Persistent glucocorticoid resistance in systemic lupus erythematosus patients during clinical remission

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ABSTRACT. Glucocorticoids (GCs) are key drugs in the treatment of systemic lupus erythematosus (SLE). GC dose reduction during remission is related to disease activity, GC dose used, length of treatment, and individual GC sensitivity. We compared GC receptor α (GRα) isoform and nuclear factor kappaB (NF-κB) messenger RNA quantitation and in vivo GC sensitivity between SLE patients during remission and healthy controls. We performed a cross-sectional study of 19 women aged 22-49 years, including 9 SLE patients in clinical remission taking ≤5 mg prednisone and 10 matched controls. We evaluated GC sensitivity using 2 cortisol suppression tests: a very-low-dose intravenous dexamethasone suppression test (VLD-IV-DST) and a low-dose oral dexamethasone suppression test. GRα and NF-κB mRNA were quantified using real-time polymerase chain reaction. Although basal cortisol and adrenocorticotropin hormone levels were similar between the groups, the percentage of cortisol reduction after the VLD-IV-DST was 56% lower in SLE patients than in controls (P = 0.014). GRα and NF-κB
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gene expression levels were similar between the groups. The low-dose oral dexamethasone test caused intense cortisol suppression in all individuals, limiting the ability of this test to discriminate individual GC sensitivity. A positive correlation was found between the extent of cortisol suppression in vivo (VLD-IV-DST) and the number of days elapsed since the last flare of lupus activity. Despite clinical remission, SLE patients displayed partial GC resistance recognized by the VLD-IV-DST. The mechanism of this resistance is unrelated to altered GRα and NF-κB mRNA expression.

Key words: Cortisol; Dexamethasone; Glucocorticoid sensitivity; Systemic lupus erythematosus

INTRODUCTION
Systemic lupus erythematosus (SLE) is an autoimmune inflammatory disease characterized by alternate periods of exacerbation and remission. The pathogenesis is complex, and the target tissue damage is caused primarily by autoantibodies and immune complexes. The abnormal immune response determines the persistence of pathogenic B and T cells (Oelke and Richard, 2002). High doses of glucocorticoids (GCs) are used to treat severe manifestations of SLE. However, some patients may be unresponsive to GCs or may respond initially but relapse when the GC dose is tapered. Additionally, toxic GC side effects are virtually universal when high doses are needed for long periods. GC dose reduction schemes during SLE remission are largely empirical and related to disease activity, GC dose used, length of treatment, clinical symptoms, and individual GC sensitivity. Elucidating the mechanisms involved in GC resistance in SLE patients is important not only to understand more fully the pathophysiology of the disease but also to identify potential treatment protocols directed at the modulation of pro-inflammatory factor expression and improvement of patient responsiveness to GCs or other medications.

In recent years, transcription factors nuclear factor kappaB (NF-κB) and activator protein 1 have been discovered to be crucial for the induction of genes involved in inflammation as well as in a wide range of diseases. These factors are present in chronic activation of the immune system, such as that in asthma, atherosclerosis, inflammatory bowel disease, and autoimmune diseases including multiple sclerosis and rheumatoid arthritis (RA) (De Bosscher et al., 2001). More recently, adaptor proteins such as transforming growth factor-activated kinase 1, NF-κB-inducing kinase, and mitogen-activated protein kinase kinase have been shown to be capable of phosphorylating the inhibitor of NF-κB kinase (IKK) complex and triggering its dissociation. This process leads to the activation of the IKK-NF-κB pathway, inducing tissue inflammation (Beck et al., 2009).

Our previous research (Cobra et al., 2009) suggested that the mechanisms involved in GC resistance in RA do not occur at the GC receptor alpha (GRα) messenger RNA (mRNA) level but as a post-receptor adaptive response. Another study (Cavalcante et al., 2010) has shown that GC sensitivity evaluated by the percentage of cortisol reduction after a very-low-dose intravenous dexamethasone suppression test (VLD-IV-DST), as well as NF-κB, IKK, c-Fos (proto-oncogene), and GRα mRNA expression, were similar in RA patients and controls.

The aim of this study was to determine and compare expression of GRα and NF-κB mRNA in SLE patients in clinical remission and healthy controls and to correlate their levels with GC sensitivity as assessed by a VLD-IV-DST and low-dose oral dexamethasone suppression test.
MATERIAL AND METHODS

Selection of subjects

We studied 9 individuals under treatment at the Rheumatology Clinic of the Internal Medicine Department of Irmandade da Santa Casa de Misericórdia de São Paulo who fulfilled the SLE classification criteria of the American College of Rheumatology (1997) (Hochberg, 1997). All SLE patients were in remission and were either not receiving prednisone treatment (3 patients) or were receiving a maximum dose of 5 mg/day.

The control group consisted of 10 apparently healthy individuals selected through clinical evaluation by history and physical examination to match subjects in the SLE group with respect to gender (all were female) and age [means ± standard deviation (SD)] in the SLE group = 30.6 ± 2.4 years and in controls 34 ± 3.5 years; P = 0.44. We also noticed that mean ± SD weight was similar among the groups: 69 ± 4.5 kg in patients and 63.9 ± 3 kg in controls (P = 0.356).

We excluded controls and SLE patients who were younger than 18 years, had acute or chronic illnesses such as endocrine disease, asthma, and inflammatory bowel disease, or had used drugs that can accelerate dexamethasone metabolism (such as phenytoin and barbiturates) during the previous 4 months, as well as those who had a basal cortisol level of <7μg/dL to assure that the hypothalamic-pituitary-adrenal axis was not suppressed. The study protocol was approved by the Local Institutional Ethics Committee, and all individuals signed a written consent form before inclusion in the study.

Clinical characteristics of SLE patients

All SLE patients had an SLE Disease Activity Index (Gladman et al., 2002) score of 0. The mean ± SD duration of SLE was 7.3 ± 5.1 years. The mean age at SLE diagnosis was 26.7 ± 8 years. The average time since the last flare of lupus activity was 840.8 ± 572.4 days. All lupus patients had a positive antinuclear autoantibody test (Arbuckle et al., 2003) and had arthritis. None of the patients had a history of aggressive manifestations of the disease, such as cerebrovascular events, thromboembolic complications, or severe kidney or hematologic involvement. Six patients were using oral prednisone at a daily dose of 5 mg along with hydroxychloroquine (400 mg/day) or diphosphate of chloroquine (250 mg/day). Two patients used only an antimalarial drug (chloroquine, 250 mg daily), and one used only enalapril for the treatment of systemic arterial hypertension. End-organ damage was demonstrated in 4 patients and was quantified by the Systemic Lupus Index of the Consensus Committee/American College of Rheumatology (Rahman et al., 2001).

VLD-IV-DST and low-dose oral dexamethasone suppression test

All patients and controls underwent a VLD-IV-DST (20 μg/m²). We recently described the use of this test for the recognition of individual GC sensitivity in various age groups (Faria et al., 2008). Briefly, after a fasting period of 10-12 h and 30 min of rest, a blood sample was obtained for cortisol and adrenocorticotropic hormone (ACTH) measurements. Peripheral blood mononuclear cells (PBMCs) were obtained at the same time. Afterward, individuals of both groups received disodium phosphate dexamethasone intravenously (Decadron®, 2 mg/mL, Prodome Chemical and Pharmaceutical, São Paulo, SP, Brazil) at a dose of 20 μg/m² body sur-
face area as a bolus. A subsequent blood sample was taken 120 min later for cortisol and ACTH measurement (Immulite 2000 Cortisol, DPC, Los Angeles, CA, USA, and IMMULITE® 2000 ACTH, Siemens Healthcare, Llanberis, UK). The in vivo assessment of pituitary GC sensitivity was represented by the percentage of cortisol reduction (F%) after VLD-IV-DST.

The recognition of hypercortisolism is usually identified using an oral low-dose dexamethasone test. We compared the accuracy of this test to that of the VLD-IV-DST to identify GC sensitivity. All individuals received dexamethasone orally at a dose of 20 µg·kg⁻¹·day⁻¹ (every 6 h) for 2 consecutive days, when a new blood sample was obtained for cortisol and ACTH measurements. The expected response of normal controls to the test is cortisol <1.8 µg/dL and ACTH <20 pg/mL when the samples are collected after 2 days of dexamethasone administered every 6 h (Liddle, 1960).

**GRα and NF-κB mRNA determination through real-time quantitative polymerase chain reaction (PCR)**

A 20-mL venous blood sample was collected from each subject in tubes containing ethylenediaminetetraacetic acid K3 (Vacutainer, Becton-Dickinson, Franklin Lakes, NJ, USA). PBMCs were obtained after the addition of 20 mL Histopaque® 1077 (Sigma, USA) and centrifugation for 30 min at 800 g, according to manufacturer recommendations. Total RNA was isolated from cells using guanidinium thiocyanate-chloroform extraction (Trizol, Invitrogen, USA). Complementary DNA (cDNA) was synthesized from 1 µg total RNA using a reverse transcription reaction (TaqMan Reverse Transcription Reagents, Applied Biosystems).

NF-κB and GRα mRNA were determined according to our previously published protocol (Sousa e Silva et al., 2010). Briefly, real-time PCR was performed for these genes and the breakpoint cluster region (BCR) gene as a normalizing gene. Primers and probes were as follows: NF-κB sense primer: 5ꞌ-AAACACTGTGAGGATGGGATCTG-3ꞌ; NF-κB antisense: 5ꞌ-CGAAGCCGACCACCATGTG-3ꞌ; GRα sense primer: 5ꞌ-GAAGGAA ACTCCAGCAGA-3ꞌ; GRα antisense primer: 5ꞌ-CAGCTAAACATCTCGGGGAAT-3ꞌ; GRα probe: 6-FAM - 5ꞌ-GCTTCCAAACATTTTTGGATAAGACCAT-3ꞌ - TAMRA; BCR sense primer: 5ꞌ-CCTTCGACGTCAATACAAAGGAT-3ꞌ; BCR anti-sense primer: 5ꞌ-CCTGCCATGGCGTGTA-3ꞌ; BCR probe: 6-FAM - 5ꞌ-TCCATCTCGCTCATCATTACCAGACA-3ꞌ - TAMRA.

In each PCR run, we used a standard curve of serial dilutions of cDNA obtained from a standardized Jurkat (E6-1 clone, ATCC) cell culture. Real-time quantitative PCR conditions were the same for both the GRα and the BCR genes as described elsewhere (Melo et al., 2004) and were carried using a TaqMan PCR Core kit (Applied Biosystems). Briefly, 1X TaqMan buffer A, 500 mM of each deoxyribonucleotide triphosphate, 4.5 mM MgCl₂, 200 nM of each primer, 0.025 U/mL AmpliTaq Gold, 2 µL cDNA, and water were incubated in a total volume of 23 µL. SYBR Green, an intercalating, nonspecific dye detection system was used for the quantitation of NF-κB. For NF-κB, primer concentrations were adjusted to 0.3 µL of each primer (final concentration, 120 nM), with a corresponding increase in water (9.9 mL) in each reaction (Cavalcante et al., 2010).

Cycle conditions on an ABI 7500 (Applied Biosystems) were the same for all genes: 95°C for 10 min (AmpliTaq Gold or Taq Platinum activation) followed by 45 cycles of 95°C for 15 s (denaturation) and 60°C for 90 s (annealing and extension). When SYBR Green detection was used, a final dissociation stage was included, and melting curves were analyzed to ensure specific amplification.
**Statistical analysis**

Calculations of expression units for GRα and NF-κB with BCR normalization were performed in MS-Excel 2000 for Windows (Microsoft, Redmond, WA, USA). Statistical analyses were carried out using SigmaStat for Windows v.3.05 (SPSS, Chicago, IL, USA) with the level of significance set at $P < 0.05$. For comparison of quantitative variables, we used the Student $t$-test (when normal distribution was confirmed by the Kolmogorov-Smirnov test) or the Kruskal-Wallis test. We compared proportions of qualitative variables using the chi-square test. Linear regression analysis was used to compare continuous variables and F%. In the evaluation of a continuous variable in 2 instances in the same individual, we used a paired $t$-test or the Wilcoxon signed-rank test. In lupus patients, the identification of variables that are associated to F% was demonstrated by multivariate linear regression analysis (backward stepwise linear regression, $F$ to remove = 3.9).

**RESULTS**

**Comparison of GC sensitivity**

Basal median cortisol concentrations were similar in SLE patients and controls (11.7 and 19.4 µg/dL, respectively; $P = 0.25$). Basal ACTH concentrations were also similar between the groups (Table 1).

**Table 1.** Basal cortisol and adrenocorticotropic hormone (ACTH) concentrations, glucocorticoid sensitivity and glucocorticoid receptor α (GRα), and nuclear factor kappaB (NF-xB) mRNA quantitation in patients with systemic lupus erythematosus (SLE) and controls.

<table>
<thead>
<tr>
<th></th>
<th>SLE</th>
<th>Controls</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal cortisol*</td>
<td>11.7 (9.9-13.3)</td>
<td>19.4 (10-26.2)</td>
<td>0.25</td>
</tr>
<tr>
<td>Basal ACTH**</td>
<td>24.1 (9.9)</td>
<td>17.7 (17.3)</td>
<td>0.33</td>
</tr>
<tr>
<td>F%*</td>
<td>23.5 (-10-47.9%)</td>
<td>53.2 (42-58.6%)</td>
<td>0.014</td>
</tr>
<tr>
<td>FO%*</td>
<td>94.8 (78.9-95.6%)</td>
<td>93.9 (90.8-98.1%)</td>
<td>0.41</td>
</tr>
<tr>
<td>GRα mRNA*</td>
<td>16.8 (9.5-32.4)</td>
<td>11.7 (7.1-128.1)</td>
<td>0.77</td>
</tr>
<tr>
<td>NF-xB mRNA*</td>
<td>29.2 (11-198.9)</td>
<td>32.0 (2.6-197.6)</td>
<td>0.67</td>
</tr>
</tbody>
</table>

F% = cortisol suppression after 120 min very-low-dose intravenous dexamethasone suppression test (VLD IV-DST); 2 patients presented increased cortisol concentrations at 120 min after VLD IV-DST, thus having negative F%. FO% = cortisol suppression after 2 days of oral dexamethasone. *Data are reported as median (p25-p75), Kruskal-Wallis. **Data are reported as means (standard deviation) $t$-test.

After performing the low-dose oral dexamethasone suppression test with the VLD-IV-DST, we observed that the values for cortisol at 120 min were similar in the 2 groups, with means ± SD of 10.3 ± 4.9 for patients and 8.9 ± 3.9 for controls ($P = 0.51$). However, the extent of cortisol suppression at 120 min in patients was lower than that in controls (23.5 vs 53.2%, respectively; $P = 0.014$). The proportion of individuals who had more than 50% of cortisol suppression was statistically lower in SLE patients than in the control group (0/9 vs 6/10, respectively; $P = 0.001$).

After 2 days of low-dose oral dexamethasone suppression testing, we found that the extent of cortisol suppression (FO%) was similar in the 2 groups, with a median of 94.8% for SLE patients and 93.9% for controls ($P = 0.414$). To check the discriminatory efficiency of FO%, we calculated the median suppression in the entire group (94.4% suppression) and compared the number of individuals who suppressed more than the median in each group: 5/10 in controls and 5/9 in SLE patients ($P = 0.81$). We also checked for agreement between greater response to the oral test...
and VLD-IV-DST (F% vs FOr%). Of the 10 individuals who suppressed more than the median in the oral test, only 3 also had more than 50% suppression in the VLD-IV-DST (P = 0.876).

Comparison of gene expression

Median mRNA values of GRα and NF-κB were similar in the 2 groups (see Table 1).

Relationship between glucocorticoid sensitivity, gene expression, and clinical findings in SLE patients

We observed no significant correlation between in vivo GC sensitivity, as assessed by suppression of cortisol after VLD-IV-DST and PBMC expression of GRα or NF-κB (r = 8.8%, P = 0.324 and r = 5.7%, P = 0.462, respectively). Similarly, no significant correlation was detected between FOr% and expression of GRα or NF-κB (r = 7.9%, P = 0.796 and r = 0.9%, P = 0.766, respectively).

The suppression of cortisol after VLD- IV-DST, which reflected pituitary GC sensitivity, did not correlate significantly with basal ACTH and cortisol levels. A multivariate linear regression analysis indicated that increased number of days since the last flare of lupus activity (SLE Disease Activity Index >6) was the only independent variable correlated with higher GC sensitivity, as evaluated with F% using the VLD-IV-DST (r = 75.6%, P = 0.019; Figure 1).

**Figure 1.** Correlation of the extent of cortisol suppression at 120 min (F%) and days elapsed since the last flare of lupus activity (SLEDAI >6) (r = 75.6%, r² = 57.1%, P = 0.019). Longer periods since last disease flare correlated positively with higher GC sensitivity in SLE patients in remission.
DISCUSSION

We studied in vivo GC sensitivity in 9 patients with SLE in clinical remission using the VLD-IV-DST and the mRNA levels of the GRα isoform and NF-κB. Although no restriction was made in relation to gender in the inclusion criteria, the patients were all females. The SLE patients had a mean age at SLE diagnosis of 26.7 years, a figure similar to that found in the literature, which asserts that the age at diagnosis of SLE is generally 15 to 40 years (Petri, 2002). For the present study, we selected SLE patients with a low degree of severity and, therefore, using low doses of prednisone (≤5 mg/day).

Controls maintained parity by age, gender, weight, and body surface area to patients, allowing an appropriate comparison between groups even with a small number of samples, especially in tests of GC sensitivity. Basal cortisol concentrations were >7 μg/dL, suggesting that the hypothalamic-pituitary-adrenal axis was not suppressed in either group at the time of sample collection (Winter and Harris, 2008). We assumed that expression of GRα and the results of the cortisol suppression tests were not influenced by a negative feedback mechanism or downregulation of GRs.

Analysis of basal cortisol and ACTH in SLE patients and controls showed similar results. After 120 min of VLD-IV-DST, the concentration of cortisol was similar between groups, whereas the ACTH level was lower in the control group. Quantification of ACTH is characterized by known methodological difficulties owing to the sensitivity of the protein to heat and its instability in plasma (Castro and Moreira, 2002). The observed difference may also have been caused by sampling variability owing to the small number of cases in each group.

Significant reduction of ACTH and cortisol concentrations occurred after VLD-IV-DST, indicating that this test was effective in establishing a spectrum of sensitivity to GC in vivo as previously demonstrated (Faria et al., 2008; Cobra et al., 2009; Cavalcante et al., 2010). However, the extent of suppression was lower in patients than in controls (23.48 vs 53.2%, respectively), indicating lower GC sensitivity in SLE patients during the remission phase of the disease. Comparing patients and controls for GC sensitivity in vivo after the oral test with dexamethasone revealed no difference between the groups, and we were unable to identify a degree of suppression that differentiated patients from controls. Analysis of cases and controls together to assess the results of cortisol suppression in the VLD-IV-DST and the oral test (F% and FOr%) showed no agreement between the 2 tests. This result may indicate that GC suppression at the time of the oral test was too intense (very high FOr% median) to create an adequate spectrum of response that could identify partial GC resistance.

Similar to studies in RA patients (Neeck et al., 2002; Cobra et al., 2009), our study found no difference between the SLE and control groups in GRα expression in PBMCs. In addition, no correlation occurred between GRα or NF-κB expression and cortisol suppression after the VLD-IV-DST in patients or controls, which correlates to observations published by Cavalcante et al. (2010). However, RA may present greater differences in inflammation between synovia and PBMCs, and due to the systemic involvement in SLE, PBMC gene expression may better reflect inflammation status. The degree of inflammation in our patients may have been insufficient for the detection of changes in mRNA expression from PBMCs, but another explanation includes the interference of previous chronic use of GCs.

We found that patients with less time since the last flare of SLE activity had a lower GC sensitivity, and this finding was the only variable that correlated with the extent of cortisol
suppression *in vivo*, even when multivariate analysis was performed. One explanation is the existence of post-receptor effect - that is, a residual inflammatory process after a disease flare, not mediated by NF-κB, that causes partial GC resistance in the central nervous system. Abnormal translocation of activated GRα to the nucleus, co-inactivation of GRα and AP-1, heterodimerization of GRα with the GRβ isoform, and altered expression or function of co-activator could be post-receptor interferents that increase GC resistance (Bamberger et al., 1996; Schaaf and Cidlowski, 2003; Matthews et al., 2004; Faria and Longui, 2006; Piotrowski et al., 2007).

Another possible explanation for the partial resistance to GCs in some SLE patients is the enhanced expression of GRβ. Piotrowski et al. (2007) demonstrated greater expression of mRNA GRβ compared to that of GRα in SLE patients with highly active disease versus patients with low degrees of systemic inflammation. The association of immune-mediated diseases and expression of GRβ has been studied by several authors, who have described GRβ acting as a dominant negative inhibitor of GRα and increased expression of this β isoform in the PBMCs of GC-resistant patients with asthma (Oakley et al., 1996, 1999; Webster et al., 2001).

Additionally, changes in GR phosphorylation can also explain GC resistance, as exemplified by the increased activity of protein kinases such as c-Jun N-terminal kinase, extracellular-signal-related kinase, and p38 mitogen-activated protein kinase (MAPK). Phosphorylation of the GR-mediated p38 MAPK is linked to a reduction in the activity of GRα transcription. Thus, the GC resistance found in some patients might be reversed by inhibitors of MAPKs (Li et al., 2004).

The occurrence of mutations causing GC resistance can cause errors in data interpretation. A study by Lee et al. (2004) reported a silent mutation at codon 766 (exon 9) in 8.3% of SLE patients. This polymorphism had been shown by Koper et al. (1997) to be correlated to a lowered response to the oral dexamethasone test in elderly patients.

Regarding the NF-κB activity, a defect in the activation of NF-κB-mediated T-cell receptors in PBMCs has been identified in SLE patients but not in RA patients and healthy controls (Wong et al., 1999). Expression of NF-κB is reportedly increased in RA and could be one of the factors involved in disease pathogenesis (Mckay and Cidlowski, 1999). However, describing the impact of alterations on NF-κB activity in SLE and explaining the numerous immunological abnormalities found in this pleomorphic disease are difficult. A possible explanation for similar NF-κB gene expression in controls and SLE patients is their low degree of systemic inflammation.

Future studies may evaluate expression of c-Fos, AP-1, and GRβ and recognize GR polymorphisms to elucidate the mechanisms of partial resistance to GCs in certain patients with SLE. Another consideration is whether SLE patients in remission with increased sensitivity to GC on a VLD-IV-DST may have their doses of prednisone decreased or interrupted without worsening their clinical status.

Despite the limitations of our study, we demonstrated for the first time in patients with SLE in clinical remission using low doses of prednisone that partial GC resistance *in vivo* was not due to alterations in expression of GRα or NF-κB. We also demonstrated that increased sensitivity to GC in SLE correlates with increased time since the last flare of disease activity.

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Conflicts of interest

The authors declare no financial or other potential conflicts of interest.

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