Increased Glucocorticoid Receptor β Alters Steroid Response in Glucocorticoid-insensitive Asthma

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Rationale: Glucocorticoids (GCs) are highly effective in the treatment of asthma. However, some individuals have GC-insensitive asthma.

Objectives: To evaluate the functional response to steroids of bronchoalveolar lavage (BAL) cells from sites of airway inflammation from patients with GC-insensitive versus GC-sensitive asthma. As well, to attempt to define the functional role of glucocorticoid receptor (GCR) β (a splicing variant, and dominant negative inhibitor of, the classic GCR α) in controlling GCR α nuclear translocation and transactivation at a molecular level.

Methods and Measurements: Fiberoptic bronchoscopy with collection of BAL fluid was performed on seven patients with GC-sensitive asthma and eight patients with GC-insensitive asthma. GCR α cellular shuttling in response to 10^{-6} M dexamethasone treatment and GCR β expression were analyzed in BAL cells by immunofluorescence staining. The effects of overexpression and silencing of GCR β mRNA on GCR α function were assessed.

Main Results: Significantly reduced nuclear translocation of GCR α in response to steroids was found in BAL cells from patients with GC-insensitive asthma. BAL macrophages from patients with GC-insensitive asthma had significantly increased levels of cytoplasmic and nuclear GCR β . It was demonstrated that GCR α nuclear translocation and its transactivation properties were proportionately reduced by level of viral transduction of the GCR β gene into the DO-11.10 cell line. RNA silencing of GCR β mRNA in human BAL macrophages from patients with GC-insensitive asthma resulted in enhanced dexamethasone-induced GCR α transactivation.

Conclusions: GC insensitivity is associated with loss of GCR α nuclear translocation in BAL cells and elevated GCR β , which may inhibit GCR α transactivation in response to steroids.

Keywords: asthma; bronchoalveolar lavage cells; glucocorticoid insensitivity; glucocorticoid receptor

Glucocorticoids (GCs) are currently the most effective agents for the treatment of inflammation (1). Although the majority of patients respond to GC therapy, up to 25% of patients demonstrate persistent tissue inflammation despite treatment with high doses of GCs (2, 3). GC insensitivity has been widely recognized as complicating the management of chronic inflammatory diseases, such as asthma, inflammatory bowel disease, and autoimmune diseases (1, 4).

The antiinflammatory effects of GCs are mediated through GC receptor α (GCR α), which acts as a ligand-dependent transcription factor (4, 5). GCs interact with GCR α in the cytoplasm.

Under GC-responsive conditions, this results in translocation of the hormone–receptor complex into the cell nucleus, and binding of the GCR α to specific DNA response elements within the promoter region of GC-responsive genes to enhance transcription of antiinflammatory genes (transactivation).

Identification of the markers of GC insensitivity is important to be able to minimize side effects from high-dose steroid therapy and prospectively to provide alternative therapeutic approaches to such patients for better treatment outcomes. In humans, alternative splicing of the ninth exon of GCR pre-mRNA results in GCR α and GCR β proteins that are divergent at the carboxyl terminus (5). The two proteins are 94% identical, but the GCR β isoform fails to bind hormone or to activate gene expression. Thus, GCR β functions as a dominant negative inhibitor of GCR α (6). GCR β has a longer half-life than that of GCR α (7), and its expression is enhanced by proinflammatory cytokines such as tumor necrosis factor α (TNF- α) and interleukin 1 (IL-1) (8), combination IL-2 and IL-4 (9), and IL-13 (10). Increased expression of the GCR β isoform relative to the ligand-binding isoform, GCR α , has been previously reported to be associated with GC insensitivity in several inflammatory cell types (10–12), making GCR β a potentially attractive marker of GC insensitivity. However, the precise physiological role of GCR β has been controversial. Several studies have found elevated GCR β levels in association with GC insensitivity in a variety of the diseases (12–16). Some investigators have argued, however, that under physiological conditions the number of GCR α copies in the cell predominates over the number of copies of GCR β , making it unlikely that it could have any functional inhibitory effect (17, 18).

Most studies on GC-insensitive asthma have used peripheral blood mononuclear cells (PBMCs) or cell lines to demonstrate potential mechanisms of GC insensitivity in asthma (8, 19–21). The current study examines the functional response to corticosteroids of bronchoalveolar lavage (BAL) airway cells from patients with GC-insensitive and GC-sensitive asthma, thus allowing us to investigate GC insensitivity at the target organ level. Because the initial step in the classic GC signaling pathway is translocation of GCR α from the cytoplasm to the nucleus, decreased nuclear translocation is a plausible molecular mechanism of GC insensitivity. Our current study was designed to address the hypothesis that individuals with GC-insensitive asthma as compared with those with GC-sensitive asthma have reduced GCR α nuclear translocation in response to GCs in BAL cells from sites of airway inflammation. As well, we attempt to define the functional role of GCR β in controlling GCR α nuclear translocation and transactivation at a molecular level.

METHODS

Subjects

Patients with a diagnosis of asthma according to American Thoracic Society criteria (22) were selected for evaluation. Patients with asthma had a baseline FEV₁ of 55 to 85% of predicted, a β_2 -adrenergic response of at least 12% of baseline FEV₁, and/or a provocative concentration of methacholine causing a 20% fall in FEV₁ not exceeding 8 mg/ml. None of the subjects had received systemic GC therapy for at least

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1 mo before bronchoscopy (Table 1). The corticosteroid response of patients with asthma was classified on the basis of their prebronchodilator morning FEV₁% predicted response to a 1-wk course of oral prednisone (40 mg/d). Patients with asthma were defined as GC insensitive if they had less than 15% improvement in FEV₁, and as GC sensitive if they showed significant improvement (> 20%). None of the subjects with asthma had evidence of other types of lung diseases. All patients who were recruited for the study were not smoking for at least 1 yr before this study. Disease severity was characterized in both groups on the basis of baseline and postbronchodilator FEV₁% predicted, number of nocturnal events per month, rescue short-acting β -agonist use, and controller medication use (Table 1). Informed consent was obtained from all patients before enrollment in this study. The Institutional Review Board of the National Jewish Medical and Research Center (Denver, CO) approved this study.

Specimen Collection

PBMCs were isolated from heparinized blood by Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ) gradient centrifugation as previously described (23). Fiberoptic bronchoscopies with BAL were performed according to the guidelines of the American Thoracic Society (24). BAL cells were filtered through a 70- μ m Nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ), spun at 200 $\times$ g for 10 min, washed two times, and resuspended in Hanks' balanced salt solution. Cytospin preparations were made, and differential counts of BAL cells were performed after staining with Diff-Quik (Scientific Products, McGraw Park, IL), counting a minimum of 500 cells.

For this study, cells were resuspended in RPMI 1640 (BioWhittaker) containing 10% heat-inactivated, charcoal-filtered, GC-free fetal bovine serum (FBS; Gemini Bio-Products, Calabasas, CA), L-glutamine (40 μ mol/L), penicillin (100 U/ml), streptomycin (100 U/ml), and N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid (20 mmol/L) buffer solution (GIBCO-BRL/Life Technologies, Rockville, MD).

Inhibition of BAL Cell Cytokine Production by Steroids

In these experiments, human BAL cells (1×10^5 /ml) were stimulated with LPS (10 ng/ml) or zymosan (100 particles/cell) in the absence or presence of 10^{-9} to 10^{-6} M dexamethasone (DEX) for 24 h. Supernatants were collected and stored at -80°C before analysis. The ability of DEX to inhibit production of IL-6 and TNF- α in culture supernatants was measured by ELISA according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN).

GCR α Nuclear Translocation

GCR α intracellular shuttling in response to 10^{-6} M DEX (Sigma, St. Louis, MO) treatment was analyzed by immunostaining. BAL cells were seeded at 1×10^6 cells/ml on poly-D-lysine-coated coverslips. The cells were treated with 10^{-6} M DEX or cultured in medium alone for 3 h, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized for 15 min at room temperature in permeabilization solution (PBS containing 0.1% [vol/vol] Tween 20, 0.1% [wt/vol] bovine serum albumin [Sigma], and 0.01% [wt/vol] saponin [Sigma]), and blocked with a commercial blocking solution (Super Block; ScyTek Laboratories, Logan, UT) for 15 min at room temperature. The cells were then incubated with an affinity-purified polyclonal antibody to GCR α (Affinity BioReagents, Golden, CO) diluted in permeabilization solution (1:250) overnight at 4°C , washed, and then incubated with secondary antibody (donkey anti-rabbit IgG, F[ab']₂ fragment, Cy3 conjugated, diluted 1:200; Jackson Laboratories, West Grove, PA). Nuclei were counterstained with 300 nM 4',6-diamidino-2-phenylindole (DAPI; Sigma) for 1 h at room temperature. The cells were then washed and mounted on slides. Purified nonimmune rabbit IgG (SouthernBiotech, Inc., Birmingham, AL) was used as an isotype control. The slides were analyzed by fluorescence microscopy (Leica Microsystems, Wetzlar, Germany) with imaging software (SlideBook; Intelligent Imaging Innovations, Denver, CO) and expressed as a nuclear:cytoplasmic ratio of the mean fluorescence intensity (MFI) of Cy3 staining (GCR) of BAL cells as described previously (20).

Analysis of GCR β Expression

GCR β expression by BAL cells was analyzed by immunofluorescence staining. Cells were fixed, permeabilized, and blocked as described above. The cells were then incubated overnight at 4°C with an affinity-purified polyclonal antibody to GCR β (Affinity BioReagents) diluted in permeabilization solution (1:750), washed, and then incubated with secondary antibody (donkey anti-rabbit IgG, F[ab']₂ fragment, Cy3 conjugated, diluted 1:200). Nuclei were counterstained with 300 nM DAPI for 1 h at room temperature. The cells were then washed and mounted on slides. Purified nonimmune rabbit IgG (SouthernBiotech, Inc.) or synthetic GCR β antibody-neutralizing peptide N(728) VMWLKPESTSHTLI(742)C (Affinity BioReagents) was used to control the specificity of staining.

Real-Time Polymerase Chain Reaction

BAL cells were preserved in RLT buffer (provided with RNeasy mini kit; Qiagen, Valencia, CA) immediately after isolation. RNA was

TABLE 1. PATIENT CHARACTERISTICS

Parameter	Subjects with Asthma	
	GC Insensitive	GC Sensitive
Number of subjects	8	7
Age, yr (mean $\pm$ SD)	40.1 $\pm$ 9.3	35.7 $\pm$ 4.0
Sex, M/F	2/6	5/2
Atopic subjects*	8/8	7/7
Active smokers	0	0
Prior smokers, no. (pack-years)	1 (2.5)	1 (< 1.0)
Prebronchodilator FEV ₁ % predicted before prednisone burst (mean $\pm$ SEM)	66.0 $\pm$ 3.3	68.1 $\pm$ 2.9
Prebronchodilator FEV ₁ % predicted after prednisone burst (mean $\pm$ SEM)	64.7 $\pm$ 3.5 [†]	83.6 $\pm$ 3.6
Postbronchodilator (albuterol) FEV ₁ % predicted (mean $\pm$ SEM)	73.4 $\pm$ 4.2	82.8 $\pm$ 5.2
Age at asthma onset, yr (mean $\pm$ SEM)	21.6 $\pm$ 5.0	10.9 $\pm$ 4.1
Duration of asthma, yr (mean $\pm$ SEM)	18.5 $\pm$ 6.3	24.6 $\pm$ 9.3
Nocturnal symptoms, events/mo (mean $\pm$ SEM)	2.5 $\pm$ 1.7	5.7 $\pm$ 4.1
Medications		
Rescue β_2 -agonists, puffs/d (mean $\pm$ SEM)	1.7 $\pm$ 0.4	2.3 $\pm$ 0.9
ICS	3/8	3/7
ICS + LABA	2/8	0/7
Systemic CS	0	0
Other maintenance medications	None	None

Definition of abbreviations: CS = corticosteroid; F = female; GC = glucocorticoid; ICS = inhaled corticosteroid; LABA = long-acting β_2 -agonist; M = male.

*Patients were defined as atopic on the basis of the degree of skin-prick responses to a set of common allergens.

[†] FEV₁ was lower in patients with GC-insensitive asthma as compared with patients with GC-sensitive asthma ($p = 0.003$).

extracted according to the guidelines of the manufacturer (Qiagen), transcribed into cDNA, and analyzed by real-time polymerase chain reaction (PCR), using the dual-labeled fluorogenic probe method and an ABI PRISM 7000 sequence detector (Applied Biosystems, Foster City, CA) as described (25). Primers and probes for human mitogen-activated protein kinase phosphatase-1 (MKP-1) mRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18S RNA were purchased from Applied Biosystems. GCR α and GCR β primers based on the sequences published by DeRijk and coworkers (26) were custom ordered from Applied Biosystems. Standard curves for MKP-1, GAPDH, and 18S RNA were generated on the basis of fluorescence data from twofold serial dilutions of total RNA from the sample providing the highest expression level. GCR α and GCR β standard curves were generated from 10-fold serial dilutions of the GCR plasmids. Quantities of each target gene in test samples were normalized to the corresponding levels of the housekeeping genes (18S RNA and GAPDH) in each sample.

Expression of GCR β in Murine DO-11.10 Hybridoma Cells

cDNA encoding the human GCR β isoform (base pairs 23–2296) was subcloned into the replication-defective murine stem cell virus (MSCV) retroviral vector as a bicistronic coding unit containing the gene encoding green fluorescent protein (GFP), followed by the encephalomyelitis virus internal ribosome entry site and the GCR β -coding region, as described (27). Phoenix packaging cells were transiently transfected with the expression vectors for GCR β and GFP, using calcium phosphate precipitation. Culture supernatants from transfected Phoenix cells producing recombinant MSCV were used to transduce DO-11.10 hybridoma cells by spinfection as described (27). DO-11.10 hybridoma cells transduced with human GCR β /GFP were sorted for GFP⁺ cells (MoFlo cell sorter; Dako, Fort Collins, CO). For further experiments, the resulting GFP⁺ cell populations were sorted for GFP^{dim} and GFP^{bright} cells. After gating on live DO-11.10 hybridoma cells according to forward and side scatter and doublet exclusion, GFP^{dim} cells were defined as the 3 to 4% of gated live cells expressing GFP at the lowest fluorescence intensity. Accordingly, GFP^{bright} cells were defined as the 3 to 4% of gated live cells expressing GFP at the highest fluorescence intensity. The sorted cells were then cultured in 10% FBS–RPMI medium. GCR α nuclear translocation in response to DEX and MKP-1 induction by DEX were evaluated in wild-type and transgenic GCR β ^{dim} and GCR β ^{bright} DO-11.10 cells.

Western Blotting

Analysis of GCR α nuclear translocation and GCR β localization in DO-11.10 cells was performed by Western blot. Western blot was also used to analyze GCR β expression in fractionated BAL macrophages from patients with asthma. Nuclear and cytoplasmic extracts from cells were prepared with an NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce Biotechnology, Rockford, IL).

Western blotting was performed as previously described (20). Membranes were blotted with anti-GCR α (P-20; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-GCR β (Abcam, Cambridge, MA) antibodies. To control the quality of nuclear and cytoplasmic protein preparation the membranes were stripped and reprobed with anti-NF-1 and anti- β -tubulin antibodies (Santa Cruz Biotechnology) as nuclear and cytoplasmic proteins, respectively.

Silencing of GCR β Expression by Specific Small Interfering RNA

Small interfering RNA (siRNA, targeting GCR β , annealed) was custom designed and synthesized by Ambion, Inc. (Austin, TX). The sequence targeting human GCR β was as follows: sense, 5'-GGCUUUUCAUUA AAUGGGAtt-3'; antisense, 5'-UCCCAUUUAAUGAAAAGCt-3'. To be able to estimate the efficiency of transfection, siRNA was labeled with a Cy5 siRNA labeling kit (Ambion) in accordance with the manufacturer's recommendations. The specificity of silencing was controlled by nonsilencing control siRNA (Ambion).

Freshly isolated BAL cells from patients with GC-insensitive asthma were transfected with siRNA in a Nucleofector device (Amata, Cologne, Germany), using a Nucleofector human monocyte kit (Amata). Briefly, 1 μ g of GCR β siRNA was added to 3×10^6 cells that had been previously washed with PBS and resuspended in 100 μ l of human

monocyte kit transfection solution. Cells were subjected to nucleofection, using the Y001 program (Amata). Control cells were either mock transfected or transfected with 1 μ g of the nonsilencing control siRNA (Ambion) (negative control). Transfected cells were immediately diluted with prewarmed monocyte growth medium (Amata) supplemented with 10% heat-inactivated, charcoal-filtered, GC-free FBS with L-glutamine (40 μ mol/L) and cultured in 24-well plates or on poly-D-lysine-coated round coverslips in 24-well plates (1 ml/well). After 20 h, transfection efficiency was estimated by fluorescence microscopy. The cells were treated with or without 10^{-6} M DEX for an additional 4 h and harvested for RNA isolation or fixed on slides. GCR β knock-down was ascertained by quantitative real-time PCR and immunostaining as described above. To estimate the effect of the GCR β silencing on steroid responses, MKP-1 induction by DEX in all test groups was analyzed as well.

Statistical Analysis

Parametric-based statistical procedures (namely, *t* tests and linear mixed models) were used for analysis of outcome variables, which tended to be symmetrically distributed and without extreme outliers. Regarding two-sample *t* tests: in cases with extreme differences in variance between asthmatic groups, the unequal variance test was used. Regarding linear mixed model analyses: a spatial exponential covariance structure was used to model within-subject repeated measures over time (because time points were unequally spaced), and a compound symmetric covariance structure was used to model within-subject repeated measures over various treatments or between sites. In the linear mixed models, two-way interactions between predictors were examined. Select intergroup comparisons (e.g., comparing patients with GC-insensitive asthma and patients with GC-sensitive asthma at specific DEX concentrations) were conducted if related main effect or interaction terms were significant ($p < 0.05$). All reported *p* values are related to two-sided tests. SAS software (version 9.1; SAS Institute, Cary, NC) was used to carry out mixed model analyses. Data are expressed as means $\pm$ SEM.

RESULTS

Subject Characteristics

The characteristics of patients who enrolled into this study are shown in Table 1. Patients were divided into GC-insensitive and GC-sensitive groups based on FEV₁% predicted responses after a 1-wk burst with oral prednisone. Patients in the GC-insensitive group did not show any improvement in FEV₁ after exposure to prednisone ($p = 0.431$, as compared with FEV₁% predicted before steroid burst); in contrast, patients in the GC-sensitive group showed significant improvement in their lung function after steroid burst ($p = 0.00008$, as compared with FEV₁% predicted before steroid burst; Table 1). During the study, the patients continued to use inhaled steroids, but were asked to withdraw them 24 h before bronchoscopy.

In terms of asthma severity both groups were equivalent (Table 1), with one ex-smoker per group. Baseline FEV₁% predicted was 66.0 ± 3.2 in the GC-insensitive group and 68.1 ± 2.9 in the GC-sensitive group ($p = 0.24$). Postbronchodilator FEV₁% predicted was 73.4 ± 4.2 and $82.8 \pm 5.2\%$, respectively ($p = 0.09$). Symptom severity measured by nocturnal events were 2.5 ± 1.7 /mo in the GC-insensitive group and 5.7 ± 4.1 /mo in the GC-sensitive group ($p = 0.24$). Group similarities were also reflected in rescue, short-acting β_2 -agonist use, 1.7 ± 0.4 versus 2.3 ± 0.9 puffs/d, respectively ($p = 0.26$). Controller medication in the GC-insensitive group occurred in five of eight patients, with three using inhaled corticosteroid alone and two using inhaled corticosteroid and a long-acting β_2 -agonist. In the GC-sensitive group, controller medication occurred in three of seven patients and consisted only of inhaled corticosteroid.

The number of total white cells in BAL samples varied between patients (mean total white cell counts for GC-insensitive and GC-sensitive groups were $[11.9 \pm 3.1] \times 10^6$ and $[17.9 \pm 1.5] \times 10^6$,

respectively). However, the percentages of macrophages, lymphocytes, neutrophils, and eosinophils did not differ between the two groups (Table 2). Macrophages composed a mean percentage of 89.1 ± 3.6 and $91.9 \pm 1.5\%$, lymphocytes composed 9.4 ± 3.6 and $6.2 \pm 1.2\%$ in BAL samples from GC-insensitive and GC-sensitive asthma study groups, respectively.

Response of BAL Macrophages to Dexamethasone

The production and suppression by DEX of proinflammatory cytokines by BAL cells from GC-insensitive and GC-sensitive patients after 24 h of stimulation with LPS or zymosan was analyzed. The presence of IL-6 and TNF- α in culture supernatants was measured by ELISA. It was found that within the GC-insensitive group production of IL-6 and TNF- α by BAL macrophages was not suppressed as effectively by DEX as compared with the GC-sensitive group (Figure 1). The reduced inhibitory effect of DEX on cytokine release was observed in BAL cells of GC-insensitive patients both after LPS and zymosan stimulation (Figure 1). Thus, suppression of cytokine production by GCs paralleled the clinical responsiveness of patients to GCs.

GC-insensitive Asthma Is Associated with Reduced GCR α Translocation

To gain insights into why GCs did not suppress cytokine production by airway macrophages from GC-insensitive groups, GCR α cellular shuttling in response to GCs was analyzed in the BAL cells of patients with asthma. GCR α nuclear translocation in BAL cells from patients with asthma was assessed in response to 10^{-6} M DEX (3 h) by immunofluorescence staining (Figure 2). In the absence of DEX, GCR α was localized mainly to the cell cytoplasm of BAL cells. This corresponded to a GCR α MFI nuclear:cytoplasmic ratio lower than 1. BAL cells from GC-sensitive patients demonstrated GCR α translocation in response to DEX ($n = 7$) (statistically significant change in the GCR α nuclear:cytoplasmic ratio was observed; $p < 0.001$ as compared with the absence of DEX). However, in BAL cells from patients with GC-insensitive asthma, GCR α failed to translocate and was still localized to the cytoplasm of studied cells ($n = 6$ patients; no difference in the GCR α MFI nuclear:cytoplasmic ratio was found; $p = 0.82$ as compared with the absence of DEX). The GCR α MFI nuclear:cytoplasmic ratio after 3 h of DEX treatment was (mean $\pm$ SD; 0.88 ± 0.10 for the GC-insensitive group, $p < 0.001$ compared with the GC-sensitive asthma group) and 1.31 ± 0.12 for the GC-sensitive patients (Figure 2).

BAL Macrophages from Patients with GC-insensitive Asthma Have Elevated Levels of GCR β Expression

Previous literature on cell lines has reported that GCR β is localized primarily to the nucleus (28, 29). However, our immunostaining studies revealed that this is not true for human

monocytes/macrophages, where GCR β is distributed equally between the cytoplasm and cell nuclei (Figure 3A). To validate the fluorescence methods regarding intracellular distribution of GCR β nuclear and cytoplasmic fractions were prepared from freshly isolated BAL macrophages. GCR β expression in these fractions was examined by Western blot. The quality of fractionation was confirmed by stripping and reprobing membranes for β -tubulin, which is known to have only a cytoplasmic distribution (Figure 3C). These experiments confirmed fluorescence microscopy data showing nuclear and cytoplasmic expression of GCR β in human macrophages. Because cell staining in BAL samples indicated that macrophages were the predominant cell type in BAL samples (Table 2), we analyzed whether GCR β can influence steroid responses of BAL macrophages.

Expression of GCR α and GCR β isoforms by BAL samples was analyzed by real-time PCR. A significant increase in GCR β mRNA expression by BAL cells from patients with GC-insensitive asthma as compared with the GC-sensitive group was found (Figure 3D). In contrast, there was no difference in GCR α expression by BAL cells from the two asthma groups (Figure 3E). This effect appeared to be BAL specific because no difference in GCR β and GCR α mRNA expression was observed in purified peripheral blood monocytes from GC-insensitive and GC sensitive asthma groups (GCR β , 0.0015 ± 0.003 and 0.0011 ± 0.003 fg/ng 18S RNA; GCR α , 0.0005 ± 0.0001 and 0.0010 ± 0.0003 pg/ng 18S RNA for monocytes from patients with GC-insensitive asthma [$n = 3$] and patients with GC-sensitive asthma [$n = 3$], respectively).

In addition, our data indicated that BAL macrophages from patients with GC-insensitive asthma have significantly higher levels of both nuclear and cytoplasmic GCR β protein expression as compared with BAL macrophages from GC-sensitive patients (GCR β MFI was 562.5 ± 53.1 vs. 167.3 ± 18.6 and 429.9 ± 45.5 vs. 104.2 ± 7.6 in the nuclear and cytoplasmic compartments of BAL macrophages in GC-insensitive [$n = 7$] and GC-sensitive asthma [$n = 6$] groups, respectively; $*p < 0.0001$ compared with the GC-sensitive asthma group; Figure 3B).

Overexpression of GCR β Controls GCR α Nuclear Translocation and Transactivation

Because GCR β can form heterodimers with GCR α , but does not engage GCs because of an absent ligand-binding site (6, 7), we explored the possibility that cytoplasmic GCR β controls GCR α nuclear translocation in response to GCs, thus influencing GCR α transactivation capacity. To more precisely demonstrate the effect of GCR β on steroid responsiveness, we compared GCR α nuclear translocation in murine DO-11.10 cells that expressed different levels of GCR β (Figure 4A). Mice have just one isoform of the GCR, that is, GCR α . Unlike humans, they do not express GCR β . Therefore mice may be considered natural

TABLE 2. BRONCHOALVEOLAR LAVAGE CELL DIFFERENTIALS FROM PATIENTS WITH GLUCOCORTICOID-INSENSITIVE ASTHMA AND FROM PATIENTS WITH GLUCOCORTICOID-SENSITIVE ASTHMA

Cell Type	Subjects with Asthma	
	GC Insensitive	GC Sensitive
Total white blood cells, $\times 10^6$, median (range)	9.36 (1.88–26.72)	18.22 (12.73–19.88)
Macrophages/monocytes, % (mean $\pm$ SEM)	89.1 ± 3.6	91.9 ± 1.5
Polymorphonuclear cells, % (mean $\pm$ SEM)	0.5 ± 0.2	0.5 ± 0.2
Lymphocytes, % (mean $\pm$ SEM)	9.4 ± 3.6	6.2 ± 1.2
Eosinophils, % (mean $\pm$ SEM)	0.9 ± 0.3	1.4 ± 0.7

Definition of abbreviation: GC = glucocorticoid.

Figure 1. Suppression of bronchoalveolar lavage (BAL) macrophage production of tumor necrosis factor α (TNF- α) and interleukin 6 (IL-6) by dexamethasone (DEX) in patients with glucocorticoid (GC)-insensitive asthma ($n = 8$; solid triangles) and patients with GC-sensitive asthma ($n = 7$; solid squares) 24 h after LPS (A and B) or zymosan (C and D) stimulation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

GCR β knockouts (30). Murine DO-11.10 cells that were virally transduced with a GFP-GCR β bicistronic construct (as described in Hauck and coworkers [27]) had both cytoplasmic and nuclear expression of GCR β , with a majority of the GCR β accumulating in the cytoplasm (Figure 4B). As shown by Western blot, we found that overexpression of GCR β inhibits GCR α nuclear translocation in response to steroids (Figure 4C).

Induction of MKP-1 by corticosteroids, measured by real-time PCR, was used to confirm these data because MKP-1 is one of the early markers induced by steroids via binding of the GCR α to glucocorticoid response element (GRE) sequences in the promoter region of the MKP-1 gene (31), and therefore reflects GC transactivation responses. We demonstrated that GCR α transactivation properties are proportionately reduced by GCR β expression: MKP-1 induction by DEX was significantly reduced in the cells that express GCR β (MKP-1 mRNA fold induction after 2 h of 10^{-7} M DEX treatment: 1.58 ± 0.05 , 1.16 ± 0.11 ,* and 0.59 ± 0.33 * in wild-type DO-11.10 cells, DO-11.10 GCR β GFP^{dim} cells, and DO-11.10 GCR β GFP^{bright} cells, respectively, $n = 3$; * $p < 0.05$ vs. wild-type cells; Figure 4D). This suggests that elevated cytoplasmic GCR β inhibits GCR α nuclear translocation in response to DEX and reduces GCR transactivation.

RNA Silencing of GCR β Expression in BAL Macrophages from Patients with GC-insensitive Asthma Enhances Their GC Response

Freshly isolated BAL macrophages from patients with GC-insensitive asthma were transfected with GCR β siRNA or nonsilencing control siRNA, or remained untreated, and were cultured for 20 h, followed by treatment for 4 h with 10^{-6} M DEX or medium to analyze DEX-induced MKP-1 production. Using Cy5-labeled siRNA oligonucleotides, the efficiency of transfection was estimated to be about 60 to 70%, 20 h after electroporation. Twenty-four hours after transfection GCR β mRNA expression was significantly suppressed as compared with nonsilencing control siRNA (Figure 5A). This effect was GCR β specific and

introduction of GCR β siRNA did not alter GCR α mRNA expression (Figure 5B). Significant enhancement of DEX-induced MKP-1 mRNA production was observed when GCR β expression by the cells was suppressed (Figure 5C) (MKP-1 fold induction by DEX was 2.7 ± 0.5 in the GCR β siRNA group [$p = 0.0168$ as compared with 1.2 ± 0.3 in the medium group and 0.9 ± 0.3 in the nonsilencing control siRNA group]).

Because GCR β silencing efficiently suppressed GCR β expression in both cytoplasmic and nuclear compartments of the cells, we cannot separate the effect of silencing on the two aspects of GCR α action—nuclear translocation and transactivation—as both these functions of GCR α can be controlled by GCR β . But the experiments performed demonstrate that inhibition of GCR β expression enhances GCR α transactivation and overall steroid responsiveness.

DISCUSSION

Although the majority of patients with asthma respond favorably to inhaled and systemic steroid therapy, up to 25% of patients with difficult-to-control asthma have poor clinical responses even to high doses of systemic GCs (2, 3). Given the increase in asthma prevalence and severity worldwide, GC insensitivity has become a challenging health problem that is costly to the health care system. Understanding the mechanisms that control GC insensitivity will be important in developing alternative therapies and in minimizing side effects from long-term systemic GC therapy, by allowing early identification of biomarkers that predict GC insensitivity (32).

The current study was performed on patients with CS-insensitive asthma and patients with GC-sensitive asthma as defined by response to a 1-wk course of oral prednisone treatment. The groups were equivalent regarding asthma severity at baseline. The GC-insensitive group was not more symptomatic, did not have worse lung function, and did not use more rescue medication. Interestingly, the two groups were markedly different in age at onset of disease (although the difference observed was

Figure 2. Impaired GCR α nuclear translocation in BAL macrophages from GC-insensitive patients (original magnification, $\times 400$; blue [DAPI], nuclear staining; red [Cy3], GCR α). Representative images of GCR α cellular shuttling in BAL macrophages from patients with GC-insensitive asthma (A) and patients with GC-sensitive asthma (B) in response to 3 h of 10^{-6} M DEX treatment *in vitro* are shown. Note the cytoplasmic localization of GCR α in the cells after DEX treatment of the patient with GC-insensitive asthma versus nuclear localization of GCR α in the cells from the patient with GC-sensitive asthma, respectively (arrows). The mean fluorescence intensity (MFI) of Cy3 staining (GCR α) of BAL cells from GC-insensitive pa-

tients (C) and GC-sensitive patients (D) was assessed by analysis software within the computer-generated masks for nuclear and cytoplasmic regions of the cells and is expressed as the GCR α (Cy3) nuclear:cytoplasmic ratio. Fifty to 100 cells were analyzed for each patient studied.

not significantly different, $p = 0.11$). Spirometry studies revealed greater postbronchodilator response in the GC-sensitive group than in the GC-insensitive group, but because of the small sample size the difference observed was not significantly different ($p =$

0.09). The latter observation was probably not due to recruitment of possible former smokers into the study because only two former smokers were involved in this study (one in each group). These differences in postbronchodilator response suggest that

Figure 3. Increased expression of GCR β in BAL macrophages of patients with GC-insensitive asthma. GCR β expression in BAL macrophages from patients with asthma was analyzed by immunofluorescence staining (A and B) (original magnification, $\times 400$; blue [DAPI], nuclear staining; red [Cy3], GCR β) and revealed significantly higher GCR β in BAL macrophages from GC-insensitive patients. The MFI of Cy3 staining (GCR β) in BAL cells from patients with GC-insensitive asthma and from patients with GC-sensitive asthma was assessed by analysis software within the computer-generated masks for nuclear and cytoplasmic regions of the cells. Forty to 60 cells were analyzed for each patient studied. Note the nuclear and cytoplasmic expression of GCR β in human macrophages (A). The nuclear and cytoplasmic distribution of GCR β in BAL macrophages was confirmed by Western blot (C). Compared with the GC-sensitive group, increased GCR β mRNA expression (D), but not GCR α mRNA expression (E), was observed by real-time polymerase chain reaction (PCR) in freshly isolated BAL cells from patients with GC-insensitive asthma.

anti- β -tubulin antibodies as nuclear and cytoplasmic proteins, respectively. (C) GCR α nuclear shuttling in response to DEX is impaired in the cells expressing GCR β . (D) GCR α transactivation properties are proportionately reduced by GCR β expression. DO-11.10 wild-type, GFP GCR β^{dim} , and GFP GCR β^{bright} cells were collected at various time points after DEX treatment. MKP-1 mRNA induction by DEX in the cell isolates as compared with medium-treated cells was analyzed by real-time PCR.

GC-insensitive, as compared with GC-sensitive, asthma is associated with decreased reversibility of airway obstruction. However, future studies powered to examine this aspect of lung function in these two forms of asthma are required before any firm conclusion can be made.

In the current article, we describe for the first time that BAL airway cells from patients with GC-insensitive asthma have reduced GCR α nuclear translocation in response to GCs. Our data in human BAL cells extend other data (Matthews and coworkers [21]) indicating that most patients respond to GCs according to the degree of PBMC GCR α nuclear translocation. Importantly, *in vitro* studies have demonstrated that there are several potential pathways for induction of GC insensitivity including overexpression of GCR β , an endogenous inhibitor of GCR α (9, 11, 12), and reduced nuclear translocation of GCR α (20, 33). It has been suggested that T-cell GC insensitivity may be associated with a failure of GCs to control mitogen-activated protein kinase activation (33, 34). In addition, *in vitro* studies have revealed that these kinases alter phosphorylation status of GCR α , thus negatively regulating its function (19, 35–37). Interaction of GCR α with heat shock proteins (38) and FK506 proteins 51 and 52 (39, 40) has been reported to control GCR α nuclear translocation. The molecular mechanism for GC insensitivity of BAL macrophages, the predominant airway cell in asthma, has not been previously studied nor have the potential relationships between various potential mechanisms of GC insensitivity been explored.

Increased expression of GCR β has been found in several diseases associated with GC insensitivity, suggesting that an imbalance between GCR α and GCR β expression is associated with GC insensitivity, leading to a reduction in the ability of GCR α to interact with the GRE. In contrast to GCR α , GCR β interacts

weakly with heat shock proteins, does not bind GCs, and is transcriptionally inactive (6, 7, 28). GCR β is known to inhibit GCR α -mediated transactivation in a dose-dependent manner (29). It has been reported that proinflammatory cytokines induce GCR β expression (8). The ability of GCR β to antagonize the function of GCR α suggests its importance in the regulation of cell sensitivity to GCs. It has been reported that GCR β competes with GCR α in the nucleus for coactivators, and that GCR α –GCR β heterodimers are transcriptionally inactive (41).

However, the measurement of GCR β expression in various diseases has resulted in contradictory findings (42–46). This may relate to the fact that such studies have focused on mixed cell populations. It is likely that GCR β can be detected more easily with a homogeneous cell population in the target organ of the disease as opposed to mixed cell populations (such as peripheral blood), in which a potentially positive signal may be hidden by high background signals coming from cells that do not upregulate GCR β expression.

Our data confirm observation by Hamid and coworkers (12) that BAL cells from patients with GC-insensitive asthma express increased levels of GCR β as shown by immunocytochemistry. Indeed, our current study found no difference in GCR β gene expression between monocytes of patients with GC-insensitive asthma and patients with GC-sensitive asthma. To our knowledge, the current report is the first to show elevated GCR β expression in BAL cells that is confirmed both by real-time PCR and immunofluorescence and to show that GCR β is evenly distributed between cell cytoplasm and nuclei in human monocytes/macrophages. This suggests, aside from the known ability of GCR β to inhibit GCR α transactivation, that it can influence GCR α nuclear translocation in response to steroids, most likely by heterodimer formation between GCR α and GCR β . Several

Figure 4. Overexpression of GCR β controls GCR α nuclear translocation and its transactivation properties. (A) Distribution of green fluorescent protein (GFP) expression in GFP-GCR β retrovirally transduced DO-11.10 cell lines. As shown previously (27), the level of GFP expression in transgenic DO-11.10 cells correlates with GCR β expression, because GCR β cDNA was subcloned into the viral vector as a bicistronic coding unit containing GFP. (B) GCR α has cytoplasmic localization in wild-type (WT) and GCR β -expressing DO-11.10 cells. GFP GCR β^{dim} transgenic cells have nuclear and cytoplasmic localization of GCR β , with the majority of protein residing in the cell cytoplasm. To control the quality of nuclear and cytoplasmic protein preparations, the membranes were re-probed with anti-NF-1 and

Figure 5. Specific GCR β siRNA enhances GCR α transactivation in BAL macrophages from patients with GC-insensitive asthma. Introduction of GCR β siRNA into the cells resulted in specific inhibition of GCR β (A) but not GCR α (B) mRNA expression by the cells as shown by real-time PCR. Data are expressed as the percentage of GCR mRNA expression as compared with such after introduction of nonsilencing control siRNA. (C) Silencing of GCR β significantly increased DEX-induced MKP-1 mRNA production by BAL macrophages. The cells were transfected with siRNA or remained untreated, and were cultured for 20 h and treated with 10^{-6} M DEX for 4 h. MKP-1 mRNA induction by DEX as compared with medium-treated cells was analyzed by real-time PCR.

studies have already demonstrated that these two GCR isoforms can interact when found in the same cell compartment, but most studies performed in cell lines have reported the presence of GCR β in the nucleus, where it has been postulated to interfere with GCR α transactivation (28, 29). At this time, we do not know what factors control GCR β subcellular localization in monocytes. However, the novel observation that cytoplasmic GCR β may interfere with GCR α nuclear translocation provides a new dimension by which GCR β may act as a dominant negative inhibitor of GCR α action independent of its effects on transactivation. Our experiments show that GCR α still remains present in larger quantities than GCR β in GC-insensitive cells, but siRNA experiments suggest that despite this, GCR β still plays a role in reduction of steroid responses, indicating that GCR β is not working solely by competing with GCR α for DNA GRE.

To evaluate how GCR β can influence GCR α function, two approaches were used: overexpression of GCR β and silencing of GCR β mRNA. We reported previously that viral transduction of GCR β cDNA into mouse hybridoma cells to induce stable expression of GCR β results in GC insensitivity of these cells (27). Furthermore, using whole cell lysates, we have shown that in such cells GCR α is complexed with GCR β (27). Using the same system in this article, we have found that retrovirally transduced DO-11.10 cells have a predominance of GCR β in the cytoplasm. Importantly, our current study demonstrates that no GCR α nuclear translocation was observed in cells that overexpress GCR β . We hypothesize that GCR α -GCR β heterodimer formation in GCR β -overexpressing cells inhibits GCR α nuclear shuttling in response to steroids. As well, we found that overexpression of GCR β abolishes GCR α transactivation capacity. As shown by real-time PCR, MKP-1 mRNA induction by steroids (as a readout of GCR α transactivation capacity) was proportionally reduced by GCR β expression.

Previous studies have proposed that a likely mechanism for the dominant negative activity of GCR β is via the formation of heterodimers with GCR α , however, the precise mechanism and structural basis of this phenomenon are only starting to be understood. Immunoprecipitation experiments from our laboratory have previously demonstrated that murine GCR α can heterodimerize with human GCR β (27). Sequence comparison of murine GCR (NP_032199) and human GCR α (NM_008173) shows 89% identity between two proteins, including a ligand-binding domain (LBD), which is known to participate in GCR protein dimerization. The human GCR α LBD structure has been determined (47). This structure suggests that the dimerization interface of GCR is distinct from that of other nuclear receptor LBD structures, in that it involves the H4 domain and β -strands 3 and 4, located on the opposite face of the folded domain from H10 to H12 and therefore structural differences in H11 and H12 domains of human GCR β do not perturb the dimer interface found in human GCR α . On the basis of the crystal structure it is believed that GCR dimerization is ligand independent because the conformational changes that occur on hormone binding are far away from the published dimer interface; the solved structure supports the possibility of heterodimerization of GCR α and GCR β (29).

To address potential confounding problems with cell lines that express nonphysiologic levels of GCR β , we investigated whether specific RNA silencing of GCR β expression in human BAL macrophages from patients with GC-insensitive asthma can enhance the response of these primary cells to steroids. Indeed, we found that GCR β has nuclear and cytoplasmic localization in human BAL macrophages, and that introduction of a specific GCR β siRNA, but not control siRNA, results in significant inhibition of both cytoplasmic and nuclear GCR β expression. Concomitant with the inhibition of GCR β expression,

MKP-1 mRNA induction by steroids was significantly enhanced in such cells (Figure 5D). These data demonstrate that GCR β does have a physiologic role in modulating steroid responsiveness in cells derived from patients with GC-insensitive asthma and that increased GCR β expression may be a therapeutic target in restoring steroid responsiveness in GC-insensitive asthma.

Conflict of Interest Statement: E.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L-b.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.T.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.J.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.J.M. received less than \$10,000 per year per company (GlaxoSmithKline, IVAX, Sanofi-Aventis, and Schering) for lecture fees and advisory board membership combined. He also received a research grant from GlaxoSmithKline for \$50,000, 2003–2005. D.Y.M.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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