A new link between steroid resistance, glucocorticoid receptor and nuclear factor kappa B p65 in idiopathic nephrotic syndrome

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Key words: glucocorticoids; sensitivity; relapse; peripheral blood cells; idiopathic nephrotic syndrome

Abstract

OBJECTIVES: The aim of our study was to investigate levels of glucocorticoid receptor (GRα), nuclear factor kappa B (NFκB) p65/p50 and inhibitor of NFκB alpha (IκBα) in the peripheral mononuclear blood cells (PMBC) of children with idiopathic nephrotic syndrome (INS).

METHODS: The expression of GRα, NFκBp65/p50 and IκBα was determined in 59 patients (age 10.2±5.1) and 25 healthy controls (CO) (age 13.1±3.4) using Western blot analysis. Patients were labeled according to their clinical sensitivity to glucocorticoids (GCs) as responders (RE), partial responders (PR), and non-responders (NR).

RESULTS: Significantly higher expressions of GRα were observed in RE than in PR, NR (p<0.01) and even CO (p<0.05). Similarly, expression of NFκBp65 was higher in RE in comparison to PR, NR and CO (p<0.05). These differences were more emphasized in the relapse: levels of GRα were significantly lower in PR than in RE and CO (p<0.01). Significant differences were also observed in expression of NFκB: RE showed significantly higher expression of NFκBp65 in comparison to PR, NR and even CO (p<0.01).

CONCLUSIONS: Lower levels of both GRα and NFκBp65 are associated with poor or no response to GCs and the difference is more pronounced in patients experiencing relapse of INS.

Abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>GCs</td>
<td>glucocorticoids</td>
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<tr>
<td>ALL</td>
<td>acute lymphoblastic leukemia</td>
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<td>INS</td>
<td>idiopathic nephrotic syndrome</td>
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<td>GRα</td>
<td>glucocorticoid receptor alpha</td>
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<td>NFκB</td>
<td>nuclear factor kappa B</td>
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<td>IκBα</td>
<td>inhibitor of NFκB</td>
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<tr>
<td>PMBC</td>
<td>peripheral mononuclear blood cells</td>
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<tr>
<td>RE</td>
<td>responders</td>
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<tr>
<td>PR</td>
<td>partial responders</td>
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<tr>
<td>NR</td>
<td>non-responders</td>
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<tr>
<td>CO</td>
<td>healthy controls</td>
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<tr>
<td>CI</td>
<td>calcineurin inhibitors</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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INTRODUCTION

Glucocorticoids (GCs) are employed in a therapy of various diseases such as asthma, bowel diseases, glomerulopathies and rheumatoid arthritis due to their anti-inflammatory and immunosuppressive effects. In addition, induction of apoptosis underlies their role in a therapy of hematological malignancies, e.g. acute lymphoblastic leukemia (ALL) or lymphomas. However, insufficient response or even resistance to GCs may occur in any of these diseases at the very beginning of therapy (primary resistance) or develop during steroid treatment (secondary resistance) (Kofler et al., 2000).

Steroid-resistant patients represent a major therapeutic dilemma especially in disorders where clinical response is closely related to prognosis, as it has been observed in idiopathic nephrotic syndrome (INS). INS is the most frequent glomerular disease in childhood and is characterized by massive proteinuria, edema and hypoalbuminemia. Clinical experience has demonstrated that patients with poor response to steroids have unfavorable prognosis and often develop an end-stage renal failure (Gbadegesin & Smoyer, 2008).

Nevertheless, in general, there is very little known about possible molecular mechanisms that might underlie resistance to GCs. The issue of steroid resistance is being widely studied in the field of hematological malignancies, particularly in ALL. The primary focus of those investigations was to correlate sensitivity to GCs with levels of glucocorticoid receptor alpha (GRα). Indeed, the majority of studies performed on this subject confirmed a link between the level of GRα in target cells and the response to GCs in ALL (Gruber et al., 2007). Nevertheless, in general, there is very little known about possible molecular mechanisms that might underlie resistance to GCs. The issue of steroid resistance is being widely studied in the field of hematological malignancies, particularly in ALL. The primary focus of those investigations was to correlate sensitivity to GCs with levels of glucocorticoid receptor alpha (GRα). Indeed, the majority of studies performed on that subject confirmed a link between the level of GRα in target cells and the response to GCs in ALL (Gruber et al., 2007). In contrast, other studies carried out in various inflammatory diseases found no differences in GRα density between steroid sensitive and resistant subjects (Honda et al., 2000; Lane et al., 1996; Onda et al., 2004; Schlaghecke et al., 1994).

Besides GRα expression, extensive interest is devoted to other transcriptional factors, e.g. nuclear factor kappa B (NFkB) (Bantel et al., 2002), which mainly consists of p65/p50 complex and inhibits the function of GRα and vice versa (Scheinman et al., 2002). Experimental data imply that the mechanism of reciprocal transcriptional repression of NFkB by GRα is mediated through the physical interaction with the p65 subunit alone (McKay & Cidlowski, 1998). Beyond this, the transcriptional activity of the NFkB p65/p50 heterodimer is regulated by GCs via up-regulation of the NFkB inhibitor alpha (IκBα) (Baueuerle & Baltimore, 1996). These observations are supported by a recent in vitro study, which suggests that targeting the NFkB pathway by enhanced expression of GRα, NFkBp65 and IκBα may sensitize cells to GCs (Oerlemans et al., 2007).

In the context of the results obtained from steroid resistance studies in ALL, the aim of our investigation was to elucidate the association between clinical sensitivity to GCs and levels of GRα, NFkB subunits and IκBα in the peripheral mononuclear blood cells (PMBC) of children with INS. Furthermore, levels of GRα, NFkB p65/p50 and IκBα were studied with regard to the activity of disease in order to characterize the pattern of proteins studied expression. In addition, all the results were compared to a control group to identify any differences between patients with INS and healthy subjects.

MATERIALS AND METHODS

Subjects and design of the study

Fifty nine patients with INS, 20 females and 39 males, aged 1–19 years (average age 10.2 years) were enrolled into the study. Patients were divided into three groups according to their response to standard therapy of INS (4-week course of daily oral prednisone at 60 mg/m²/day followed by 40 mg/m²/day on alternate days for an additional four weeks): i) patients with complete remission of proteinuria after only GCs were labeled as “responders” (RE), ii) as “partial responders” (PR) were labeled patients with only partial remission achieved after only GCs (proteinuria fluctuated between 166 mg/1.73m² and < 2 g/1.73 m²) (Ehrich et al., 2008), these patients required additional medications to achieve disease control, iii) “non-responders” (NR) failed to achieve even partial remission after 8 weeks of only steroid therapy.

Moreover, patients were subdivided according to the onset/relapse and remission of the disease. An onset or a relapse of the disease was diagnosed when proteinuria had exceeded 50 mg/m²/kg. Blood samples had been drawn from relapsing patients before therapy of the actual relapse was initiated. More detailed clinical and immunosuppressive treatment characteristics of subjects are listed in Table 1 and Table 2.

As a control group, blood samples were collected from 25 healthy controls (CO), 12 females and 13 males, aged 8–18 years (average age 13.1 years) without any acute disease or a drug history in last two months.

Informed consent was obtained from parents or guardians of all children before enrollment in this study. The study was approved by the Ethic committee of the Safarik University School of Medicine in Kosice, Slovakia.

Blood cells preparation

Peripheral blood was collected in EDTA tubes and processed within 1 hour. The PMBC were isolated from all patients and CO by density centrifugation using the Ficoll PM 400 (Sigma-Aldrich, USA). Each sample of venous blood was diluted 1:1 with phosphate-buffered saline (PBS) and density gradient centrifugation performed at 1600 rpm for 30 min. at 20 °C. Viability and number of cells were determined using trypan blue.

Western blot analysis

Cell lysates for GRα, NFkB p65, NFkB p50 and IκBα detection were prepared as described before (Haarman et al., 2008).
Briefly, the suspension of cells was promptly frozen in liquid nitrogen, frozen pellet was resuspended in lysis buffer (1.2% Igepal, Sigma-Aldrich, USA in PBS; Protease inhibitor cocktail, Sigma-Aldrich, USA; phenylmethylsulfonylfluoride, Serva, Germany; aprotinin, Serva, Germany) and lysates were clarified by microcentrifugation. Protein concentration was measured using Bio-Rad D_2 Protein Assay (Bio-Rad, USA). Following protein measurement, sample buffer (0.5 mol·L^{-1} Tris-HCl, pH=6.8; 1% glycerol, 4% sodium dodecyl sulfate (SDS), 0.005% bromphenol blue) supplemented with 5% β-mercaptoethanol was added and lysates were boiled. Immunoblotting was performed as described before (Haarman et al., 2004): twenty micrograms of the total cell lysates were separated on a 7.5% (GRα) and 12.5% (NFκBp65/p50, IκBα and β actin) polyacrylamide gel containing SDS and electroblotted onto a nitrocellulose membrane (Pall Gelman Laboratory, USA). Afterwards membranes were washed and incubated in non-fat dry milk (Laktino, Promil, Czech Republic) to block any non-specific antibody binding. The following primary antibodies were used: anti-GRα (Santa-Cruz, USA, sc-1003, 1:500), anti-NFκBp65 (Santa-Cruz, USA, sc 8008, 1:500), anti-NFκBp50 (Santa-Cruz, USA, sc-8414, 1:1000), anti-IκBα/MAD3 (BD Transduction Laboratories, USA, 1:1000) and as a control for protein loading anti-β actin (Santa-Cruz, USA, sc-47778, 1:3000). As secondary antibodies goat-anti-rabbit-HRP (Santa-Cruz, USA, sc-2004, 1:2000) and goat-anti-mouse-HRP (Dako, USA, p0447, 1:2000) antibodies were added. Membranes were washed and proteins were visualized by enhanced chemiluminescence (Pierce, USA) according to the manufacturer's instructions on X-ray film (Pierce, USA). The signal intensity of GRα, NFκBp65/p50 and IκBα was determined densitometrically (software Quantity One, Bio-Rad, USA) and normalized to β actin. Levels of GRα, NFκBp65, NFκBp50 and IκBα were expressed relative to an internal standard the value of which was set at 1.

**Statistics**

Statistical analysis was performed using SPSS for Windows 15.0 (SPSS, Inc., USA) and GraphPad Prism 5 (GraphPad Software, Inc., USA). Based on normality tests, the results were analysed using ANOVA or Kruskal Wallis for multiple comparisons. Significant differences were further confirmed by t-test or Mann-Whitney U test to compare the medians of the two groups. A p-value of 0.05 or less was considered statistically significant.

**RESULTS**

**Decreased levels of GRα and NFκBp65 are associated with steroid insensitivity**

To determine the correlation between levels of the proteins investigated and any response to treatment with GCs, we analyzed the quantity of GRα, NFκBp65/p50 and IκBα in the whole cell lysates of the PMBC of patients and healthy controls using Western blot (Figure 1). Significantly higher expressions of GRα were observed in a group of RE than PR, NR and even CO (p=0.0039; 0.0024 and 0.0221; respectively) (Figure 2A).

Similar differences were detected in expression of NFκBp65 subunit between RE and PR, NR and CO (p=0.048; 0.002 and 0.022; respectively) (Figure 3A). In contrast, no significant differences were found in the expression of either NFκBp50 or IκBα across all the groups studied (data not shown).

**Decline in GRα and NFκBp65 expression is pronounced in the relapse of INS**

To gain further insight into whether the level of the proteins studied may vary in association with the activity of disease, the expression of GRα, NFκBp65/p50 and IκBα were compared within clinical subgroups with the remission and relapse of INS. No differences...
Interestingly, expression of GRα and NFκBp65 did not differ across RE in remission and relapse. However, in a group of PR expression of both GRα and NFκBp65 was significantly higher in patients in remission than in those in relapse ($p=0.001$ and $0.0024$; respectively) (Figure 4A and 4B). Since only one NR was in remission at the time of blood collection, no statistical analysis was performed in this case.

Similar to results observed across clinical groups, no differences in the expression of the p50 subunit of NFκB were found in relation to the activity of disease (data not shown). However, levels of IκBα differed significantly within the RE group, where patients in the relapse exhibited significantly higher levels of the protein than those in remission ($p=0.0175$) (Figure 4C).

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Relative expression of the proteins studied (mean±SEM)*</th>
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<tr>
<td>RE total</td>
<td>0.59±0.09 0.81±0.11 0.63±0.063 0.33±0.08</td>
</tr>
<tr>
<td>RE in remission</td>
<td>0.67±0.11 0.65±0.17 0.56±0.09 0.15±0.07</td>
</tr>
<tr>
<td>RE in relapse</td>
<td>0.5±0.14 0.95±0.14 0.7±0.08 0.5±0.09</td>
</tr>
<tr>
<td>PR total</td>
<td>0.28±0.07 0.51±0.09 0.67±0.21 0.27±0.06</td>
</tr>
<tr>
<td>PR in remission</td>
<td>0.49±0.11 0.76±0.11 0.55±0.08 0.32±0.09</td>
</tr>
<tr>
<td>PR in relapse</td>
<td>0.07±0.04 0.24±0.1 0.82±0.43 0.22±0.08</td>
</tr>
<tr>
<td>NR total</td>
<td>0.21±0.07 0.21±0.07 0.58±0.14 0.32±0.15</td>
</tr>
<tr>
<td>NR in remission</td>
<td>-- -- -- --</td>
</tr>
<tr>
<td>NR in relapse</td>
<td>0.15±0.07 0.14±0.08 0.64±0.16 0.32±0.17</td>
</tr>
<tr>
<td>CO</td>
<td>0.35±0.06 0.47±0.08 0.88±0.17 0.37±0.06</td>
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* correlated to internal standard included in each experiment value of which was set at 1
A new link between steroid resistance, glucocorticoid receptor and nuclear factor kappa B p65 in idiopathic nephrotic syndrome

Fig. 2. Relative expression of the glucocorticoid receptor (GRα) in the peripheral mononuclear blood cells (PMBC) of patients with idiopathic nephrotic syndrome (INS) labeled as responders (RE), partial responders (PR) and non-responders (NR) to glucocorticoids (GC). A – comparison of clinical subgroups and healthy controls (CO). B – comparison of clinical subgroups in remission and CO. C – comparison of clinical subgroups in relapse of INS and CO. *p<0.05; **p<0.01; ***p<0.001; (Mann-Whitney test)

Fig. 3. Relative expression of the nuclear factor kappa B p65 subunit (NFκBp65) in the peripheral mononuclear blood cells (PMBC) of patients with idiopathic nephrotic syndrome (INS) labeled as responders (RE), partial responders (PR) and non-responders (NR) to glucocorticoids (GC). A – comparison of clinical subgroups and healthy controls (CO). B – comparison of clinical subgroups in remission and CO. C – comparison of clinical subgroups in relapse of INS and CO. *p<0.05; **p<0.01; (Mann-Whitney test)
**DISCUSSION**

For the first time this study links low levels of both GRα and NFκBp65 subunit with resistance to GCs in INS. Importantly, we have illustrated that the difference is more pronounced when patients with partial and no response to steroids experience relapse.

Miscellaneous effects of GCs are mediated through the GRα localized in the cytoplasm, which is transferred to the nucleus after ligand binding (Davies et al., 2002; Wochnik et al., 2005) and subsequently initiates the transcription of genes regulating cell death and inflammation (Herr et al., 2007). Therefore, it is not surprising that several studies have shown that sensitivity to GCs is partially dependent on the level of receptors found within a cell (Gross et al., 2009). Concurrent with GC responsiveness in cancer (Sanchez-Vega & Gandhi, 2009), GRα expression is positively correlated with sensitivity to steroids in INS in our study (p<0.01) and might provide a further evidence for supporting the earlier observations in proteinuric adults (Han et al., 2008; Tanaka et al., 1992). However, our findings could not confirm previously published data obtained from nephrotic children (Haack et al., 1999; Wasilewska et al., 2003). Several factors that might contribute to those different conclusions should be considered. First, in comparison to the study of Wasilewska et al., (2003), which involved only steroid sensitive patients and healthy controls, children with partial and/or no response were also enrolled in our cohort. Second, our study divided patients in clinical subgroups (RE, PR and NR), that differed from those used by Haack and colleagues (1999).

Interestingly, we also observed that NFκBp65 levels significantly correlated with the steroid response of nephrotic children (p<0.05), which is consistent with experimental data published by Aviles et al., (2004). The mode of mutual inhibition exhibited by GRα and the NFκBp65/p50 complex is conveyed via physical interaction of the receptor and the p65 subunit (Ray & Prefontaine, 1994). Moreover, stochiometric interaction is required for transcriptional activity of NFκB, thereby its function might be impaired by the imbalanced expression of the p65 and p50 subunits (Perkins, 2000). These findings were supported by in vitro experiment using Cos-1 cells transfected with only p50 homodimers, in which no response to GCs was determined (McKay & Cidlowski, 1998). Therefore, insufficient expression of NFκBp65 might be involved in a poor response or resistance to GCs, as it has been observed in our study.

It has been noted that steroid treatment can increase expression of GRα (autoinduction) and that phenomenon might modulate sensitivity to GCs (Eisen et al., 1988); whereas failure of autoinduction in T-cell lines detected after exposure to steroids observed was closely related to resistance (Schmidt et al., 2006). Regarding our clinical observations, the influence of GCs on the measured levels of GRα might be ruled out mainly on

**Fig. 4.** Relative expression of the glucocorticoid receptor (GRα) and the nuclear factor kappa B (NFκB) p65 in the peripheral mononuclear blood cells (PMBC) of patients with idiopathic nephrotic syndrome (INS) labeled as partial responders (PR) to glucocorticoids (GC). **A** – comparison of levels of GRα in PR in the remission and relapse. **B** – comparison of levels of NFκBp65 in PR in the remission and relapse. **C** – comparison of levels of IκBα in RE in the remission and relapse. **p<0.01;** (Mann-Whitney test).
the basis that all but one responsive patients were not being treated with steroids at the time of blood collection. Furthermore, certain individual children using a long-term treatment with partial and no response to GCs expressed slightly higher levels of GRα than those not undergoing treatment. Similar explanation might be applied in the case of NFkB65 expression, which is also influenced by GCs (De Bosscher et al., 2003).

It has been demonstrated that down-regulation in GRα expression in malignant cells between diagnosis and relapse is associated with resistance to steroid therapy (Pui et al., 1984; Bloomfield et al., 1981), hence we also focused on levels of GRα, NFkB65/p50 and IκBα in the PMBC at the state of relapse and remission.

Based on this knowledge we theorized whether nephrotic children with various clinical responses to GCs show significant differences in levels of GRα and NFkB related proteins while experiencing relapse.

Whereas expressions of GRα, NFkB65/p50 and IκBα were comparable for patients in remission, to our surprise, levels of both GRα and NFkB65 significantly differed when patients were in the relapse. In particular, a very significant decline in GRα and NFkB65 expression was observed in relapsing PR compared to relapsing RE (p<0.01, both proteins). The same significant difference was found in levels of NFkB65 between NR and RE in the relapse (p<0.01). In addition, both GRα and NFkB65 were dramatically down-regulated in PR with relapse when compared to the same clinical subgroup in remission (p<0.01, both proteins). Due to the fact that achieving remission in corticosteroid-resistant patients is very rare, assessment of this specific subgroup could not be performed.

Conceivably, one may think whether the lower levels of GRα and NFkB65 expression seen in our cohort of nephrotic patients might underlie onset and/or relapse of INS. The finding was dominantly pronounced in a group of PR and partially also in NR, therefore, it is more likely that the lower expression of these proteins is related to the response of these patients to steroid treatment in a further course of disease as it has been suggested in pediatric patients with ALL (Pui & Costlow, 1986).

Levels of p50 subunit and IκBα were determined in the PMBC of our patients and compared with sensitivity to GCs in general, as well as in respect to the activity of disease. Yet, no differences in protein levels were found between patients with various steroid response and/or healthy controls. Concurrently, the same results were observed for the p50 subunit in the study of Aviles et al., (2004). In regard to IκBα expression, experimental investigations have shown increased levels of this protein in tumor and airway cells with in vitro sensitivity to GCs (Oerlemans et al., 2007; Kang et al., 2006). However, to our best knowledge no study focusing on IκBα levels and steroid sensitivity in INS patients has yet been published. For illustration, Sahali et al., (2001) suggested a link between a lower expression of the cytosolic IκBα levels in the PMBC of patients with steroid sensitivity experiencing relapse of INS, possibly caused by increased proteasome degradation. In contrast, we observe up-regulation of IκBα in relapsing RE in comparison to the same clinical subgroup in a remission. That finding corresponds with the role of IκBα in the NFkB-GRA loop (Oerlemans et al., 2007).

In conclusion, our study supports the importance of both GRα and NFkB65 levels in the PMBC in the clinical response to GCs in children with INS. This pivotal observation was even more pronounced in patients with partial and/or no response to GCs experiencing relapse. Our current results encourage further clinical studies in order to find a reliable and effective tool that would indicate the response of nephrotic children to steroids. Such a diagnostic tool may help clinicians to tailor and optimize immunosuppressive treatment of INS or other steroid-treated diseases and thereby avoid the unwanted effects, which are frequently associated with steroid therapy.

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REFERENCES


