Glucocorticoid receptor isoforms in steroid-dependent asthma

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KEY WORDS
- glucocorticoid receptor β isoform
- glucocorticoids
- severe asthma
- steroid-dependent asthma

INTRODUCTION
Inhaled glucocorticoids (GCs) are the most effective controller medications in the treatment of asthma, because they inhibit activation of lymphocytes, production of cytokines, and infiltration of bronchial mucosa by inflammatory cells. However, there is a small proportion of patients who require chronic treatment with systemic GCs to achieve good clinical control of the disease. This phenotype of asthma is called “steroid-dependent” because of the requirement for continuous treatment with oral steroids. Very few patients are unresponsive to systemic GCs, and such phenotype is classified as true GC-resistant asthma.

The relative unresponsiveness to GCs, which underlies the steroid-dependence, may result from decreased sensitivity of inflammatory cells to GCs. Such mechanism was extensively studied in GC-resistant asthma. It has been found that decreased sensitivity to GCs was reflected by a reduced inhibition of lymphocyte proliferation in vitro. Additionally, asthmatic patients with GC resistance were characterized by elevated expression of glucocorticoid receptor (GR) β isoform in blood lymphocytes and airway cells, which could hamper GC response.

Human GR gene expresses 2 splicing isoforms, depending on the use of alternative exons 9α or 9β (FIGURE 1). A less abundant GRβ isoform has a truncated ligand binding domain, does not bind GCs, and was found to be a dominant-negative inhibitor of the classic GRα isoform. It has been proposed that GC resistance could result from the imbalance between these 2 splicing isoforms.

ABSTRACT
INTRODUCTION
Ineffective response to glucocorticoids (GCs) in severe asthma may result from enhanced T-cell activation, immune dysregulation, or altered expression of glucocorticoid receptor (GR).

OBJECTIVES
The aim of the study was to analyze the expression of GR isoforms and in vitro sensitivity of lymphocytes to GCs in severe, steroid-dependent asthma.

PATIENTS AND METHODS
We analyzed the immunophenotype of peripheral blood lymphocytes, the effect of dexamethasone (DEX) on lymphocyte activation and proliferation, and the levels of GRα and GRβ mRNA in peripheral blood lymphocytes of 11 healthy subjects, 15 moderate asthmatics, 11 severe asthmatics on low-dose oral GCs, and 14 severe asthmatics with suboptimal symptom control on high-dose oral GCs.

RESULTS
The average level of GRβ mRNA in lymphocytes was more than 300-fold lower than GRα, and this ratio was comparable in all groups. Lymphocytes from steroid-dependent asthmatics were sensitive to steroids in in-vitro activation assays, as evidenced by a significant decrease in activation antigen (CD25, CD69) expression, and inhibition of mitogen-induced proliferation upon incubation with DEX. The results of in vitro functional assays were similar in all groups and did not correlate with the GRα/GRβ ratio.

CONCLUSIONS
Steroid dependency in severe asthma is not associated with GRβ upregulation in lymphocytes or abnormal T-cell reactivity in the presence of GCs. These data suggest that testing for the expression of GRα and GRβ isoforms in blood lymphocytes will not be useful in predicting sensitivity to GCs in severe asthma.
The study was approved by the Ethics Committee of the Jagiellonian University, Kraków, Poland, and informed consent was obtained from all participants.

Lymphocyte phenotyping

Aliquots of EDTA-anti-coagulated blood were double stained with monoclonal antibodies against CD3, CD4, CD69, or HLA-DR (all from BD Biosciences, Franklin Lakes, NJ, United States). Samples were fixed with FACS Lysing Solution (FLS, BD Biosciences), washed in phosphate buffered saline, and analyzed by flow cytometry (Coulter EPICS XL, Beckman Coulter, Fullerton, CA, United States).

Lymphocyte activation and proliferation assays

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA-anticoagulated blood with histopaque gradient centrifugation (Sigma-Aldrich, St. Louis, MO, United States). PBMC were cultured briefly in flat-bottom wells (37°C) to deplete monocytes. Nonadherent peripheral blood lymphocytes (PBL) were resuspended in RPMI-1640 medium containing 10% fetal calf serum and cultured in 96-well plates with phytohemagglutinin (PHA) at a final concentration of 10 μg/ml, for a given time interval.

To analyze the effect of dexamethasone (DEX) on T-cell activation, control, PHA (10 μg/ml), or PHA + DEX (1 × 10⁻⁵ M) stimulated samples were collected after 24 hours and double stained with anti-CD3 or anti-CD4, and one of the following antibodies (all from BD Biosciences): anti-CD25, -CD69, -HLA-DR, and -CD40L. Samples were fixed and analyzed by flow cytometry. Data are presented as a relative difference of PHA + DEX though little is known whether the GRα/GRβ ratio could determine the level of GC responsiveness in steroid-dependent asthma.

The aim of the present study was to analyze whether unresponsiveness to GCs observed in steroid-dependent asthma results from an imbalance between GR isoforms, and whether it is mirrored by impaired lymphocyte reactivity to GCs. To verify this hypothesis, we selected a group of severe asthmatics treated with different doses of systemic GCs and examined the alterations in GRα and GRβ mRNA expression in lymphocytes, together with steroid effects on lymphocytes in in-vitro functional assays.

**PATIENTS AND METHODS**

**Patients and study design**

A total of 25 patients with severe persistent asthma fulfilling the American Thoracic Society criteria were included into the study. All patients were current nonsmokers and did not have asthma exacerbation in the 2 preceding months. Clinical data were collected using the asthma control diary questionnaire during a 1-week entry period in order to include only patients with stable disease. Severe asthmatics were stratified into 2 groups according to the control questionnaire score and oral-GC dose: group 1 – severe persistent asthma with a very good disease control on low-doses (4–8 mg/d) of oral GCs (severe asthma low-dose GCs, n = 11); group 2 – severe asthma with a partial control on moderate or high doses (12–24 mg/d) of oral GCs (severe asthma high-dose GCs, n = 14). These 2 groups were compared to group 3 (moderate asthma, n = 15) and group 4 (healthy controls, n = 11). This nomenclature was used throughout the whole study.

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treated samples in comparison with maximal PHA stimulation.

To analyze the influence of DEX (1 × 10⁻⁹ to 1 × 10⁻³ M) on lymphocyte proliferation, PBLs were collected after 72 hours, stained for 15 minutes with 7-aminoactinomycin-D and FITC-CD3, and immediately counted by flow cytometry. Data are presented as a percentage change in the absolute number of viable lymphocytes, when compared to baseline samples (% of baseline) or control (% of control). All reagents other than antibodies were purchased from Sigma-Aldrich.

**Expression of glucocorticoid receptor mRNA isoforms** Total cellular RNA was extracted from 6 × 10⁶ PBMCs with TRI Reagent (Sigma-Aldrich) and reverse transcribed (AMV-reverse transcriptase, Amresco, Solon, OH, United States). GR isoforms were amplified using primers (all from TIB Molbiol, Poznań, Poland) corresponding to the 7/8 exon junction (5’-CTTCTCTCTTCAGTTCTAAAGGAC-3’), 9α exon (5’-GATTGGTGATGATTTCATCTC-3’), or 9β exon (5’-GGGATGAAAATCAGATTAATGTG-3’). Real-time polymerase chain reaction (PCR) (iCycler, BioRad, Hercules, CA, United States) with SYBR-Green I (Amresco) and integral standards (isolated GRα amplification product) was used for the quantification of GRα variant (annealing 66°C). Due to a very low amount of specific amplification product of GRβ, a quantitative analysis of PCR products was used to quantify this isoform. cDNA or diluted integral standards were amplified in duplicates for 27 to 42 cycles (annealing 63°C). PCR products were separated using electrophoresis and photographed. 

The transcripts of GR isoforms were normalized with human β-actin mRNA (primers from Integrated DNA Technologies, Coralville, IA, United States). GRα and GRβ mRNA copy numbers were calculated using corrected Ct values and equations of the relevant standard curves. Assuming that a 2-fold change in the expression of a particular transcript is biologically significant, this study had ~0.34 power in detecting such a difference (P < 0.05).

**Statistical analysis** Statistical analysis and curve fitting were performed with GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA, United States). Data are presented as medians with interquartile range (IQR) [25–75]. Group characteristics were determined with the Friedman test, Mann-Whitney U test, or Fisher’s exact test. Differences between ≥3 groups were analyzed with the analysis of variance, Kruskal-Wallis test, or paired two-way tests if required. EC₅₀ values, corresponding to concentration of DEX associated with 50% inhibitory effect, were calculated using nonlinear regression module. Spearman’s rank sum test or linear regression were used to analyze correlations. P < 0.05 was considered statistically significant.

**RESULTS Subject characteristics** The basic characteristics of the study participants are shown in Table 1. Clinical parameters of asthmatic patients are summarized in Table 2. Asthmatic patients did not differ with respect to asthma duration; however, the majority of patients from high-dose GCs group were diagnosed at adulthood (79%). The frequencies of atopy and aspirin hypersensitivity were similar in the studied groups. As expected, symptom scores were higher in the high-dose GCs group (P < 0.01); additionally, they were treated with oral GCs for a longer period of time. Asthmatics requiring high-dose GCs had baseline forced expiratory volume in 1 second significantly lower when compared with the other groups (P < 0.05). C-reactive protein levels tended to be elevated in both groups of severe asthmatics (Table 1).

**Phenotype of peripheral blood lymphocytes** White blood cell and neutrophil counts were significantly increased in steroid-dependent asthmatics treated with high doses of oral GCs in comparison with healthy subjects and moderate...
controls (P < 0.01) and moderate asthma (P < 0.05). The percentage of HLA-DR+ cells weakly correlated with asthma control scores (r = 0.38). Basic characteristics of peripheral blood leukocytes are summarized in Table 3.

**Lymphocyte activation and response to dexamethasone** Stimulation of peripheral blood lymphocytes with PHA led to a rapid increase in the expression of activation-dependent antigens. However, no significant correlation was found between CD3/CD69+ and asthma control or lung function tests. The proportion of CD3/HLA-DR+ lymphocytes was also significantly increased in high-dose GCs asthma as compared with healthy controls (P < 0.01) and moderate asthma (P < 0.05). The percentage of HLA-DR+ cells weakly correlated with asthma control scores (r = 0.38). Basic characteristics of peripheral blood leukocytes are summarized in Table 3.

**Table 3** Characteristics of peripheral blood leukocytes

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy subjects</th>
<th>Moderate asthma</th>
<th>Severe asthma low-dose GCs</th>
<th>Severe asthma high-dose GCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>15</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>white blood cells, × 10³/µl</td>
<td>5.7 (4.6–6.6)</td>
<td>5.7 (4.7–6.6)</td>
<td>7.7 (5.9–8.1)</td>
<td>8.8 (5.9–10.9)a,c</td>
</tr>
<tr>
<td>neutrophils, × 10³/µl</td>
<td>3.6 (2.9–4.4)</td>
<td>3.3 (2.9–3.9)</td>
<td>4.6 (4.3–5.5)</td>
<td>6.7 (3.8–7.6)a,c</td>
</tr>
<tr>
<td>lymphocytes, × 10³/µl</td>
<td>1.8 (1.43–2.02)</td>
<td>1.46 (1.3–2.21)</td>
<td>1.65 (1.34–2.46)</td>
<td>1.82 (1.13–2.61)</td>
</tr>
<tr>
<td>T-cells, % of lymphocytes</td>
<td>70.4 (64.6–76.4)</td>
<td>69.1 (68.1–75.0)</td>
<td>74.2 (65.8–78.6)</td>
<td>69.4 (64.1–78.8)</td>
</tr>
<tr>
<td>CD3/CD4+, % of lymphocytes</td>
<td>45.2 (43.4–48.5)</td>
<td>43.6 (39.5–48.8)</td>
<td>39.1 (37.3–45.2)</td>
<td>41.1 (27.8–44.5)</td>
</tr>
<tr>
<td>CD3/CD69+, % of CD3+</td>
<td>4.2 (2.7–6.3)</td>
<td>2.7 (1.7–4.2)</td>
<td>3.5 (2.2–5.8)</td>
<td>3.4 (2.6–7.8)a</td>
</tr>
<tr>
<td>CD3/CD69+, cells/µl</td>
<td>52 (35–78)</td>
<td>52 (19–42)</td>
<td>51 (33–68)</td>
<td>42 (25–90)c</td>
</tr>
<tr>
<td>CD3/HLA-DR+, % of CD3+</td>
<td>7.7 (3.6–8.3)</td>
<td>6.3 (5.1–8.3)</td>
<td>10 (6.5–12.3)</td>
<td>11.2 (7.6–15.6)a,d</td>
</tr>
<tr>
<td>CD3/HLA-DR+, cells/µl</td>
<td>60 (38–123)</td>
<td>74 (49–114)</td>
<td>87 (76–166)</td>
<td>135 (81–190)c</td>
</tr>
</tbody>
</table>

Compared with healthy controls: a P < 0.05  b P < 0.01  c P < 0.01  d P < 0.05  e P = 0.09

Data presented as medians (IQR)

**Abbreviations:** WBC – white blood cells, others – see Table 1
Addition of DEX resulted in a marked ($P < 0.001$) decrease in surface expression of CD40L, CD69, and CD25, and led to a smaller but significant ($P < 0.05$) inhibition of HLA-DR (FIGURE 2A). Steroid-dependent asthmatics treated with high-dose GCs were characterized by smaller inhibition of CD40L expression by DEX when compared to the low-dose GCs group ($P < 0.05$) and controls ($P < 0.05$). Regarding other activation-dependent antigens, the effect of steroids was comparable between the groups (FIGURE 2A).

PHA stimulation led to a rapid proliferation of lymphocytes and resulted in a 2 to 3-fold increase in the absolute number of viable T-cells (after 3 days) in all studied groups ($P < 0.05$), except for severe asthmatics treated with high doses of oral GCs (FIGURE 2B, control data-set). In these patients, peripheral blood T-cells were thus hyporesponsive in an in vitro proliferation assay.

Addition of DEX to mitogen-stimulated PBLs led to a further inhibition of T-cell proliferation that was dose-dependent and highly significant in all studied groups (FIGURE 2B). However, the EC$_{50}$ concentration of DEX was increased in the high-dose GCs group (21.8 [4.3–191.0] nM, $P < 0.05$) in comparison with healthy controls (0.9 [0.3–8.3] nM) and other asthmatic patients.
Expression of glucocorticoid receptor mRNA isoforms

In all analyzed subjects, the median number of GRα cDNA molecules averaged to 0.82 × 10⁶ [0.37–1.84] per 1 μg of total RNA, and was considerably higher than GRβ (3.75 × 10³ (moderate: 1.2 [0.7–5.6] nM; severe, low-dose GCs: 10.6 [0.9–41.9] nM). Therefore, lymphocytes from severe steroid-dependent asthmatics required a 5 to 10-fold higher concentration to obtain comparable inhibitory effect in the assay.

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FIGURE 3  Expression of GR mRNA isoforms  A  Absolute number of GRα (left) and GRβ (right) mRNA transcripts in peripheral blood mononuclear cells (expressed as copy number per 1 μg of total RNA) in healthy controls and asthmatic patients  B  GRα/GRβ mRNA copy number ratio and its relation to disease severity. Data presented as medians (interquartile range, min–max bars)  C  Correlation between the GRα/GRβ ratio and asthma symptom scores  D  Correlation between GRα/GRβ ratio and selected parameters of in vitro assays

Inhibition of proliferation

Expression of CD25

Expression of CD69

Effect of dexamethasone

(moderate: 1.2 [0.7–5.6] nM; severe, low-dose GCs: 10.6 [0.9–41.9] nM). Therefore, lymphocytes from severe steroid-dependent asthmatics required a 5 to 10-fold higher concentration to obtain comparable inhibitory effect in the assay.
Additionally, Torrego et al. indicated that one GRβ mRNA molecule was outnumbered by approximately 250 molecules of GRα. There were no significant differences in the abundance of GR mRNA isoforms (Figure 3A) as well as the GRα/GRβ ratio (Figure 3B). There was a positive correlation between the copy number of GRα and GRβ (r = 0.68, P < 0.01). The expression of GR isoforms did not correlate with asthma symptoms, lung function tests, or oral GCs dose (Figure 3C). Moreover, there was no association between GRα/GRβ ratio and the sensitivity of lymphocytes to DEX in all in vitro assays (Figure 3D).

**Discussion** In this study, we tested the hypothesis that steroid-dependency in asthma results from an altered ratio in the expression of GRα and GRβ isoforms in lymphocytes. Such mechanisms were already suggested in rare phenotypes of severe GC-resistant asthma, and associated with poor asthma control. We investigated both the expression of GR isoforms and the sensitivity of lymphocytes to steroids in in-vitro functional assays in stable patients with severe persistent asthma stratified according to the treatment and symptom scores. This way we focused our analysis on patients with most severe asthma and true steroid-dependency.

All known biological responses of GCs are mediated by only 1 intracellular glucocorticoid receptor; however, in the processes of alternative splicing 2 major mRNA isoforms, GRα and GRβ, are generated (Figure 1). There is growing evidence that high expression of GRβ could be responsible for the development of insensitivity to GCs in a variety of inflammatory disorders. In severe asthmatics with GC-resistant phenotype, an increased level of GRβ was found both in peripheral blood lymphocytes and airway inflammatory cells.

Our data indicate that GRβ mRNA is expressed at very low quantity in PBMCs and does not seem to be up-regulated in steroid-dependent asthma. GRα variant exceeded 200 to 400-fold over GRβ, and their ratio did not differ between the groups. Similar results have already been reported by Gagliardo et al. who detected extremely low levels of both GRβ mRNA and protein in PBMCs isolated from severe asthmatics. Additionally, Torrego et al. found that expression of GRβ mRNA in PBMCs was approximately 600-fold lower than GRα in both asthmatic patients and controls, and it did not change upon cytokine stimulation. The predominance of GRα in lymphocytes was also observed in chronic inflammatory conditions and lymphoproliferative disorders. Results from other studies indicated that GRβ variant might be up-regulated upon in vitro treatment of lymphocytes with proinflammatory cytokines. Similar mechanisms could be responsible for the induction of GRβ in active inflammatory diseases. Therefore, increased expression of GRβ, which has been reported in unstable or GC-resistant asthma, could be a consequence of ongoing inflammation in exacerbated disease. Additionally, it has been shown that β isoform did not interfere with GR-mediated transpression and GRα excess seems to overcome its inhibitory action. Therefore, it is unlikely that the presence of low amount of GRβ could confer steroid resistance in circulating lymphocytes.

Unresponsiveness to GCs could be detected with in vitro assays. Indeed, a weaker inhibitory effect of GCs on T-cell proliferation was reported to be a characteristic feature of true GC-resistant asthma, and correlated with poor clinical response to steroids. To analyze whether lymphocytes in steroid-dependent asthmatics are hyporesponsive to GCs, we tested the effectiveness of a potent steroid (DEX) in an in vitro functional assay. We first estimated its inhibitory effect on T-cell activation by studying the expression of early and late activation antigens. In our assay, both the maximal expression of these antigens and the inhibitory effect of DEX were comparable between the groups, suggesting that GCs efficiently inhibit T-cell activation in steroid-dependent asthma. Interestingly, in the high-dose GCs group, the surface expression of CD40L was only partially inhibited by DEX, indicating that in severe steroid-dependent asthma, CD40L might be refractory to GC-mediated regulation. However, functional studies are needed to validate this hypothesis.

The inhibitory effect of DEX on T-cell proliferation was observed in all subjects, but it was lower in high-dose GCs group. It could indicate a presence of relative unresponsiveness to GCs, a phenomenon similar to that observed in true GC-resistant asthma. However, it is worth noting that in the current study, the baseline mitogenic responses of lymphocytes were already substantially decreased in patients who were treated with higher doses of oral GCs.

Altogether, our results indicate that in severe asthmatics, despite the need for long-term use of oral GCs, we observed normal response of blood lymphocytes to steroids in the majority of in vitro assays. Furthermore, the expression of GR variants in lymphocytes was unaltered in steroid-dependent asthmatics, with huge predominance of GRα over GRβ, indicating that chronic steroid treatment does not interfere with splicing events involved in the expression of β isoform. What is more important, the GRα/GRβ ratio did not correlate with the results of functional assays, indicating that GRβ could not act as an inhibitor of GRα, if the latter was present in a large excess. Finally, our data suggest that measuring the expression of GRα and GRβ isoforms in blood lymphocytes will not be useful in assessing the sensitivity to GCs in severe asthma, at least in those patients who are already treated with oral steroids. Whether testing for the expression of GRβ mRNA in airway samples might help predict the response to GCs requires further studies.
REFERENCES


Izoformy receptora glikokortykosteroidowego w astmie steroidozależnej

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SŁOWA KLUCZOWE
astma ciężka, astma steroidozależna, glikokortykosteroidy, izoforma β receptora glikokortykosteroidowego

STRESZCZENIE
WPROWADZENIE Nieskuteczna odpowiedź na glikokortykosteroidy (GKS) w astmie przewlekłej ciężkiej może wynikać z nadmiernej aktywacji limfocytów T, nieprawidłowej regulacji immunologicznej lub zmian w ekspresji receptora glikokortykosteroidowego (glucocorticoid receptor – GR).

CELE Celem badania była ocena ekspresji izoform receptora GR i wrażliwości limfocytów na GKS in vitro u chorych na astmę ciężką steroidozależną.

PACJENTI I METODY Do badania włączono 11 zdrowych ochotników, 15 chorych na astmę przewlekłą umiarkowaną, 11 chorych na astmę przewlekłą ciężką leczonych małymi dawkami GKS i 14 chorych na astmę częściowo kontrolowaną leczonych dużymi dawkami GKS. U chorych badano immunofenotyp limfocytów, skuteczność deksametazonu w hamowaniu aktywacji i proliferacji limfocytów, oraz poziom ekspresji mRNA GRα i GRβ w limfocytach krwi obwodowej.

WYNIKI Średni poziom ekspresji izoformy GRβ w limfocytach był >300 razy niższy niż izoformy GRα i był podobny we wszystkich badanych grupach. Limfocyty chorych na astmę ciężką steroidozależną charakteryzowały się prawidłową wrażliwością na GKS w testach aktywacji in vitro. Po dodaniu deksametazonu obserwowano wyraźne zmniejszenie ekspresji antygenów związanych z aktywacją (CD25, CD69) oraz zahamowanie proliferacji limfocytów wywołanej mitogenami. Wyniki testów czynnościowych in vitro były podobne w badanych grupach i nie korelowały z wartością stosunku GRα/GRβ.

WNIOSKI Steroidozależność w ciężkiej astmie oskrzeliowej nie jest związana z nadmierną ekspresją izoformy β receptora GR w limfocytach krwi ani z nieprawidłową reaktywnością limfocytów T na GKS. Dane te wskazują, że badanie poziomu ekspresji izoformy GRα i GRβ w limfocytach nie jest pomocne w przewidywaniu skuteczności steroidoterapii w ciężkiej astmie.