reduced dexamethasone repression of LPS-stimulated GM-CSF release. p38 MAPK phosphorylation of $CD2^+$ T cells occurred on serine residues. The ability of dexamethasone to modulate IL-10 release was also inhibited by IL-2 and IL-4 cotreatment. These effects were also inhibited by SB203580.
Conclusion: These data show that p38 MAPK inhibitors may have potential in reversing glucocorticoid insensitivity and reestablishing the beneficial effects of glucocorticoids in patients with severe asthma. (J Allergy Clin Immunol 2002;109:649-57.)

Key words: *Human, inflammation, p38 MAP kinase, glucocorticoid receptor, asthma, cytokine release, PBMCs*

Glucocorticoids are effective anti-inflammatory drugs in several diseases, such as asthma.[1] Asthma is characterized by airway inflammation involving eosinophils, macrophages, mast cells, and $CD4^+$ T lymphocytes and is characteristically suppressed by glucocorticoids. Although glucocorticoids are effective in controlling asthma in the majority of patients, there is a minority of patients who respond less well, needing high doses of inhaled and oral glucocorticoids, with a small proportion demonstrating complete resistance.[2] These patients account for a large proportion of the high costs involved in treating asthma.[3] Impaired glucocorticoid responsiveness has been most extensively studied in asthma but has also been reported in other inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and transplantation rejection.[2]

Glucocorticoids act by binding to a cytoplasmic glucocorticoid receptor (GR), which rapidly translocates into the nucleus. Within the nucleus, the GR binds as a dimer to specific glucocorticoid response elements on DNA within the promoter region of glucocorticoid-responsive genes, thus enhancing their transcription (transactivation).[4] Glucocorticoids also prevent other transcription factors, such as activator protein 1 and nuclear factor κB, from activating their target genes by means of direct protein-protein interactions, which is termed transrepression, a mechanism that involves deacetylation of acetylated core histones.[5] In addition, glucocorticoids may also play a role in repressing the action of proinflammatory kinase cascade systems, such as the extracellular regulated kinase (ERK) and the Jun N-terminal kinase (JNK) mitogen-activated protein kinases (MAPKs).[6-9]

A spectrum of glucocorticoid responsiveness in airway inflammatory diseases may exist, reflecting several mechanisms caused by either disease activity itself or by the effects of therapy, with the glucocorticoid-resistant asthmatic subjects at one extreme of this spectrum. At a

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Supported by the Wellcome Trust, the National Asthma Campaign, the Clinical Research Committee (Brompton Hospital), and Glaxo-Wellcome. E.I. is supported by the South African Pulmonology Society through a Grant from Boehringer Ingelheim (S.A.).
Received for publication August 9, 2001; revised December 4, 2001; accepted for publication December 18, 2001.
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0091-6749/2002 $35.00 + 0 **1/83/122465**
doi:10.1067/mai.2002.122465

Abbreviations used
ATF-2: Activated transcription factor 2
ERK: Extracellular regulated kinase
GR: Glucocorticoid receptor
ICS: Inhaled corticosteroid
JNK: Jun N-terminal kinase
Kd: Dissociation constant
MAPK: Mitogen-activated protein kinase
PMA: Phorbol 12-myristate 13-acetate

molecular level, resistance to the anti-inflammatory effects of glucocorticoids can be induced by several mechanisms.[10] The reduction in glucocorticoid responsiveness observed in cells from these subjects has been ascribed to a reduced number of GRs, altered affinity of the ligand for GRs, reduced ability of the GR to bind to DNA, or increased expression of inflammatory transcription factors, such as activator protein 1, that compete for DNA binding.[11-15]

Bronchoalveolar lavage of a group of corticosteroid-resistant subjects revealed an increased number of cells expressing IL-2, IL-4, and IL-13 mRNA compared with numbers seen in steroid-sensitive asthmatic subjects.[16] This suggested that the profile of cytokine expression may underlie the poor responsiveness to glucocorticoids in these patients. Indeed, a combination of IL-2 and IL-4 or IL-13 alone can induce a reduction in the affinity of nuclear GRs in PBMCs and has been associated with an increase in GRβ expression.[13,15,16]

We have examined this mechanism for reduced glucocorticoid responsiveness in asthma further by studying whether glucocorticoid binding to the GR is impaired in a group of steroid-dependent asthmatic subjects. These patients require continuous oral corticosteroid therapy in addition to high-dose inhaled corticosteroids (ICSs) for symptom control. We have investigated the mechanism by which the combination of IL-2 and IL-4 could reduce GR affinity and the anti-inflammatory actions of glucocorticoids. IL-2 and IL-4 activate intracellular pathways, such as the ERK, JNK, and p38 MAPK cascades,[17] and STAT6,[18] respectively. Using inhibitors, we have examined the role of these kinases in modulating glucocorticoid responsiveness and show that activation of p38 MAPK results in GR phosphorylation and that p38 MAPK inhibitors increase the anti-inflammatory effects of glucocorticoids.

METHODS

Patients

Nine asthmatic subjects whose symptoms were controlled with ICSs (0.8 μg/d [95% CI, 0.2-1.3 μg/d], mean fluticasone equivalents), 19 steroid-dependent asthmatic subjects (poorly controlled on inhaled and oral steroids), and 6 healthy nonasthmatic subjects were studied. All subjects were matched in terms of age and atopic status (Table I). No patients were current smokers. Asthmatic subjects were defined as patients who have shown a 15% increase in FEV_1 to 200 μg of inhaled albuterol administered through a metered-dose inhaler or significant diurnal variation in peak expiratory flow rate of greater than 15%. All patients were taking inhaled albuterol on an as-required basis. The mean daily doses of albuterol administered through a metered-dose inhaler were 0.4 ± 0.1 mg/d and 1.3 ± 0.6 mg/d for the ICS and steroid-dependent group, respectively. The ICS group had a mean baseline FEV_1 of 2.31 ± 0.3 L (76% ± 5% of predicted value). Steroid-dependent patients, however, had a baseline FEV_1 of 52% ± 3% of predicted value and were receiving high-dose ICS therapy (1500 ± 140 μg of fluticasone or equivalent) together with a maintenance dose of oral prednisolone (20 ± 2.8 mg) for the control of their asthma. All blood samples were collected in the morning before medication and at least 24 hours after oral prednisolone. One hundred milliliters of blood was taken for each assay. Six ICS patients had a course of 40 mg of oral prednisolone for 7 days, and 8 steroid-dependent patients supplemented their daily dose of oral prednisolone to a total of 40 mg/d. FEV_1 was measured before and after this trial of prednisolone, and blood was taken for GR binding experiments. The study was approved by the Ethics committee of Royal Brompton Hospital, and written informed consent was obtained from all subjects.

PBMC isolation

PBMCs were isolated by means of Ficoll-Hypaque (Pharmacia-Biotek, St Albans, United Kingdom) density centrifugation. Viability was assessed by means of trypan blue exclusion and was consistently greater than 97%. Cells at a concentration of 1×10^6/mL in RPMI with HEPES (20 mmol/L) supplemented with 10% FCS, penicillin, streptomycin, and amphotericin B were cultured for 48 hours at 37°C in medium alone and in the presence of IL-2 (50 IU/mL; R&D Systems, Abingdon, United Kingdom) and IL-4 (50 IU/mL, R&D Systems) or IL-13 (50 IU/mL, R&D Systems). Thereafter, cells were harvested and washed in fresh RPMI. The effects of the signal-transduction pathway inhibitors wortmannin (5 nmol/L), PD098059 (10 μmol/L), Ro318220 (10 nmol/L), SB203580 (1 μmol/L; all from Calbiochem, Nottingham, United Kingdom) and IL-10 (3 ng/mL, R&D systems) on IL-2– and IL-4-stimulated modulation of GR binding characteristics were investigated. In some cases $CD2^+$ T cells were isolated by using negative selection beads (Dynal, Bromborough, United Kingdom) according to the manufacturer's instructions and treated with IL-2/IL-4 in the presence of SB203580 (1 μmol/L).

Tritiated dexamethasone binding assay

GR binding characteristics of PBMCs (1.5×10^6 cells per study point) were assessed before and after 48-hour incubation with ^3H dexamethasone (81.0 Ci/mmol, Amersham), as described previously. Separation of nuclear and cytosolic fractions was achieved exactly as described by Kam and colleagues.[13] Specific binding for each concentration was calculated after correcting for nonspecific binding. Binding isotherms were analyzed with the PRISM curve-fitting program (GraphPad Software, San Diego, Calif).

GM-CSF and IL-10 release

PBMCs (1×10^6/mL) were incubated with medium alone or IL-2 and IL-4 (50 IU/mL) for 48 hours in the presence of 1% FCS and thereafter stimulated with LPS (10 ng/mL; Sigma, Poole, United Kingdom) or phorbol 12-myristate 13-acetate (PMA)/PHA for 20 hours at 37°C. Plates were centrifuged, supernatants were collected, and GM-CSF and IL-10 were measured with a sandwich ELISA. GM-CSF and IL-10 concentrations in the culture supernatant were measured by using a specific ELISA calibrated with human recombinant GM-CSF (0-200 pg/mL, R&D Systems) or IL-10 (0-200 pg/mL, R&D Systems), essentially as previously described.[19] The concentration-dependent effects of dexamethasone on cytokine release were also studied.

TABLE I. Characteristics of asthmatic and healthy subjects

	Steroid-dependent subjects	ICS subjects	Healthy subjects
No. of subjects	19	9	6
Age (y), mean (95% CI)	46 (40-52)	48 (38-58)	32 (27-38)
Sex (M/F)	4/16	4/5	3/3
Baseline FEV_1 before BD (% predicted)	52 (45-59)†§	76 (65-87)‡	91.6 (82-100)
FEV_1 response after albuterol (% predicted)‖	36 (24-48)*	18 (12-24)	—
Duration of asthma (y)	29 (21-37)	36 (25-48)	—
Inhaled steroids (mg/d)¶	1.5 (1.2-1.8)†	0.8 (0.2-1.3)	—
Years on systemic steroids	6.8 (3.9-9.7)	—	—
Maintenance prednisolone (mg/d)	20 (14-26)	—	—
Atopy (positive/negative)#	11/3	7/2	2/4
Smoking pack-years in smokers	14 ± 4	13 ± 4	—

Results are expressed as means and 95% CIs.
BD, Bronchodilator.
*$P < .05$ and †$P < .01$ compared with ICS group.
‡$P < .05$ and §$P < .001$ compared with healthy subjects.
‖Albuterol dose of 200 μg.
¶Expressed as dose equivalent to fluticasone propionate.
#Atopy is defined as positive skin prick test responses to at least 3 common allergens.

Metabolic labeling of human PBMCs with phosphorous 32–labeled orthophosphate

PBMCs were freshly isolated and washed with fresh media before incubating with IL-2 and IL-4 in the presence or absence of SB203580 for 48 hours at 37°C in RPMI/10% FCS containing 1 mCi/mL ^{32}P-labeled orthophosphate (Amersham).[20] At the end of this time period, cells were washed extensively with fresh media before extraction of soluble proteins. All solutions contained complete protease inhibitor tablets (Boehringer, Lewes, United Kingdom) to prevent protein degradation.

Immunoprecipitation and Western blotting

PBMCs or $CD2^+$ T cells were incubated with medium or IL-2/IL-4 in the presence or absence of SB203580 (1 μmol/L). In separate experiments ^{32}P-labeled orthophosphoric acid (Amersham) was added to all cells. After 48 hours, cells were harvested and washed repeatedly before cells were lysed, and immunoprecipitation was carried out as previously described,[21] with stringent immunoprecipitation conditions and 5 μg of anti-human GRα/β antibody (Santa Cruz, Wembley, United Kingdom) or 5 μg of preimmune serum (Dako, Cambridge, United Kingdom). Western blot analysis was performed as previously described[22] by using specific GRα or GRβ antibodies (Affinity Bioreagents, Cambridge, United Kingdom). HeLa cell extracts (Santa Cruz) were used as positive controls for GRα and GRβ. Phosphorylated activated transcription factor 2 (ATF-2) and control nonphosphorylated ATF-2 antibodies were obtained from Upstate Biotechnology (Buckingham, United Kingdom). Anti-phosphoserine antibodies were obtained from Santa Cruz. All solutions contained complete protease inhibitor tablets (Boehringer) to prevent protein degradation.

Data analysis

Data are expressed as means ± SEM of independent observations. The results obtained before or after drug treatment were compared by means of ANOVA. Median effective concentration calculations were performed with the GraphPad Prism program.

RESULTS

GR ligand binding of PBMCs

There was no significant difference in the cell profile of the PBMCs isolated from any subject group (data not shown). The ability of dexamethasone to bind to GR was decreased in the nucleus in subjects with steroid-dependent asthma. Steroid-dependent subjects (Kd, 39.8 ± 4.6 nmol/L) had a greater Kd compared with those with ICS-controlled asthma symptoms (Kd, 25 ± 0.9 nmol/L; $P < .05$) or healthy subjects (Kd, 6.79 ± 0.8 nmol/L; $P < .001$). In addition, there was a significant difference in Kd between the ICS group and the healthy group ($P < .05$; Fig 1, *A*). No differences in Kd were detected in the cytoplasm, however. The number of receptors was higher in the nucleus than in the cytoplasm, although there was no difference in nuclear receptor numbers between subject groups (steroid-dependent group, 4430 ± 566/cell; ICS group, 2780 ± 357/cell; healthy subjects, 3080 ± 509/cell; Fig 1, *B*).

Response to oral prednisolone

Six patients in the ICS group had a baseline percent predicted FEV_1 of 71.3 ± 4%, and this was not significantly changed by a week of 40 mg of oral prednisolone (74.8% ± 12% of predicted value). Eight steroid-dependent patients had their oral prednisolone increased to 40 mg for 1 week. These patients demonstrated a mean improvement of 20% ± 0.4% in FEV_1 after a 1-week course of 40 mg of prednisolone (55% ± 5% of predicted value improved to 68% ± 7%; Fig 1, *C*). Treatment ICS and steroid-dependent asthmatic subjects with prednisolone (40 mg/d) for 1 week did not significantly affect the receptor number or Kd of ligand binding (Fig 1, *D* and *E*). The altered ability of steroid-dependent and steroid-sensitive asthmatic subjects to respond to steroids may therefore relate to their reduced nuclear GR affinity for ligands. Because prednisolone did not affect this altered affinity, this further suggests that reduced glucocorticoid responsiveness in steroid-dependent subjects is not caused by prednisolone-induced downregulation of GR expression.

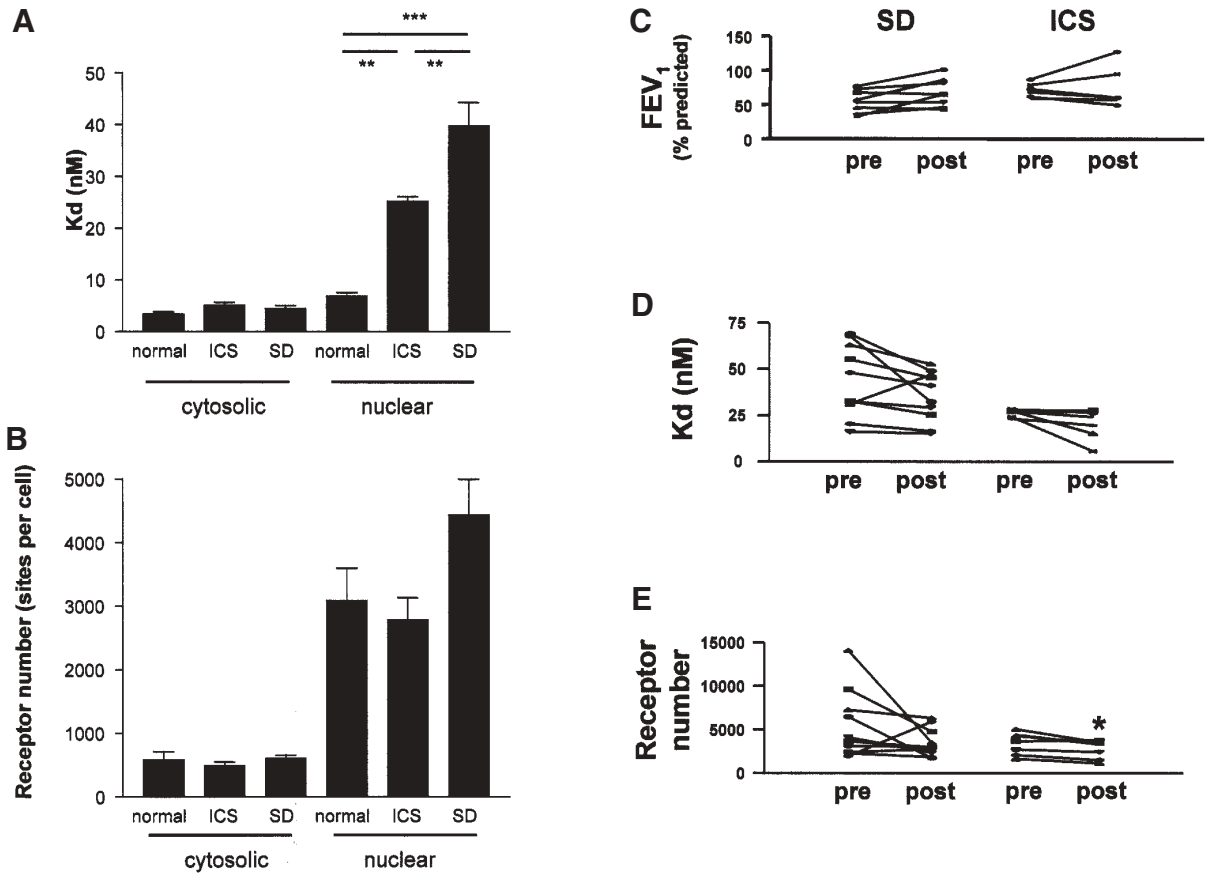


FIG 1. GR binding parameters of PBMCs isolated from healthy subjects and patients with severe asthma and the effects of prednisolone. Ligand-binding affinity *(Kd)* in cytosolic and nuclear compartments **(A)** was measured in 6 healthy subjects, 19 oral steroid–dependent *(SD)* asthmatic patients, and 9 asthmatic patients whose symptoms were controlled with ICSs *(ICS)*. The receptor number in cytosolic and nuclear compartments was also measured in these subjects **(B)**. Data are expressed as means ± SEM (**$P < .01$, ***$P < .001$). The effect of prednisolone (40 mg/d for 7 days) on percent predicted FEV_1 **(C)**, GR affinity **(D)**, and receptor number **(E)** in steroid-dependent and ICS subjects is also shown. Data are expressed as individual data points (*$P < .05$).

IL-2/IL-4 alters GR ligand binding in vitro

Treatment of PBMCs from healthy subjects for 48 hours with combined IL-2 and IL-4 (each at 50 IU/mL), but not with IL-2 or IL-4 alone, leads to a decrease in GR ligand affinity within the nucleus (9.4 ± 1.4 vs 40 ± 0.6 nmol/L, $P < .0001$). The number of receptor sites per cell was not significantly altered. The mean Kd after IL-2/IL-4 was similar to that seen in glucocorticoid-dependent asthmatic subjects (Fig 2, *A*). Treatment of cells with media or IL-2 or IL-4 alone had no effects on receptor affinity or number (data not shown). IL-13 (50 IU/mL) alone also reduced GR ligand-binding affinity at 48 hours (12.4 ± 1.6 vs 38.9 ± 1.8 nmol/L, $P < .05$).

p38 MAPK regulates IL-2/IL-4 alteration of GR ligand-binding affinity

We therefore explored the possible mechanisms for reduced steroid function caused by IL-2/IL-4 treatment by using inhibitors of signaling pathways to examine their effects on GR affinity. Inhibition of the p38 MAPK pathway with SB203580 (1 μmol/L) completely prevented the IL-2/IL-4–mediated reduced receptor affinity in healthy subjects (Fig 2, *A*). A similar effect was also seen with SB203580 (1 μmol/L) on IL-13–induced changes in ligand-binding affinity (38.9 ± 1.8 vs 11.4 ± 0.9 nmol/L, $P < 001$). We confirmed that IL-2/IL-4 activated p38 MAPK in these cells by examining ATF-2 phosphorylation, a known downstream substrate for this kinase. ATF-2 phosphorylation was inhibited by SB203580 (1 μmol/L) at both 1 and 24 hours (Fig 2, *B*).

Incubation of cells from steroid-dependent subjects in media alone for 48 hours resulted in a reversion of the altered nuclear Kd back to levels seen in healthy subjects (Fig 2, *C*). This effect was prevented by treatment of cells with IL-2/IL-4 but not by treatment with either cytokine alone (data not shown). SB203580 (1 μmol/L) prevented IL-2 and IL-4 from maintaining a low binding affinity in steroid-dependent asthmatic subjects, with a reduced

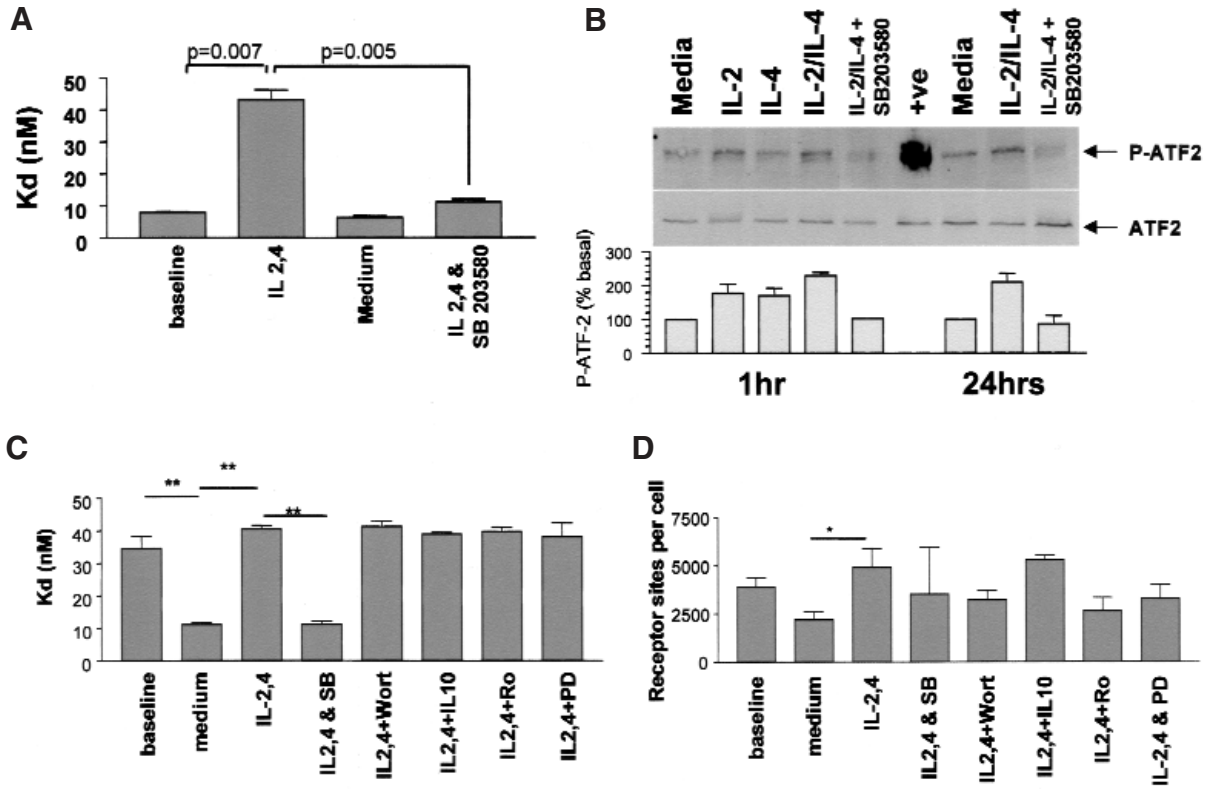


FIG 2. IL-2/IL-4–induced attenuated ligand-binding affinity is mediated by p38 MAPK. **A**, IL-2 and IL-4 (both at 50 IU/mL) reduce ligand-binding affinity *(Kd)* in healthy subjects after 48 hours. This effect is not seen with medium alone and is blocked by the p38 MAPK inhibitor SB203580 (1 μmol/L). **B**, Western blot analysis showing that IL-2/IL-4 treatment (both at 50 IU/mL) stimulates phosphorylation of ATF-2 *(P-ATF-2)* at 1 and 24 hours. This effect is inhibited by SB203580 (1 μmol/L). Equal loading was confirmed by measuring the expression of nonphosphorylated ATF-2 in the same samples. Results are representative of 3 separate experiments. Densitometric analysis of the data is shown in the panel below. **C**, Effect of 48 hours' incubation with medium alone, IL-2/IL-4 (both at 50 ng/mL), IL-2/IL-4 and SB203580 (*SB*; 1 μmol/L), wortmannin (*Wort*; 5 nmol/L), IL-10 (3 ng/mL), Ro318220 (*Ro*; 10 nmol/L), or PD098059 (*PD*; 10 μmol/L) on GR ligand-binding affinity (Kd). **D**, GR receptor numbers of PBMCs isolated from steroid-dependent subjects. Results are expressed as means ± SEM for 8 steroid-dependent patients and compared with values obtained at baseline (**P* < .05, ***P* < .01).

binding affinity at baseline (Fig 2, *C*). In contrast, inhibition of the ERK MAPK pathway by PD098059 (10 μmol/L) had no effect on the altered receptor affinity. Inhibition of phosphoinositol 3 hydroxy kinase by wortmannin (5 nmol/L) or treatment with IL-10 (10 ng/mL) also failed to modulate the effect of IL-2 and IL-4 on receptor affinity. Ro318220 (10 nmol/L), a specific protein kinase C inhibitor, similarly had no effect on affinity (Fig 2, *C*). IL-2 and IL-4 cotreatment restored the reduced levels of receptor per cell in PBMCs from steroid-dependent subjects seen after 48 hours' incubation in medium alone (Fig 2, *D*). This action of combined IL-2 and IL-4 treatment was not affected significantly by any of the inhibitors studied (Fig 2, *D*).

IL-2/IL-4 phosphorylates GR and inhibition by SB203580

To further investigate the effect of the p38 MAPK pathway on GR function, we examined whether GR, a phosphoprotein, could be a target for this kinase. GR was immunoprecipitated from whole-cell lysates from IL-2/IL-4–treated PBMCs in the presence of ^{32}P-labeled orthophosphate, size fractionated by means of SDS-PAGE, and transferred to a nylon membrane. Autoradiography of the resultant membrane showed the presence of 2 phospho-labeled bands that migrated at the same position as GR, as determined by using Western blotting, with the lower band being more heavily phosphorylated. It was not possible, with this technique, to determine whether these bands reflected GRα and GRβ or GR-A and GR-B isoforms or combinations of both. GR phosphorylation was inhibited by SB203580 (1 μmol/L; Fig 3, *A*). The other inhibitors used above did not affect GR phosphorylation (data not shown). Previous studies have suggested that IL-2/IL-4 inhibits steroid actions by inducing GRβ expression.[23] We therefore examined our samples for

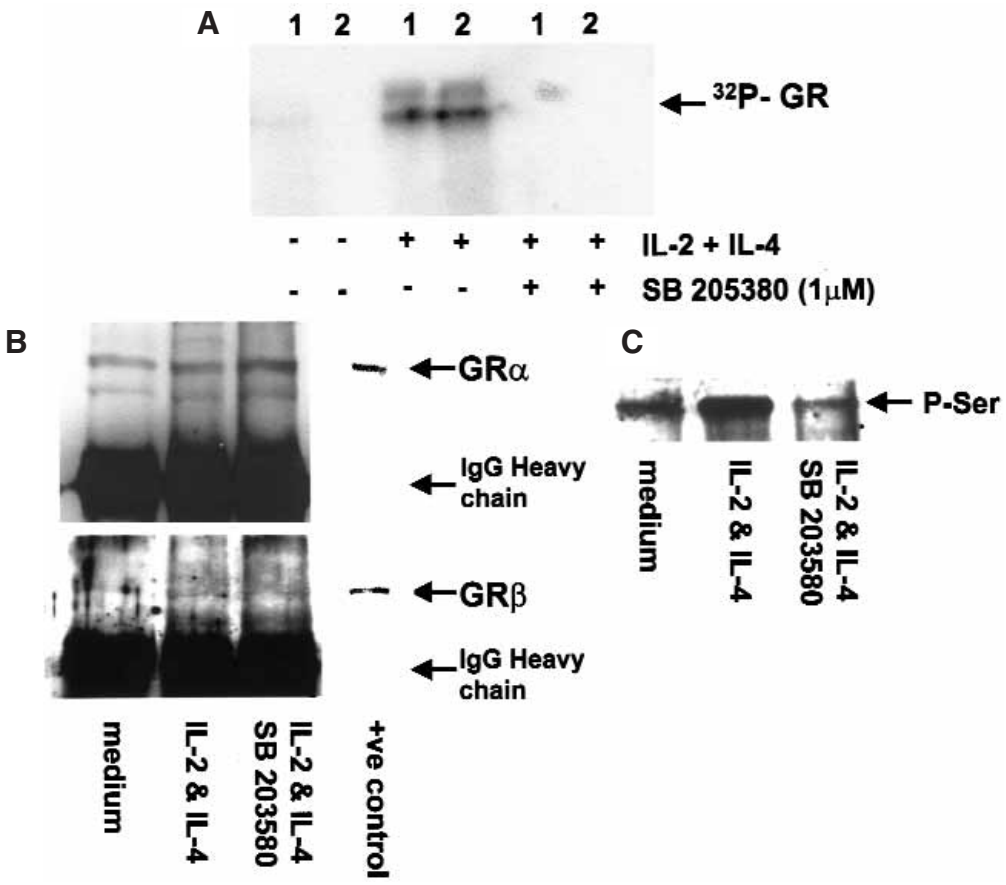


FIG 3. IL-2/IL-4 induces p38-mediated GR phosphorylation. PBMCs isolated from healthy subjects were treated with IL-2/IL-4 (both 50 IU/mL) for 48 hours in the presence of ^{32}P-labeled orthophosphate. GR was immunoprecipitated with a GRα/β antibody and fractionated with SDS-PAGE. **A**, Autoradiograph of gel showing induction of ^{32}P incorporation into GR by means of IL-2/IL-4 treatment was inhibited by SB203580 (1 μmol/L). **B**, *upper panel*, Western blot analysis of immunoprecipitated GR showing no induction of GR expression after incubation of cells with IL-2/IL-4 or with SB203580. *Lower panel*, No expression of GRβ was detected with a GRβ-specific commercially available antibody (Affinity Bioreagents). Results are representative of 3 independent experiments. Positive controls from HeLa cell extracts for GRα and GRβ are also shown. The lower intense bands are due to detection of the 50-kd IgG heavy chain and are used as loading controls. **C**, Western blot analysis of IL-2/IL-4–induced serine phosphorylation of immunoprecipitated GRα/β obtained from CD2$^+$ T cells. The effect is inhibited by means of cotreatment with SB203580 (1 μmol/L).

GRβ expression. Using a GRα/β-specific antibody, we immunoprecipitated GR from IL-2/IL-4–treated PBMCs from healthy subjects and performed Western blot analysis for GRα using an anti-GRα antibody or for GRβ using a GRβ-specific antibody. Using the GRα antibody, we detected 2 bands at approximately 90 to 100 kd that were not altered by IL-2/IL-4 stimulation. In addition, using commercially available antibodies, we were unable to detect any GRβ expression in GRα/β immunoprecipitates from these cells (Fig 3, *B*). This may reflect the lack of sensitivity of the antibody for GRβ rather than GRβ degradation because it was able to detect a GRβ positive control. In both panels the major band seen at 50 kd is the IgG heavy chain, a carryover from the immunoprecipitation antibody, which was used to control for loading.

It is likely that only a subset of cells responds to IL-2/IL-4 treatment. To further investigate this, we treated CD2$^+$ cells with IL-2/IL-4 as above in the presence and absence of SB203580 (1 μmol/L). IL-2/IL-4 induced an increase in immunoprecipitated GR serine phosphorylation, which was inhibited by SB203580 (Fig 3, *C*). GR was also seen to be phosphorylated on threonine and tyrosine residues, but this was not altered by IL-2/IL-4 or by SB203580 (data not shown).

Effect of IL-2/IL-4 on GM-CSF and IL-10 release

In concordance with the data from Larsson et al,[24] we also found that combined IL-2/IL-4 treatment of cells resulted in functional inhibition of dexamethasone actions. IL-2 and IL-4 treatment increased basal levels of GM-CSF from just above the level of detection (66 ± 8 vs 23 ± 14 pg/mL). In these experiments GM-CSF release was not further enhanced by addition of LPS, but

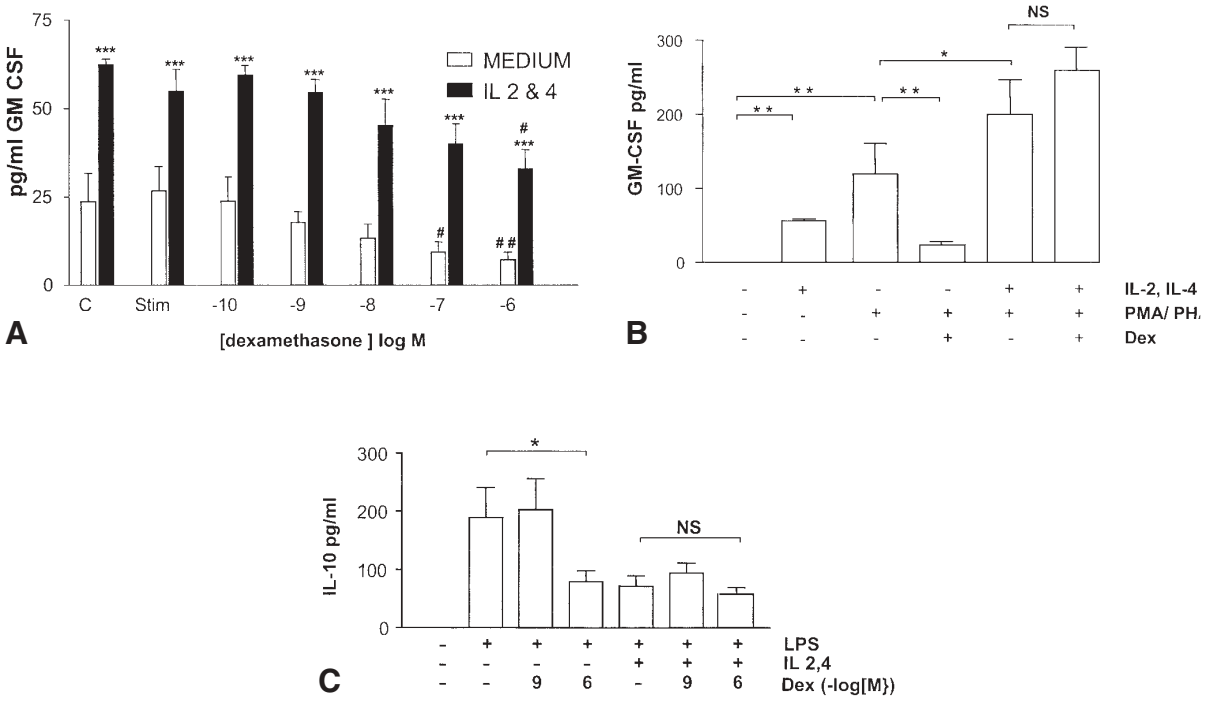


FIG 4. Effect of IL-2 and IL-4 treatment on cytokine release. **A**, Effect of dexamethasone on GM-CSF production in LPS-stimulated (10 ng/mL) cultures of PBMCs from normal subjects pretreated with IL-2 and IL-4 (50 IU/mL). ***$P < .001$ compared with medium alone. ##$P < .01$ compared with stimualted cells. #$P < .05$ compared with stimuated cells. **B**, Effect of dexamethasone on GM-CSF production in PMA/PHA-stimulated cultures of PBMCs from normal subjects pretreated with IL-2 and IL-4 (both at 50IU/mL). **$P < .01$. *$P < .05$. *NS,* Not significant. **C**, Effect of IL-2 and IL-4 cotreatment on dexamethasone inhibition of LPS-induced IL-10 release in PBMCs from normal subjects. *$P < .05$. Results are expressed as means ± SEMs of at least 5 independent experiments.

the ability of dexamethasone to maximally inhibit GM-CSF release, as well as the concentration-response curve (median effective concentration, 4.5×10^{-10} vs 0.8×10^{-8}; $P < .05$), was impaired (Fig 4, *A*). PMA/PHA–stimulated cells after IL-2/IL-4 incubation showed a similar inhibition of the ability of dexamethasone to repress GM-CSF release. In these experiments the basal levels of GM-CSF release in the cells was below the level of detection (Fig 4, *B*). IL-2/IL-4 cotreatment reduced expression of the anti-inflammatory cytokine IL-10 and reduced the ability of dexamethasone to modulate IL-10 production (Fig 4, *C*).

DISCUSSION

The ability of dexamethasone to bind to GR was decreased according to the degree of asthma severity. This difference was detected only in the nucleus and not in the cytoplasm, possibly reflecting an effect of a nuclear protein masking the GR ligand-binding site or in an altered conformation of the activated GR. This altered affinity of dexamethasone for GR may reflect either an intrinsic defect in the GR within these patients or may relate to changes in the receptor induced by the increased level of inflammation in subjects with more severe asthma. The reversal of the reduced binding affinity by means of incubation with healthy media suggests that the later is a more likely possibility. Our results confirm the differences in Kd between asthmatic and healthy subjects at baseline originally described by Leung and coworkers.[13,23,25] We also confirmed that diminished GR affinity could be induced in vitro by incubating PBMCs with IL-2/IL-4 or IL-13.

Combined treatment of cells with IL-2 and IL-4 resulted in increased expression of the proinflammatory cytokine GM-CSF and reduced expression of the anti-inflammatory cytokine IL-10. In addition, we show that the ability of dexamethasone to modulate IL-10 production and inhibit LPS- or PMA/PHA–stimulated GM-CSF release was reduced in PBMCs pretreated with IL-2 and IL-4. This suggests that the balance of cytokine production favoring a proinflammatory state may contribute to asthma severity. We have shown that a possible molecular basis for this effect is the phosphorylation of the GR after activation of the p38 MAPK pathway. We also show that the effect of IL-2/IL-4 on GR phosphorylation was

seen in $CD2^+$ T cells in which the GR was phosphorylated on serine residues. Because there is a consensus p38 MAPK phosphorylation sequence at serine 226 within the human GR, it is possible that this is a direct effect of p38 MAPK on the GR. Other potential targets may be proteins involved in GR activation, such as hsp90, and basal transcription factors or other downstream kinases may target the GR.

In contrast to data from Gagliardo et al,[26] we were unable to see any change in GR expression after oral prednisolone treatment. This may reflect the lower dose of prednisolone used in this study, the reduced time of administration (40 mg/d for 7 days compared with 1 $mg \cdot kg^{-1} \cdot d^{-1}$ for 10 days), or both. We detected 2 bands for GRα/β, suggesting that the lower band may be GRβ, but we were unable to detect GRβ with a specific antibody, suggesting a lack of GRβ expression in cells from steroid-dependent patients, as described by Gagliardo et al. However, the most highly phosphorylated band we detected after metabolic labeling of the GR was the lower band, which may reflect GRβ phosphorylation, and the failure to detect GRβ with a GRβ-specific antibody may reflect the insensitivity of the antibodies used. Thus we cannot rule out a role for GRβ in these events.

Inhibition of the p38 MAPK pathway by SB203580 prevented both the IL-2/IL-4– and IL-13–induced decrease in receptor affinity and subsequent anti-inflammatory actions of dexamethasone. Inhibition of other signal-transduction pathways postulated to be involved in modulating GR functions (ERKs, phosphoinositol 3 hydroxy kinase, protein kinase C, and IL-10) all failed to modulate IL-2/IL-4 actions on GR, however. p38 MAPK responds primarily to stressful and inflammatory stimuli, such as TNF-α, IL-1-β, and LPS, which may also be expected to modulate GR action. Indeed, IL-1β (20 ng/mL) and LPS (100 μg/mL) reduce GR binding affinity in BEAS-2B cells.[27]

GR is a phosphoprotein that contains consensus sequences for numerous kinases, such as protein kinase A, caesin kinase II, protein kinase C, cyclin-dependent kinases, glycogen synthase kinase 3, and MAPKs.[28] The MAPKs phosphorylate and activate transcription factors that regulate cell proliferation and inflammation, effects opposite to those observed with glucocorticoids. These activation pathways can interact with GR, modifying each others' actions and occurring at the level of the transcription factors themselves.[29] Recent evidence suggests that GR can inhibit the activity of both ERK and JNK enzymes, thus limiting the inflammatory response.[6-9] Rogatsky et al[30] have, in turn, shown reciprocal inhibition of rat GR reporter gene activity by JNKs by means of a direct phosphorylation of serine 246 through the consensus nonpolar-X-Ser/Thr-Pro motif, whereas ERK can inhibit GR action by means of an indirect effect, possibly through phosphorylation of a cofactor.

Our data cannot determine whether this is a direct or indirect effect of p38 MAPK or whether GR phosphorylation alters ligand-binding affinity directly, but the data in Fig 2 clearly show that SB203580 can modify ligand-binding parameters. Previous data have shown that reversible serine-threonine phosphorylation of GR may influence the binding of the hormone to the cytoplasmic GR complex, GR nuclear translocation, GR binding to glucocorticoid response elements, and formation of an activated transcriptional complex.[31] Our data suggest that IL-2/IL-4 may alter the functional activity of GR through phosphorylation of a serine residue, possibly serine 226, by means of p38 MAPK within the nucleus. The resultant effect is to change GR binding characteristics and probably also its ability to interact with components of the transcription apparatus. This may result from either a change in GR conformation caused by association of distinct cofactors or partial blocking of the ligand-binding domain caused by association of the GR with nuclear transcriptional modulating proteins. Similar results have been seen after nitric oxide treatment of GR, whereby nitrosylation of GR at an hsp90 interaction site modified ligand binding.[32]

The GR phosphorylation site serine 226 and the sequences immediately surrounding it are highly conserved, suggesting functional conservation for transcriptional antagonism between the GR and p38 MAPK. Leucine-rich sequences flanking serine 226 may be involved in nuclear export,[33] or this region may form a surface that interacts with factors involved in transcriptional regulation.[34] Interactions between the rat GR and the transcriptional complex involve several domains within the N-terminal region of GR, including a leucine-rich domain surrounding the p38 MAPK site.[34] Serine 226 of the human GR lies within one such domain, and thus phosphorylation of serine 226 may alter or disrupt the protein-protein interactions regulating GR action.

GR phosphorylation by p38 MAPK provides a novel mechanism for downregulation of GR activity in T cells in response to proinflammatory stimuli, leading to relative glucocorticoid resistance or dependence, and may have clinical implications for the use of p38 MAPK inhibitors in these subjects. Enhanced activation of this pathway as a result of stimulation by inflammatory mediators, such as IL-2 and IL-4, that are elevated at the site of inflammation in corticosteroid-resistant asthma may modify GR directly, leading to recruitment of distinct GR-associated proteins and resulting in reduced glucocorticoid function. The requirement for p38 MAPK activation to induce reduced glucocorticoid responsiveness may explain why these patients do not experience corticosteroid-induced side effects. These data suggest that SB203580 and other p38 MAPK inhibitors may have potential not only as anti-inflammatory agents in their own right but also as agents that restore corticosteroid responsiveness and therefore act as steroid-sparing agents. Targeted treatment with low doses of p38 MAPK inhibitors may reverse glucocorticoid resistance and improve the anti-inflammatory effects of glucocorticoids in these glucocorticoid-dependent or glucocorticoid-resistant patients with asthma, who are usually unresponsive to other currently available therapies.

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