Nuclear bioavailability of the glucocorticoid receptor in a pediatric asthma cohort with variable corticosteroid responsiveness

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BACKGROUND: Despite the overall effectiveness of glucocorticoids (GCs) in the treatment of asthma, a large proportion of patients do not fully respond to this medication. The objective of the present study was to investigate the potential molecular mechanisms responsible for corticosteroid insensitivity in pediatric asthma.

METHODS: Asthmatic children were classified as good (GSR) or poor corticosteroid responders (PSR) based on the changes in pulmonary function following GC treatment. Immortalized B-cells derived from patients at two ends of the spectrum of GC responsiveness (five each) were grown in culture and treated with hydrocortisone (10^{-6} M). Baseline and temporal changes in GC receptor (GR) protein and mRNA were evaluated by western blot and quantitative reverse transcription PCR respectively. The effect of GC treatment on GR nuclear levels was assessed by western blots.

RESULTS: Cells derived from PSR asthmatics displayed lower GR protein levels when compared to GSR. Moreover, in PSR cells GC-induced nuclear translocation of GR was short-lived and homologous downregulation of GR mRNA and protein was faster than in GSR.

CONCLUSION: Our data demonstrate the existence of a novel mechanism of GC insensitivity resulting from limited GR nuclear bioavailability as a consequence of decreased baseline GR protein expression and more rapid hormone-induced downregulation.

Asthma is the most prevalent chronic disease in children, affecting 7.1 million Americans under the age of 18 and more than 15% of the Canadian pediatric population of ages 4–11 (1,2). It is characterized by persistent airway inflammation, reversible airflow obstruction, and enhanced airway hyper-responsiveness (3). Due to their potent anti-inflammatory effects, inhaled glucocorticoids (GCs) are the most commonly used anti-inflammatory drug for the treatment of asthma (4). Overall, GCs reduce symptoms, prevent exacerbations, and decrease asthma-associated mortality (3).

However, there is significant variability in GC responsiveness and up to 40% of asthmatics fail to show improved lung function following inhaled corticosteroid treatment (5). While some GC-insensitive patients do respond to higher doses than normally prescribed, the administration of these higher doses for prolonged periods can have marked adverse effects. Moreover, poor clinical responses have been observed even among asthmatics receiving high doses of systemic corticosteroids (6). The economic burden of asthma on healthcare systems is significant, particularly due to the mismanagement of severe asthmatics (3,7). Therefore, a better understanding of the molecular mechanisms responsible for GC insensitivity is necessary in order to tailor patient-specific treatment, increasing drug effectiveness while avoiding side effects.

Most of the anti-inflammatory effects of GCs result from transcriptional regulation of target genes through the GC receptor (GR), which, in the absence of ligand, is sequestered in the cytoplasm (4). Once activated, GR translocates into the nucleus with the aid of nuclear transport receptors. Multiple mechanisms have been proposed to contribute to variable corticosteroid responsiveness, including altered expression and dysfunctional nuclear translocation of GR (reviewed in ref. (7)). We previously identified importin 13 (IPO13), a nuclear import receptor for GR in the lung (8). Additionally, since corticosteroids induce GR downregulation, it is reasonable to speculate that differences in temporal activation of this negative feedback mechanism could also add to variations in corticosteroid sensitivity (9).

To explore the potential molecular mechanisms responsible for GC insensitivity in pediatric asthma, immortalized B-cell lines (lymphoblastoid cell lines) were derived from asthmatic children classified as good (GSR) or poor corticosteroid responders (PSR) (see Table 1). Cell lines derived from patients at the two ends of the spectrum of GC responsiveness were grown in culture and treated with GCs. We hypothesized that PSR cells would present decreased expression of genes involved in the GC pathway and/or altered nuclear translocation of GR

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in response to GC treatment. Herein, we report that lymphoblastoid cells derived from PSR children showed diminished GR protein expression at baseline as well as faster homologous downregulation and nuclear translocation of GR in response to corticosteroids when compared to GSR lymphoblastoid cells.

RESULTS

PSR Lymphoblastoid Cells Express Abnormal Levels of GR

Protein

We assessed GR protein levels by western blots. Lymphoblastoid cells derived from GSR asthmatics had significantly higher levels of GR protein when compared to PSR cells ($P < 0.05$, Figure 1a),

Table 1. Patient clinical characteristics

Subject	Class	Age	Sex	Race	12 mo FEV ₁ change
1	Good	7	Female	Caucasian	46.88
2	Good	12	Female	Caucasian	22.22
3	Good	9	Male	Caucasian	30.67
4	Good	13	Male	Caucasian	50.00
5	Good	12	Female	Caucasian	37.70
6	Poor	5	Female	Caucasian	−1.96
7	Poor	11	Male	Caucasian	−12.00
8	Poor	11	Male	Caucasian	−9.21
9	Poor	5	Male	Caucasian	−12.71
10	Poor	8	Male	Caucasian	−17.50

suggesting that the amount of GR protein could be a predictor of GC response. To verify this association, a Pearson correlation analysis was conducted between GR protein levels and change in lung function over time. GR protein levels in GSR and PSR lymphoblastoid cells positively correlated with change in lung function after 2 ($P = 0.03$, $r = 0.672$, Figure 1b) and 12 ($P = 0.0319$, $r = 0.676$, Figure 1c) months of inhaled corticosteroid treatment.

PSR Lymphoblastoid Cells Display Altered GC-Induced Nuclear Translocation of GR

Having identified decreased GR protein expression in PSR cells, we next wished to examine if these reduced receptor levels affected GR nuclear translocation. To determine if lymphoblastoid cells derived from PSR asthmatics displayed abnormal nuclear translocation of GR in response to GC treatment, cells were grown in culture and treated with corticosteroids for 15, 30 min, and 2 h. Nuclear protein fraction and cytospin slides were prepared and western blots and immunofluorescence assays were performed. Corticosteroid treatment caused the accumulation of GR protein in the nucleus of GSR and PSR lymphoblastoid cells. However, there was a marked difference in temporal regulation of nuclear import. In lymphoblastoid cells derived from GSR children, GC addition to culture media caused a rapid (15 min) nuclear translocation of GR. High nuclear GR protein levels were maintained by 30 min and continued to increase until at least 2 h following GC treatment ($P < 0.05$, Figure 2a,c). In PSR lymphoblastoid cells, nuclear GR also increased 15 min after GC addition to the media ($P < 0.001$, Figure 2b,d). However, the

Figure 1. (a) Relative GR protein expression by western blot in lymphoblast cell lines of good (GSR) and poor corticosteroid responders (PSR). Means ($n = 5$) are presented $\pm$ SEM, $*P < 0.05$. Representative western blot showing baseline GR protein levels in GSR and PSR lymphoblastoid cells. Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control. Correlation between the change in FEV₁ following (b) 2 or (c) 12 mo of inhaled corticosteroid treatment and baseline GR protein levels in lymphoblastoid cells from asthmatic children. $P = 0.03$, $r = 0.672$ and $P = 0.0319$, $r = 0.676$ respectively, $n = 10$.

large influx of GR was short-lived and by 30 min the levels of GR protein decreased to less than half of those observed at 15 min ($P < 0.001$, **Figure 2b,d**). These results were confirmed by immunofluorescence experiments (**Figure 2e,f**).

Interestingly, we noted that lymphoblastoid cells derived from children classified as PSR had higher levels of GR mRNA at baseline ($P < 0.05$, **Figure 3**).

Effects of GC Exposure on GSR and PSR Lymphoblastoid Cells

Having established that GSR and PSR lymphoblastoid cells display differences in GR protein and hormone-induced nuclear translocation, we next wished to evaluate the response of these cells to sustained GC exposure.

To determine the effects of GC on GR expression, lymphoblastoid cells were treated with GC for 30 min, 2 and 24 h. We first assessed GR mRNA expression by quantitative reverse transcription PCR. In GSR lymphoblastoid cells, GC had no effect on the levels of GR mRNA in the early time points (30 min and 2 h). However, after 24 h of GC addition to the media, GR mRNA levels decreased ($P < 0.001$, **Figure 4a**). On the other hand, in PSR lymphoblastoid cells, 2 h of GC treatment led to decreased levels of GR mRNA, an effect that was maintained after 24 h ($P < 0.001$, **Figure 4b**).

To determine the effects of GC on cellular GR protein levels, western blots were conducted using total protein extracts. In GSR lymphoblastoid cells, GR protein levels decreased after

Figure 2. Nuclear GR protein levels over time by western blot in (a) GSR and (b) PSR lymphoblast cell lines following glucocorticoid (GC) treatment (solid bars) and respective controls (open bars). Means ($n = 5$) are presented $\pm$ SEM, * $P < 0.05$, ** $P < 0.001$ as compared to respective control or as indicated. Representative western blot for nuclear GR protein in (c) GSR and (d) PSR lymphoblastoid cells following GC treatment. TBP was used as a nuclear protein loading control. Representative immunofluorescence image showing GR (red color) translocating into the nucleus (blue color) of (e) GSR and (f) PSR lymphoblastoid cells after 0, 0.25, and 2 h after the addition of GC to the media (Scale bar, 10 μ m). TBP, TATA-binding protein.

2 h of corticosteroid treatment and were even lower after 24 h ($P < 0.05$, **Figure 5a,c**). In contrast, PSR lymphoblastoid cells displayed reduced levels of GR protein after 30 min of GC addition to the media ($P < 0.001$, **Figure 5b,d**) and reached its lowest point after 24 h of treatment ($P < 0.001$).

GC Effects on GR Nuclear Import

Given that PSR lymphoblastoid cells displayed a faster down-regulation of GR in response to GC, we next wished to determine the effects of this agent on GR nuclear influx. Nuclear extracts were obtained from GSR and PSR lymphoblastoid cells after treatment with GC for 30 min, 2 and 24 h. Consistent

with our previous experiment, corticosteroid treatment caused a large influx of GR into the nucleus of GSR lymphoblastoid cells that peaked at 2 h after the addition of corticosteroids to the media ($P < 0.001$, **Figure 6a,c**). In PSR lymphoblastoid cells, GC also caused an increase in GR nuclear protein, but the levels never exceeded those observed at 30 min ($P < 0.001$, **Figure 6b,c**).

To determine if the observed differences in GR nuclear translocation between GSR and PSR lymphoblastoid cells was due to differential expression of IPO13, we assessed the levels of this importin by quantitative reverse transcription PCR and western blot after GC treatment. No significant differences in the levels of IPO13 mRNA were found neither between GSR and PSR lymphoblastoid cells at baseline (0.94 vs. 1.02, $P = 0.316$) or after treatment with GC (fold change from time 0, 30 min: 1.03 vs. 0.93, $P = 0.121$; 2 h: 0.96 vs. 0.87, $P = 0.473$; 24 h: 0.84 vs. 0.78, $P = 0.156$). Similarly, no differences in IPO13 expression were found at the protein level.

DISCUSSION

It has been proposed that diminished receptor levels and defective transport into the nucleus may be associated with reduced corticosteroid responsiveness in asthma (10–13). We show that lymphoblastoid cells derived from PSR asthmatic children had lower GR protein expression than GSR lymphoblastoid cells. Interestingly, we noted that the PSR cells had higher baseline levels of GR mRNA. Peripheral blood mononuclear cells from severe asthmatics, which tend to be less GC responsive, express higher levels of GR α mRNA at baseline than peripheral blood mononuclear cells of mild stable asthmatics and healthy individuals (14,15). The reduced levels of GR protein without concomitant levels of mRNA, suggest that lymphoblastoid cells derived from PSR children may have defective translational and/or post-translational regulation of GR, leading to low amounts of protein. It should be noted that even though we used an anti-GR α/β antibody in all experiments, only one

Figure 3. Relative GR mRNA expression by qRT-PCR in lymphoblast cell lines of good (GSR) and poor corticosteroid responders (PSR). Means ($n = 5$) are presented $\pm$ SEM, $*P < 0.05$. qRT-PCR, quantitative reverse transcription PCR.

Figure 4. Fold change of GR mRNA from time 0 by qRT-PCR in (a) GSR and (b) PSR lymphoblast cell lines following treatment with glucocorticoid (solid bars), and respective controls (open bars). Means ($n = 5$) are presented $\pm$ SEM, $*P < 0.001$ as compared to respective control. qRT-PCR, quantitative reverse transcription PCR.

Figure 5. Fold change in total cellular GR protein expression from time 0 by western blot in (a) GSR and (b) PSR lymphoblast cell lines following 0.5, 2, and 24 h treatment with glucocorticoid (GC, solid bars) and respective controls (open bars). Means ($n = 5$) are presented $\pm$ SEM, * $P < 0.05$, ** $P < 0.001$ as compared to respective control. Representative western blots for total GR in cellular extracts of (c) GSR and (d) PSR lymphoblastoid cells after 0, 0.5, 2, and 24 h treatment with GC. Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control.

Figure 6. GR protein levels over time by Western blot in nuclear extracts from (a) GSR and (b) PSR lymphoblast cell lines following treatment with glucocorticoid (GC, solid bars) and respective controls (open bars). Means ($n = 5$) are presented $\pm$ SEMs, * $P < 0.05$, ** $P < 0.001$ as compared to respective control. Representative western blots for GR in nuclear extracts of (c) GSR and (d) PSR lymphoblastoid cells after 0, 0.5, 2, and 24 h treatment with GC. TBP was used as a nuclear protein loading control. TBP, TATA-binding protein.

single band corresponding to GR α was detected in western blots. This is consistent with previous reports that failed to detect the GR β isoform in peripheral blood mononuclear cells of healthy and asthmatic individuals (14,16).

Due to its involvement in vital cellular functions, the GC pathway is tightly controlled to maintain cell homeostasis. It is

reasonable to speculate that in face of low amounts of GR, PSR cells might turn on a compensatory mechanism by which transcription of the receptor is stimulated and/or the half-life of the messenger increased, explaining the discrepancy observed between protein and mRNA expression. Furthermore, it is possible that GR mRNA degradation and ongoing protein

translation are linked, in a way that when translation is inhibited the mRNA is stabilized (17).

GR-induced transcriptional responses are proportional to the number of receptor molecules present in the cell (18). These results at the cellular level seem to have a physiological effect and are consistent with our findings that increased amounts of GR protein are present in GSR lymphoblastoid cells and correlate with better therapeutic outcome. Although the levels of GR protein could explain the phenotypic differences between GSR and PSR children *per se*, it is unlikely that corticosteroid responsiveness is solely the result of variance in receptor expression.

We also investigated the hormone-induced nuclear translocation of GR, a key step in the normal function of the GC pathway. Interestingly, there was a clear temporal difference in the nuclear localization of GR between GSR and PSR lymphoblastoid cells that was not dependent on the expression of the nuclear transport receptor IPO13. After GC addition to the culture media, GR nuclear levels peaked at 2 h in GSR lymphoblastoid cells and at 15 min in PSR cells. It is possible that the temporal differences in nuclear GR are a reflection of the initial levels of cellular GR protein. While in GSR lymphoblastoid cells, there is sufficient number of receptors available for activation, keeping a constant nuclear influx over time, in PSR cells the reservoir of GR in the cytoplasm gets saturated only after 15 min of treatment. Even though this explanation is supported by the cellular expression levels of GR, we cannot rule out the possibility that the observed differences in nuclear translocation are a result of dysregulation of import receptors other than IPO13. Several other nuclear transport receptors, including importin 7 and importin α/β , have been shown to be able to transport GR into the nucleus (19).

GSR and PSR lymphoblastoid cells not only show a difference in baseline GR expression but more importantly they display variance in the homologous down regulation of the receptor mRNA and protein. Consistent with previous reports, corticosteroid treatment led to decreased expression of GR, which occurred earlier and was more pronounced at the protein than at the mRNA level (9). This is not surprising, since homologous downregulation of the receptor involves direct destabilization of the existing protein while decreased levels of GR mRNA are believed to be mainly a result of reduced transcription (9, 20). We show that GR expression decreased more rapidly in cells derived from PSR asthmatics at both the protein and mRNA levels, suggesting that the negative feedback mechanism in PSR children has a lower GC threshold for activation. Even though the existence of this mechanism is necessary to limit the effects of long-term exposure to the hormone, if it gets activated too soon it could be maladaptive, making cells unresponsive to further corticosteroid treatment (9). PSR lymphoblastoid cells have low expression of GR protein and when treated with corticosteroids these levels decrease at higher rates than in GSR cells, affecting the bioavailability of active GR in the nucleus. These results further support the hypothesis that the differences in GR nuclear influx between

GSR and PSR lymphoblastoid cells is a result mainly of receptor levels in the cytoplasm and not a consequence of defects on the actual nuclear translocation mechanism.

Steroid treatment leads to a twofold decrease in GR protein half-life reducing cellular responsiveness to ligand (9,21). Residues in the PEST degradation motifs of GR get ubiquitinated upon GC binding, targeting the receptor for rapid degradation by the proteasome (22,23). Other studies have provided further evidence for the involvement of the proteasomal system in limiting GC responsiveness; for example, treatment with a proteasome inhibitor increases GR transactivation and overcomes corticosteroid resistance in certain cancer cells (23–25).

The differences between GSR and PSR lymphoblastoid cells in all parameters assessed were mainly evident during the early time points after treatments. The GC pathway is tightly regulated and it is likely that small variations on GR expression/translocation early on have a crucial effect on normal corticosteroid response. Despite our limited sample size, our results support the idea that corticosteroid insensitivity in asthmatic children results, at least in part from increased degradation of GR, a defect that becomes more apparent after corticosteroids induce the downregulation of the receptor. Our findings have implications for the development of strategies for therapeutic intervention in the pediatric setting where GC treatment is ineffective.

METHODS

CAMP Lymphoblastoid Cell Lines

The Childhood Asthma Management Program (CAMP) is a multicentered, randomized, double-blind clinical trial, testing the safety and efficacy of long-term anti-inflammatory drugs in the treatment of asthma. The design and methodology of the trial as well as the analysis of the primary outcomes have been published elsewhere (26,27). Approval was obtained from the institutional review boards at each of the CAMP study centers (University of Toronto; Children's Hospital, Seattle, WA; University of New Mexico; Kaiser San Diego; Children's Hospital, Washington University, St Louis; Harvard Pilgrim Health Care, Boston MA; National Jewish Hospital, Denver CO; Allergy Center, Johns Hopkins University, Baltimore MD). Informed assent and consent was obtained from participants and their parents or guardians.

Patients were classified as good (GSR) or poor corticosteroid responders (PSR) if they showed a positive ($>20\%$ increase) or negative ($\leq 0\%$) change in FEV₁ following 12 mo of budesonide treatment respectively. Patients at the two extremes of the spectrum of GC responsiveness (five each) were selected to conduct this study (Table 1). Blood was collected in a 10 ml "Yellow top" ACD vacutainer vial during a normal follow-up visit during the CAMP Continuing Studies 2 (CAMP CS/2) cohort study. B-cells were isolated and transformed into lymphoblastoid cell lines by Epstein-Barr virus at the Partners HealthCare Center for Personalized Genetic Medicine (Harvard Medical School, Boston, MA).

Cell Culture and Treatment

Lymphoblastoid cells were plated at 200,000 cells/ml and grown in Roswell Park Memorial Institute medium (Invitrogen, Burlington, ON, Canada) containing 10% fetal bovine serum (HyClone, Logan, UT), penicillin (100 units/ml), and streptomycin (100 μ g/ml) (Invitrogen) until day 4. Cells were then serum-starved for 24 h in Roswell Park Memorial Institute medium containing 10% charcoal-stripped fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μ g/ml) before being treated with hydrocortisone (10^{-6} M), for 0.25, 0.5, 2, and 24 h.

Protein Isolation and Western Blots

Total protein was isolated using KPO4 buffer and nuclear and cytoplasmic fractions were obtained using buffers provided in NE-PER kit (Pierce Biotechnology, Rockford, IL) and following manufacturer's instructions. Fifteen micrograms of total protein or nuclear fraction were boiled for 5 min in sodium dodecyl sulfate-loading buffer before being electrophoresed on 7.5% sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene difluoride membrane (Biorad, Hercules, CA). Membranes were blocked overnight at 4 °C with 10% milk/phosphate-buffered saline (PBS)-Tween and probed with anti-GR, anti-IPO13 anti-glyceraldehyde 3-phosphate dehydrogenase (total loading control), and anti-TATA-binding protein (nuclear loading control; Santa Cruz Biotech, Santa Cruz, CA) for 1 h. After several washes in PBS-Tween buffer, membranes were incubated with HP-conjugated secondary antibodies. The ECL Plus Western Blotting Detection System was used for protein detection (GE Healthcare, Buckinghamshire, UK).

RNA Isolation

Cells were pelleted down and lysed using 1 ml Trizol reagent (Invitrogen) and total RNA was isolated following manufacturer's protocol. RNA was resuspended in RNase-free water and traces of DNA were removed using the Turbo DNase-free kit (Ambion, Austin, TX).

Quantitative Real-Time RT-PCR

Quantitative real-time reverse transcription-PCR (qRT-PCR) was performed on the Mx4000 QPCR system (Stratagene, La Jolla, CA) with the QuantiTect SYBR green RT-PCR kit (Qiagen, Mississauga, ON, Canada) as previously described (28). Gene-specific primers for SYBR green detection of the GR, IPO13, and glyceraldehyde 3-phosphate dehydrogenase were designed. Synthesis of cDNA was prepared from an initial 200 ng/ml of RNA. RNA was incubated at 65 °C for 5 min with 1 mg/ml of random primers and 10 mmol/l dNTPs. To this, 5× first-strand buffer, 1 mmol/l DTT, 1 ml of RNase OUT, and 1 ml of Superscript II (Invitrogen) was added and incubated at 42 °C for 1 h and at 70 °C for 15 min. qRT-PCR was performed in 25 ml reactions for 40 cycles with 1 µl of cDNA. Results were analyzed using the delta-delta cycle threshold method.

Cytospins and Immunofluorescence

An aliquot of cell suspension was taken at each time point and cells were pelleted down. Cells were then resuspended in cold saline, cytospin slides (Cytospin 4; Shandon, Pittsburgh, PA) prepared, air dried, and fixed. For immunofluorescence experiments, slides were rehydrated through a series of decreasing ethanol washes, rinsed with PBS-0.03% Triton, and incubated in warm 10 mmol/l sodium citrate for antigen retrieval. Slides were then incubated in H₂O₂ and methanol for 20 min to block endogenous peroxidase activity. To block nonspecific binding, slides were incubated in PBS-0.03% Triton containing 5% normal goat serum and 1% bovine serum albumin. GR primary antibody (Santa Cruz Biotech) was incubated overnight at 4 °C and the following day in corresponding fluorescent-conjugated secondary antibody for 30 min at room temperature. Slides were washed with PBS-0.03% Triton and mounted with pro-long anti-fade media containing 4',6-diamidino-2-phenylindole (Invitrogen).

Statistical Analysis

All results are presented as mean ± SEM. Statistical significance was determined by Student *t*-test or two-way ANOVA followed by pair-wise group comparisons using Student-Neuman-Keuls test. Correlations were analyzed by Pearson Product-Moment Correlation. All statistical analyses were conducted using SigmaPlot-Systat Software. Significance was defined as *P* < 0.05.

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