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Dose- and time-dependent glucocorticoid receptor signaling in podocytes

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Guess A, Agrawal S, Wei C, Ransom RF, Benndorf R, Smoyer WE. Dose- and time-dependent glucocorticoid receptor signaling in podocytes. *Am J Physiol Renal Physiol* 299: F845–F853, 2010. First published July 14, 2010; doi:10.1152/ajprenal.00161.2010.—Glucocorticoids (GC) are the primary therapy for idiopathic nephrotic syndrome (NS). Recent evidence has identified glomerular podocytes as a potential site of GC action in this disease. The objectives of this study were to determine the presence of key components of the glucocorticoid receptor (GR) complex and the functionality of this signaling pathway in podocytes and to explore potential opportunities for manipulation of GC responsiveness. Here, we show that cultured murine podocytes express key components of the GR complex, including the GR, heat shock protein 90, and the immunophilins FKBP51 and FKBP52. The functionality of GR-mediated signaling was verified by measuring several GC (dexamethasone)-induced responses, including 1) increases in mRNA and protein levels of selected GC-regulated genes (FKBP51, phenol sulfotransferase 1, α B-crystallin); 2) downregulation of the GR protein; 3) increased phosphorylation of the GR; and 4) translocation of the GR into the nuclear fraction. Dexamethasone-induced phosphorylation and downregulation of GR protein were also demonstrated in isolated rat glomeruli. Podocyte gene expression in response to dexamethasone was regulated at both the transcriptional and posttranscriptional levels, the latter also including protein degradation. Short-term, high-dose GC treatment resulted in similar changes in gene expression and GR phosphorylation to that of long-term, low-dose GC treatment, thus providing a molecular rationale for the known efficacy of pulse GC therapy in NS. Induction of FKBP51 and downregulation of the GR represent negative feedback mechanisms that can potentially be exploited to improve clinical GC efficacy. Collectively, these findings demonstrate the presence of key molecular components of the GR signaling pathway and its functionality in podocytes and identify novel opportunities for improving clinical GC efficacy in the treatment of NS.

nephrotic syndrome; dexamethasone; FKBP51

NEPHROTIC SYNDROME (NS) is among the most common kidney diseases seen in children and adults. The most commonly used treatment for NS is oral glucocorticoids (GC), which effectively induce remission in the majority of children. However, GC have severe side effects, and ~20% of patients develop or present with resistance to GC therapy (9, 12). Despite their frequent use, neither the target cells nor the mechanism of action of GC in inducing remission in NS have been clearly identified. In addition, the molecular basis for the development of clinical resistance to GC therapy is unclear in the majority of patients.

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The known anti-inflammatory and immunosuppressive activities of GC have historically been taken as indirect evidence, suggesting that their mechanism of action in NS involves inhibition of soluble mediators released by T lymphocytes. However, recent evidence has suggested that glomerular podocytes may also be a direct target of GC in NS. An *in vivo* histological study reported that podocytes express the glucocorticoid receptor (GR), with its translocation into the nucleus following GC treatment, a prerequisite for GR-mediated signaling (43). More recently, *in vitro* studies have reported that GC protect cultured podocytes against injury via actin filament stabilization and prevent apoptosis induced by puromycin aminonucleoside (PAN), confirming direct effects on podocytes (22, 36). Functional responses to GC in cultured murine and human podocytes have also been suggested by expression analyses reporting several GC-regulated genes (23, 42). However, only recently has GR expression been verified in cultured murine and human podocytes by RT-PCR and Western blotting, respectively (35, 42). Aside from these reports, little else is known about GR-mediated signaling in podocytes or how abnormalities in this pathway in podocytes might contribute to clinical steroid resistance in NS.

Treatment of NS typically involves sustained administration of GC orally. However, high-dose intravenous GC pulse therapy has also been used with success in the treatment of NS, especially in children refractory to the standard sustained oral therapy (6, 16, 27, 33). This GC pulse therapy was reported to be reasonably effective and safe, with less cumulative toxicity than sustained oral treatment at lower dosages.

The molecular actions of GC include genomic and various nongenomic mechanisms. The canonical genomic mechanism involves the activation of the GR, which must be bound to the heat shock protein and chaperone HSP90 to keep the GR in a high-affinity steroid-binding conformation (21). This GR complex contains a number of additional components, including the HSP90-binding immunophilins (typically FKBP51 or FKBP52), which are critical for regulating GR activity. For genomic mechanisms, it usually takes hours or days before the responses of cells or tissues become evident. Nongenomic effects may also be mediated by the GR, or may be independent of the GR (15, 27, 30, 31). Additionally, nongenomic mechanisms may modulate the activity of the GR. Examples of this include the proteasome-mediated degradation or phosphorylation of the GR (11, 37). Some nongenomic effects of GC (e.g., modification by phosphorylation) take only minutes to hours to become evident.

The objectives of this study were to determine the presence of key components of the GR complex and the functionality of this signaling pathway in podocytes in response to pharmacological concentrations of GC. Additionally, the molecular responses of podocytes to selected GC treatments designed to

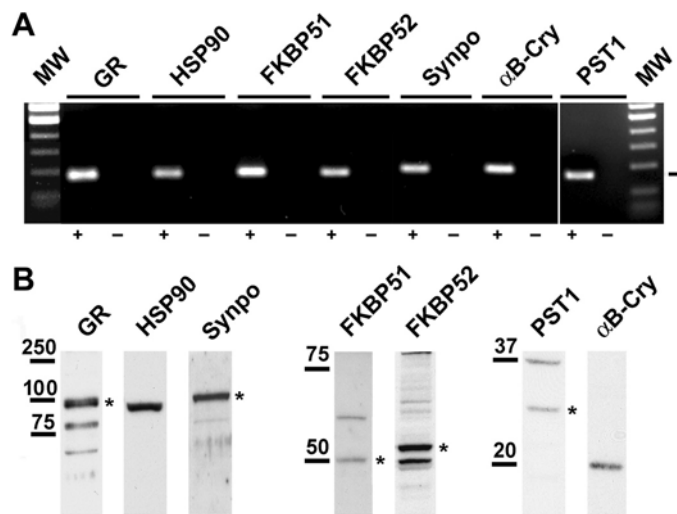


Fig. 1. Expression of selected genes in cultured podocytes. A: RT-PCR demonstrated the mRNA expression of key members of the glucocorticoid receptor (GR) complex, glucocorticoid-regulated genes and of the podocyte differentiation marker synaptopodin (Synpo). +/-, Presence or absence of the reverse transcriptase in RT-PCR. PCR primers were designed to produce amplicons of ~200 bp (bar). B: SDS-PAGE/Western blotting demonstrated the expression of corresponding proteins in podocytes. The relevant bands are labeled with asterisks. The positions of molecular mass markers are indicated on the left of each group of blots (in kDa).

mimic sustained oral vs. pulse intravenous GC treatments of patients were compared.

MATERIALS AND METHODS

Podocyte culture and treatments. The conditionally immortalized mouse podocyte cell line MPC-5 was cultured as previously described (19, 28). Briefly, podocytes were grown at 33°C with 100% relative humidity and 5% CO₂ atmosphere in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml glutamine (GIBCO, Carlsbad, CA), and 10 U/ml mouse γ -interferon (Sigma, St. Louis, MO) in cell culture flasks (Greiner, Monroe, NC) coated with rat tail collagen type I (BD Biosciences, Bedford, MA).

For differentiation, podocytes were trypsinized and 12,000 cells/well were seeded into collagen-coated six-well culture plates (Greiner) containing the same medium without γ -interferon. Differentiation was induced by transferring the cells to 37°C. Cells were allowed to differentiate for 10–14 days. Twenty-four hours before dexamethasone (Dex) treatments, podocytes were incubated in RPMI 1640 medium supplemented with charcoal-stripped fetal bovine serum (GIBCO). Cells were treated for either 1 h (short-term) or up to 5 days (long-term) with 1, 10, or 100 µM Dex, 20 µM RU486 (both

from Sigma), or with DMSO (vehicle control) as indicated, and gene expression was monitored for up to 5 days. Cells were pretreated with RU486 for 30 min before select Dex treatments.

RNA extraction and RT-PCR. Total RNA was extracted from podocyte cultures using TRIzol reagent (Invitrogen, Carlsbad, CA) followed by RNeasy (Qiagen, Germantown, MD) column purification as specified by the manufacturers. Purity and yield were determined by measuring the absorbance at 260 and 280 nm. cDNA was synthesized from 1 µg total RNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Negative control reactions contained no reverse transcriptase. Subsequently, the gene expression in podocytes as specified in Fig. 1A and Table 1 was determined by RT-PCR using the MyCycler thermal cycler (Bio-Rad). The reaction mixture contained 12.5 µl HotStart Plus Taq polymerase Master Mix (Qiagen), 0.5 µM of each primer, 0.5 µl template cDNA, and PCR-grade water in a total volume of 25 µl. The nucleotide sequences of the primers used are given in Table 1. Thermal cycling conditions consisted of 1 cycle at 95°C for 5 min, 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 20 s, and a final elongation cycle at 72°C for 10 min. PCR products were resolved on 1.5% agarose gels according to standard procedures and visualized under a UV transilluminator.

Expression of mRNAs was measured by quantitative RT-PCR (qRT-PCR) using an iQ5 thermal cycler (Bio-Rad). The PCR reaction mix contained 12.5 µl iQ SYBR-Green Supermix (Bio-Rad), 0.5 µM of each primer, 0.5 µl cDNA, and PCR-grade water in a total volume of 25 µl. Thermal cycling conditions consisted of 1 cycle at 95°C for 3 min, 40 cycles at 95°C for 10 s and 55°C for 10 s, and a final elongation cycle at 72°C for 10 min, followed by melting curve analysis. Standard curves for each PCR run were plotted using three to four serial dilutions of selected cDNA samples. Each sample was analyzed in triplicate in individual assays performed on two or more occasions. Results were normalized to the housekeeping gene β -actin and expressed as fold-change (means \pm SD) relative to time-matched vehicle control treatments (20). Statistical significance was determined by two-way ANOVA with the Bonferroni/Dunnett post hoc test. Probability values were considered significant at $P < 0.05$ and indicated by asterisks or plus signs, as indicated in the figure legends.

Electrophoresis, Western blotting, and densitometry. Proteins were extracted with buffer A (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; protease and phosphatase inhibitor cocktails from Sigma) from treated podocyte cultures at the times indicated. After the protein concentrations were measured using the Bradford assay (Bio-Rad), β -mercaptoethanol and bromophenol blue were added yielding 2 and 0.01% final concentrations, respectively. Fifteen micrograms of total protein was loaded in each lane of 10% SDS-PAGE gels. After the run, proteins were transferred to nitrocellulose membranes and visualized with specific primary and secondary antibodies. The following primary antibodies were used at the indicated dilutions in 5% nonfat milk powder in 0.1% Tween 20/PBS: rabbit polyclonal anti-GR (dilution 1:200), goat polyclonal anti-FKBP51 (dilution 1:200), goat polyclonal anti-lamin B1 (dilution 1:200), and rabbit polyclonal anti- α -synaptopodin (1:200), all from Santa Cruz Biotechnology, Santa

Table 1. Genes, transcripts, proteins, and primers as analyzed or used in this study

Gene	Transcript	Protein/Enzyme Name (IUPAC-IUB Classification)	Forward Primer	Reverse Primer	Amplicon Size, bp
<i>Nr3c1</i>	NM_008173	Glucocorticoid receptor	5'-gctgacgtgtggaagctgta	5'-tcgagcttccaggttcattc	169
<i>Hsp90aa1</i>	NM_010480	Hsp90 α	5'-ggcaagaccttggtgtctgt	5'-gatgtcaccagtcgggttga	173
<i>Fkbp5</i>	NM_010220	FKBP51, FKBP5 (EC 5.2.1.8)	5'-cttgaccaccgctatggttt	5'-tcccttctctttcaccgatgg	171
<i>Fkbp4</i>	NM_010219	FKBP52, FKBP4 (EC 5.2.1.8)	5'-aaggagagcattccatcgtg	5'-gttgctctgctccagcttct	176
<i>HspB5</i>	NM_009964	α B-Crystallin, HspB5	5'-ttctctggagagaccctgttg	5'-ccccagaaccttgactttga	207
<i>Sult1a1</i>	NM_133670	Phenol sulfotransferase 1 (EC 2.8.2.1)	5'-agacgcactcaccctgttct	5'-agttagccatgggttctcc	172
<i>Synpo</i>	NM_177340	Synaptopodin	5'-tccttctccaccgggaat	5'-agggggacattggtggag	199
<i>Actb</i>	NM_007393	β -Actin	5'-cttggtgatggaatcctgtgg	5'-gtacttgcctcaggaggag	214

IUPAC-IUB classification for enzymes is indicated where applicable.

Cruz, CA; rabbit polyclonal anti-phospho (Ser211) GR (dilution 1:1,000), and rabbit polyclonal anti-Hsp90 (dilution 1:1,000), both from Cell Signaling Technology, Danvers, MA; mouse monoclonal anti-FKBP52 (dilution 1:1,000, Hi52C, kind gift from Dr. M. Cox, El Paso, TX); rabbit polyclonal anti-phenol sulfotransferase 1 (PST1; dilution 1:500, Abcam, Cambridge, MA); rabbit polyclonal anti- α B-crystallin (α B-Cry; 1:5,000, Assay Designs, Ann Arbor, MI); and mouse monoclonal anti-GAPDH (1:10,000, Millipore, Billerica, MA). Secondary antibody solutions contained either peroxidase-conjugated goat anti-rabbit (dilution 1:10,000), goat anti-mouse (dilution 1:10,000), or donkey anti-goat IgG antibodies (dilution 1:10,000, all from Jackson ImmunoResearch Laboratories, West Grove, PA). Antibody binding was visualized with the ECL chemiluminescence system (GE Healthcare Bio-Sciences, Piscataway, NJ) and detected by exposure to X-ray film.

Protein expression experiments were performed at least three times, and Western blots of replicate samples were performed at least twice for each experiment. X-ray films were scanned using a calibrated ArtixScan M1 transillumination scanner (Microtek Lab, Cerritos, CA) controlled by the ScanWizard Pro program (version 7.042) using standard settings. Densitometric analysis of the integrated band density was performed using ImageJ (version 1.39; standard settings), which is available at <http://rsb.info.nih.gov/ij/>. Equal loading of protein samples of each sample set was according to the GAPDH signal obtained on the Western blots, resulting in essentially invariable GAPDH signals (not shown). All densitometry graphs show time-dependent changes in the amount of the specified proteins relative to the *time 0* controls.

Cell fractionation. Harvested podocytes were processed for fractionation into cytosolic and nuclear fractions using the NE-PER kit, according to the manufacturer's instructions (Thermo Scientific, Rockford, IL).

Isolation, incubation, and analysis of glomeruli. Four kidneys from two female rats (~200 g) were harvested (Institutional Animal Care and Use Committee approval AR07-00057), and glomeruli were isolated under sterile conditions as previously described (28). Approximately 100,000 glomeruli were collected, which is consistent with the determined number of >20,000 glomeruli/rat kidney (17). Preparations were assessed by microscopic inspection and routinely contained ~95% glomeruli. For incubation, ~18,000 glomeruli were resuspended in 500 μ l DMEM/F12 (1:1)/HEPES medium supplemented with 8% charcoal-stripped FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.29 mg/ml glutamine (GIBCO), and incubated in microcentrifuge tubes with perforated lids at 37°C in a 5% CO₂ humidified atmosphere.

Following 1-h preincubation, isolated glomeruli were treated with either 1 or 100 μ M Dex for long-term (24 h) or short-term (1 h) exposures, respectively. Controls were treated identically with the vehicle only. Following 1-h short-term treatment, the glomeruli were washed twice in PBS and incubation was continued in steroid-free medium. After 24-h incubation, glomeruli were harvested by pelleting and washing twice with PBS and then extracted in *buffer A* containing protease and phosphatase inhibitors. The glomeruli were disintegrated by three freeze-thaw cycles followed by sonication, and the resulting protein extract was analyzed by standard SDS-PAGE/Western blotting after boiling in the presence of 1% β -mercaptoethanol.

RESULTS

Podocytes express key components of the GR complex and are responsive to GC. We confirmed by RT-PCR using specific primers (Table 1) that mRNAs encoding key components of the

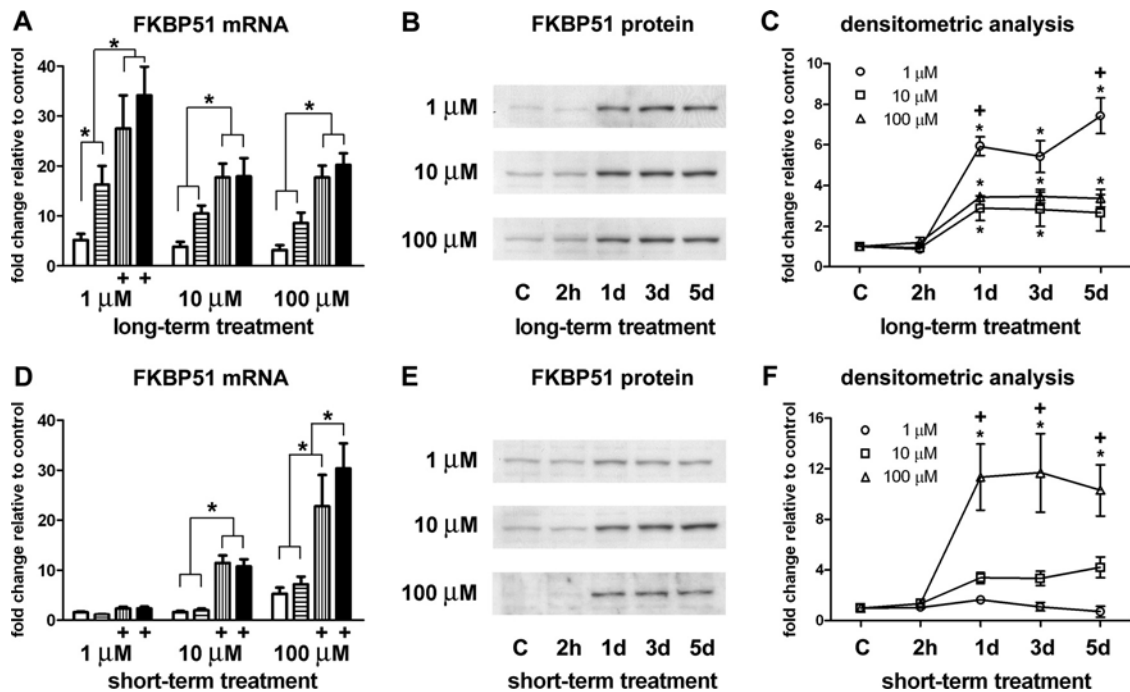


Fig. 2. Induction of FKBP51 following dexamethasone (Dex) treatment. **A:** qRT-PCR demonstrated that long-term treatments with each concentration of Dex strongly induced FKBP51 mRNA, with 1 μ M Dex being most effective. **B and C:** Western blotting demonstrated the accumulation of FKBP51 protein after 1 day of each long-term Dex treatment. **D:** qRT-PCR demonstrated that 10 and 100 μ M Dex short-term treatments induced FKBP51 mRNA after 3 days. **E and F:** Western blotting demonstrated the accumulation of FKBP51 protein after 1 day short-term treatment, with 10 and 100 μ M Dex being most effective. Note that in controls and, in some conditions (1 μ M Dex, short-term treatment), the expression of FKBP51 was low and at or below its detection limit, which caused some variability not reflecting actual changes. White bars, analysis at 2 h; horizontally striated bars, analysis at 1 day; vertically striated bars, analysis at 3 days; black bars, analysis at 5 days. Asterisks (\star) indicate significant differences between samples taken at different times, either between the specified groups (A and D) or compared with untreated control cells (C and F). Plus signs (+) indicate significant differences to the corresponding samples of both other sample groups, taken at the same time. Detection of GAPDH served as a loading control (data not shown; B and E).

GR complex (GR, HSP90, FKBP51, FKBP52) were present in extracts of differentiated podocytes (Fig. 1A). In addition, we demonstrated the presence of mRNA encoding synaptopodin, a marker of podocyte differentiation (18, 44). We also demonstrated the expression of genes known to be regulated by GC in rodents and/or primates which served as reporters for GC signaling, including the phenol sulfotransferase 1 (PST1) and the small heat shock protein α B-Cry, in addition to FKBP51 and the GR themselves (1, 4, 34, 41). The expression of corresponding proteins was confirmed by SDS-PAGE followed by Western blotting (Fig. 1B). The names of all genes and proteins, their GenBank accession numbers, and IUPAC-IUB classifications (if applicable) as analyzed in this study are given in Table 1.

In summary, differentiated podocytes contain key elements of the GR-mediated signaling pathway, a precondition for the cellular response to this class of steroids. Additionally, they express genes that are known to be regulated by GC.

GC induce changes in expression of selected genes in podocytes. The effects of GC on the expression of FKBP51, PST1, α B-Cry, and the GR were studied in greater detail at the mRNA and protein levels, using qRT-PCR and SDS-PAGE/Western blotting, respectively. Gene expression was measured in podocytes treated with three different concentrations of the synthetic GC Dex. The Dex concentrations were selected either to be consistent with clinical doses of GC as used in the standard treatment of glomerular diseases (1 μ M) or to mimic supra-pharmacological doses as used in pulse steroid treatment (100 μ M) (27, 42). The Dex concentration of 1 μ M falls within the range of prednisolone concentrations found in the serum of patients treated with doses of 0.5–2 mg \cdot kg⁻¹ \cdot day⁻¹, which are typical doses for the clinical treatment of nephrotic syndrome

(22, 5). High-dose intravenous pulse methylprednisolone therapy delivers \sim 100 times more steroids in 1 h and can produce serum levels of 60–200 μ M (26). Cells were treated with Dex using either long-term (Dex remains in the medium throughout the treatment period) or short-term exposure (1 h Dex treatment followed by washing and incubation in steroid-free medium). In both settings, samples were analyzed for gene and protein expression 2 h, 1 day, 3 days, and 5 days after the start of treatment.

Long-term Dex treatments markedly induced FKBP51 and PST1 mRNAs at all concentrations in a time-dependent manner, with 1 μ M Dex inducing the strongest response (\sim 35-fold at 5 days; Figs. 2A and 3A). Surprisingly, short-term treatment resulted in a similarly strong induction of FKBP51 and PST1 mRNAs at 100 μ M Dex, while lower Dex concentrations resulted in a moderate induction (10 μ M Dex) or had no effect (1 μ M Dex) (Figs. 2D and 3D). These mRNA results were reflected in the protein analyses for FKBP51, although the scale of induction was less pronounced. Similar to the gene expression results, long-term treatments resulted in increased amounts of FKBP51 protein, in which 1 μ M Dex achieved a significantly greater induction than 10 or 100 μ M (Fig. 2B–C). Short-term treatments also resulted in pronounced increases in FKBP51 protein, with 100 μ M Dex being significantly more effective than 1 or 10 μ M (Fig. 2, E and F). The upregulation of PST1 mRNA was not reflected in the protein analysis. A weak induction of PST1 protein was observed, with significance established after 5 days of 100 μ M Dex for both long- and short-term treatments (Fig. 3, B, C, E, and F). The strong and sustained induction of FKBP51 and PST1 mRNAs and proteins (FKBP51 only) by 100 μ M Dex several days after its

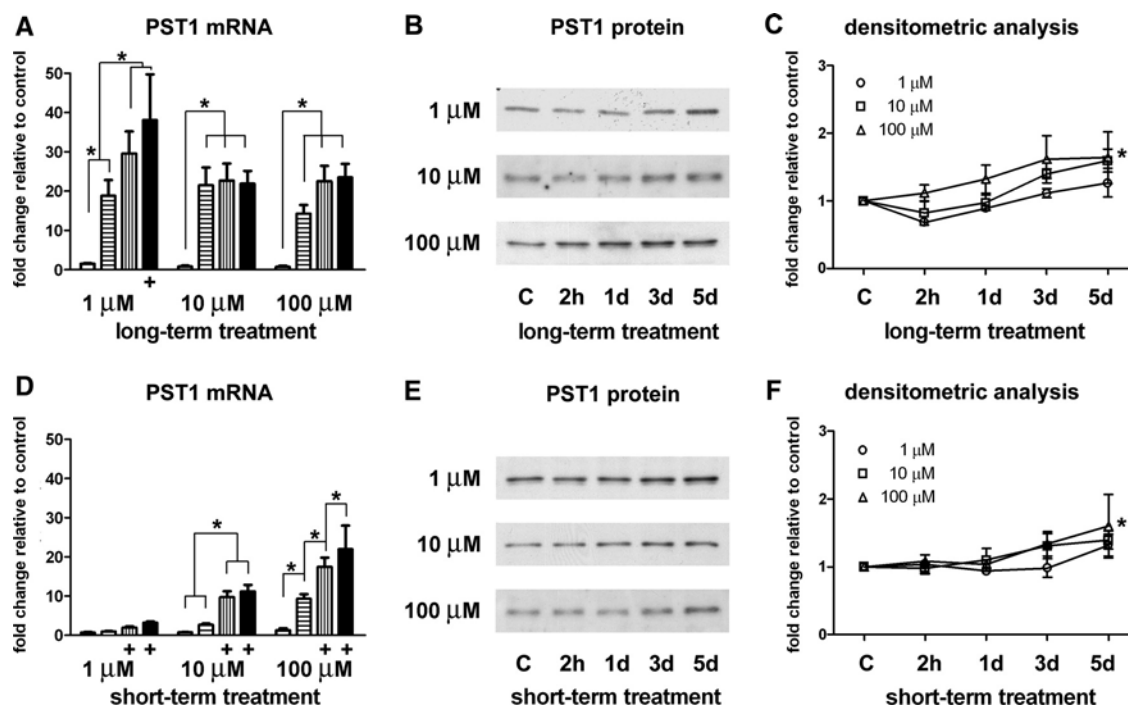


Fig. 3. Induction of phenol sulfotransferase 1 (PST1) following Dex treatment. *A*: qRT-PCR demonstrated that long-term treatment with each concentration of Dex strongly induced PST1 mRNA with 1 μ M Dex being most effective. *B* and *C*: Western blotting demonstrated the accumulation of PST1 protein after 3 days of each long-term Dex treatment. *D*: qRT-PCR demonstrated that 10 and 100 μ M Dex short-term treatments induced PST1 mRNA after 3 days. *E* and *F*: Western blotting demonstrated the accumulation of PST1 protein after 3 days of 100 μ M Dex short-term treatment. The fills of the columns and the use of the indicators for statistical significance are as in Fig. 2. Detection of GAPDH served as a loading control (data not shown; *B* and *E*).

removal was an unexpected finding and suggested that similar expression profiles can result from very disparate exposures to GC. Induction of FKBP51 represents a negative feedback mechanism of GC signaling which can potentially be exploited to improve the efficacy of GC treatment of patients with NS (cf. DISCUSSION).

In contrast to FKBP51 and PST1, induction of α B-Cry mRNA by Dex was less pronounced (maximal \sim 2-fold increase), albeit a slight increase in mRNA abundance was detectable within 5 days in all tested conditions (Fig. 4, *A* and *D*). Surprisingly, a significant induction of α B-Cry was detected at the protein level after 3 days of 1 μ M Dex long-term (Fig. 4, *B* and *C*) and 100 μ M Dex short-term treatments, with each concentration being significantly more effective than the other two, respectively (Fig. 4, *E* and *F*).

To assess the specificity of the induction of FKBP51, PST1, and α B-Cry the expression of several other genes with relevance for GC signaling was determined, including the GR, HSP90, and FKBP52. Based on the most effective conditions identified in Figs. 2–4, we elected to compare long-term, low-dose and short-term, high-dose Dex treatments. At the mRNA level, no significant changes were detectable after 3 days with 1 μ M Dex long-term or 100 μ M Dex short-term treatments for all three genes (Fig. 5, *A* and *D*). Consistent with this, the protein levels of HSP90 and FKBP52 did not change detectably (Fig. 5, *B* and *E*).

The GR protein level in podocyte extracts, however, decreased significantly after 1 day of either treatment (Fig. 5, *B*, *C*, *E*, and *F*), despite the constant mRNA level. This down-regulation of the GR apparently represents another negative

feedback mechanism regulating GC signaling which could potentially be exploited to improve the efficacy of GC treatment (cf. DISCUSSION). The activity of the GR is also regulated by phosphorylation at various sites. In the mouse sequence, phosphorylation at Ser220 in particular is thought to be important for GR activity (11). Phosphorylation at this site significantly increased after 2 h of either treatment and returned to baseline levels thereafter (Fig. 5, *B*, *C*, *E*, and *F*). Taking into account the decreasing amount of total GR, the fraction of phosphorylated GR clearly increased following Dex treatment. Thus two obviously opposing effects of regulation of GR activity appear to interfere in response to Dex treatment.

Involvement of the GR in response to Dex. The cellular response to GC is mediated through at least two distinct pathways; genomic events involving the GR and nongenomic, GR-independent events (21, 30). To determine whether the above podocyte responses were GR dependent, the mRNA and protein expression of both FKBP51 and PST1 were measured after 3 days of Dex treatment in the presence or absence of 20 μ M RU486, a competitive GR antagonist (13). Induction of FKBP51 mRNA and protein by either 1 μ M Dex long-term treatment or 100 μ M Dex short-term treatment was effectively inhibited by RU486 (Fig. 6, *A* and *C*, *top*). The fact that 20 μ M RU486 significantly blocked the effects of 100 μ M Dex is consistent with its higher association constant for binding to the GR than that of Dex (10).

Induction of PST1 mRNA by 1 μ M Dex long-term treatment was also profoundly inhibited by RU486, whereas its induction by 100 μ M Dex short-term treatment was only moderately affected by this drug (Fig. 6*B*). At the protein level, the effect

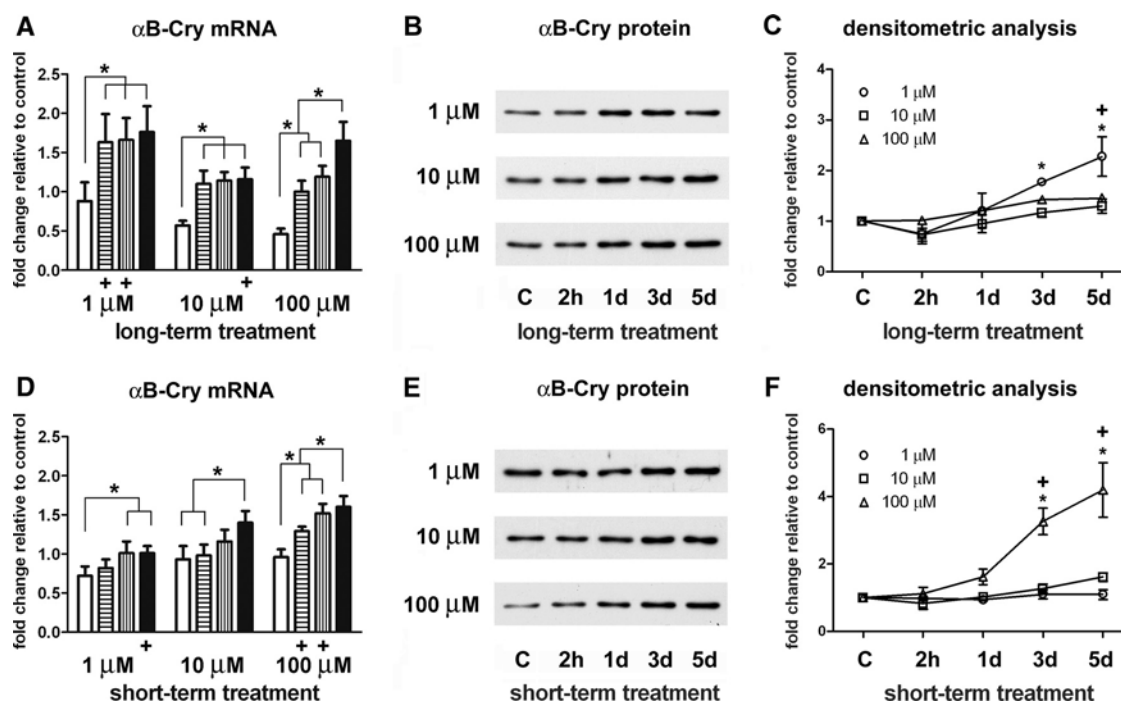


Fig. 4. Induction of α B-crystallin (α B-Cry) following Dex treatment. *A*: qRT-PCR demonstrated that long-term treatment with each concentration of Dex moderately induced α B-Cry mRNA within 5 days approximately to the same extent. *B* and *C*: Western blotting demonstrated that the accumulation of α B-Cry protein was apparent after 3 days of 1 μ M Dex long-term treatment. *D*: qRT-PCR demonstrated that short-term treatments with 10 and 100 μ M Dex slightly induced α B-Cry mRNA within 5 days. *E* and *F*: Western blotting demonstrated that the accumulation of α B-Cry protein was apparent after 3 days of 100 μ M Dex short-term treatment. The fills of the columns and the use of the indicators for statistical significance are as in Fig. 2. Detection of GAPDH served as loading control (data not shown; *B* and *E*).

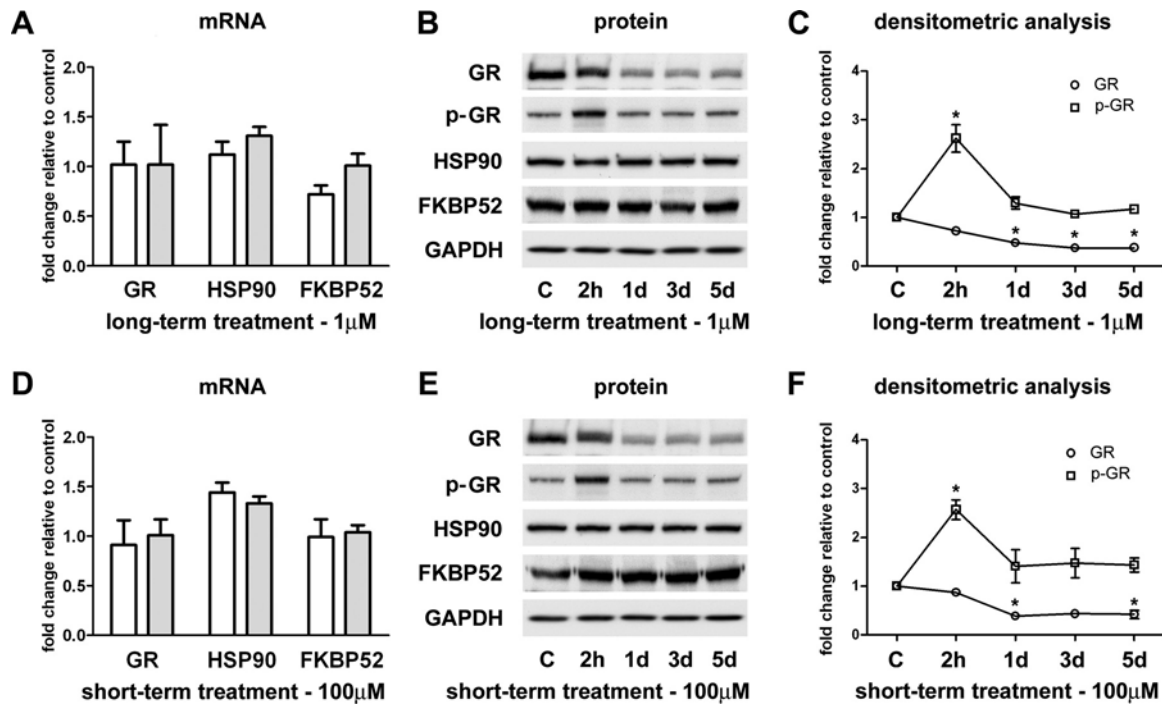


Fig. 5. Expression of GR, HSP90, and FKBP52 and phosphorylation of GR in podocytes following Dex treatment. *A*: qRT-PCR demonstrated that 1 μM Dex long-term treatment for 1 and 3 days had no major effect on mRNA expression. *B* and *C*: Western blotting demonstrated that 1 μM Dex long-term treatment resulted in reduced GR protein after 1 day, while increased phosphorylation of the GR was apparent after 2 h. HSP90 and FKBP52 remained constant. *D*: qRT-PCR demonstrated that 100 μM Dex short-term treatment for 1 and 3 days had no major effects on mRNA expression. *E* and *F*: Western blotting demonstrated that 100 μM Dex short-term treatment for 2 h through 5 days resulted in reduced GR protein after 1 day, while increased phosphorylation of the GR was apparent after 2 h. HSP90 and FKBP52 remained constant. The use of the indicators for statistical significance (*C* and *F*) are as in Fig. 2. Detection of GAPDH served as a loading control (*B* and *E*). White bars, analysis at 1 day; gray bars, analysis at 3 days.

of RU486 on accumulation of PST1 was either minor (1 μM Dex long-term treatment) or nondetectable (100 μM Dex short-term treatment) (Fig. 6*C*, middle). These results suggest that PST1 is in part regulated posttranscriptionally and independently of the GR, in contrast to FKBP51, which is regulated by the GR at the transcriptional level.

Dex induces nuclear translocation of the GR. According to the canonical model, steroid receptors are activated in the cytoplasm by a HSP90-based assembly complex before they translocate into the nucleus and *trans*-activate gene expression (21). To verify activation of the GR in response to Dex at the level of translocation under different experimental conditions, differentiated podocytes were treated either long-term with 1 μM Dex, short-term with 100 μM Dex, or with vehicle alone. After 72 h, podocyte extracts were separated into cytosolic and

nuclear fractions. In control cells, we found the GR partitioned predominantly with the cytosolic fraction, while either long-term or short-term treatments with Dex caused the GR to partition predominantly with the nuclear fractions, with no major difference between either treatment (Fig. 7). Thus both long-term, low-dose and short-term, high-dose GC treatments activated the GR in podocytes to approximately the same extent.

Isolated rat glomeruli respond to GC. Finally, we examined whether the identified responses of cultured podocytes to Dex are also representative of intact glomeruli. Rat glomeruli were isolated as previously described (2, 29), with microscopic inspection used to confirm that the glomeruli were intact (representative example is shown in Fig. 8*A*). Following Dex treatment, isolated glomeruli revealed both downregulation

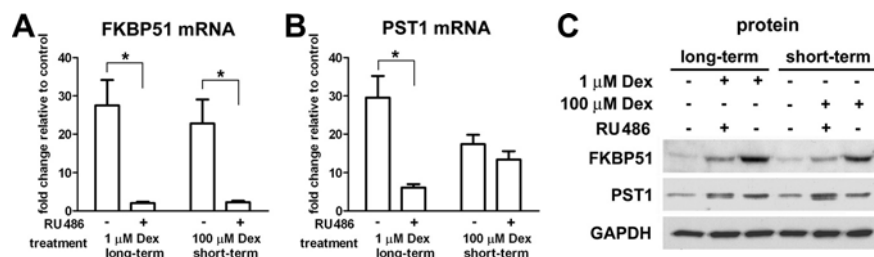


Fig. 6. Effect of RU486 on the expression of FKBP51 and PST1 in podocytes. *A*: qRT-PCR demonstrated significant inhibition by RU486 of the induction of FKBP51 mRNA after 3 days of either 1 μM Dex long-term or 100 μM Dex short-term treatments. *B*: qRT-PCR demonstrated that RU486 significantly reduced the induction of PST1 mRNA following 1 μM Dex long-term treatment, yet had only a minor effect on PST1 mRNA following 100 μM Dex short-term treatment. *C*: Western blotting demonstrated that RU486 significantly reduced accumulation of FKBP51 protein following 3 days of either treatment, yet had no effect on the accumulation of PST1 protein. Brackets with asterisks indicate significant differences (*A* and *B*). Detection of GAPDH served as a loading control (*C*).

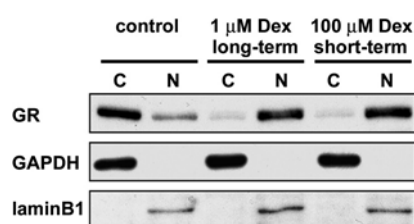


Fig. 7. Effect of Dex on the translocation of GR into the nuclear fraction. Seventy-two hours after 1 μ M Dex long-term or 100 μ M Dex short-term treatment of podocytes, the distribution of the GR shifted from a predominant cytosolic (C) to a nuclear (N) location. GAPDH and lamin-B1 served as markers for the cytosolic and nuclear fractions, respectively.

and increased phosphorylation of the GR protein after 1 day, both in short-term and long-term treatments (Fig. 8B), very similar to the responses seen in cultured podocytes. Given the limitations for incubation time related to the in vitro cultivation of glomeruli, the genomic responses, however, could not be determined.

Taken together, some of the molecular responses of cultured podocytes could be verified in isolated glomeruli, suggesting that the podocyte responses identified in this study may be representative of glomeruli and possibly also of intact kidneys.

DISCUSSION

In the current study, we established the presence of key components of the GR-mediated signaling pathway in cultured podocytes, including the GR, HSP90, and the immunophilins FKBP51 and FKBP52. Evidence of functional GC signaling in podocytes was also demonstrated by multiple GC-induced responses, including 1) upregulation of FKBP51, PST1, and α B-Cry; 2) downregulation of the GR; 3) temporary phosphorylation of the GR; and 4) translocation of the GR from the cytosolic to the nuclear fraction. Additional findings include the differential regulation of expression of the studied genes, the operation of negative feedback regulatory mechanisms of GC signaling (see below), and an unexpected similarity in the podocyte's response to GC following either short-term, high-dose treatment or long-term, low-dose treatment with Dex. Collectively, these findings identify novel opportunities for improving the clinical efficacy of GC in the treatment of patients with NS.

The cellular response to GC is remarkably complex. The canonical model of genomic GC action includes transformation, activation, and translocation of the GR into the nucleus, resulting in *trans*-activation of responsive genes (21). The complexity is increased by the existence of nongenomic GR-dependent and GR-independent mechanisms, by various modifications of the GR itself (including phosphorylation and sumoylation), and by regulated degradation of the GR (11, 15, 30, 31, 37). The findings of this study lend support to this concept of complex regulation of gene expression at different levels. At the mRNA level, expression of both FKBP51 and PST1 was strongly induced (>20-fold after 5 days), similar to previous reports in other experimental systems (4, 34, 14), while induction of α B-Cry at the mRNA level was only moderate in all settings (<2-fold). In contrast to the strong mRNA induction for FKBP51 and PST1, the accumulation of the corresponding proteins in response to GC was more moderate. Nonetheless, a pronounced accumulation of FKBP51 protein was observed with all Dex concentrations in the long-term treatments, and with 10 and 100 μ M in the short-term treatments.

Taken together, these findings suggest a complex and differentially regulated gene expression in podocytes following GC exposure.

At the mRNA level, short-term treatments exhibited clear concentration dependence on the induction of FKBP51 and PST1, with 100 μ M Dex inducing the strongest response. In contrast, long-term treatments did not exhibit such concentration dependence, with the lowest Dex concentration (1 μ M) inducing the strongest response. The reason for this unexpected response pattern is not known, although a number of explanations are plausible. First, activation of negative feedback mechanisms (e.g., downregulation of the GR or induction of FKBP51; see below) may occur during long-term exposures. Alternatively, in long-term treatments 1 μ M Dex may result in saturation of the GR, preventing a further increase in response to higher Dex concentrations. This would be distinct from the short-term treatments, in which 1 μ M Dex may not saturate the GR.

The observed partial insensitivity of PST1 mRNA induction to the GR antagonist RU486 in short-term treatments suggests the presence in podocytes of GC-induced signaling events that are independent of the GR. This response pattern identifies a difference between long- and short-term treatments, since in long-term treatment conditions PST1 mRNA induction was found to be largely GR dependent (although a minor GR-independent fraction of this response was also detectable). Interestingly, at the protein level, RU486 had no detectable effects following either treatment, despite the pronounced inhibition of PST1 mRNA synthesis in long-term treatment conditions. Apparently, PST1 expression in response to GC involves complex regulatory mechanisms which may include posttranscriptional regulation.

Dex also downregulated the expression of the podocyte GR itself. Although none of the treatments altered the GR mRNA level to a significant extent, the amount of GR protein decreased markedly after 1 day of either treatment, suggesting the activity of posttranscriptional processes such as GR degradation or diminished translation. Such posttranscriptionally autologous downregulation of the GR represents a negative feedback mechanism that has also been observed in other cells, e.g., in mouse embryonic fibroblasts, and for other genes, e.g., the cyclooxygenase-2 (15, 41).

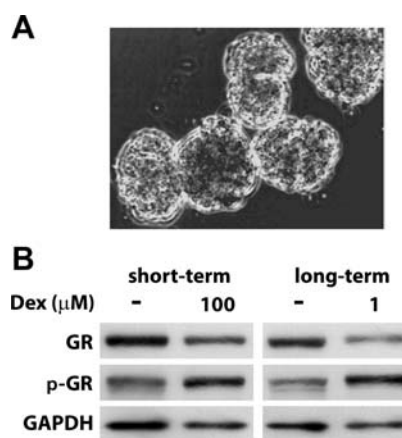


Fig. 8. Response of isolated rat glomeruli to Dex. A: image demonstrates integrity of isolated glomeruli; B: Western blotting of glomerular extracts demonstrated that both 1 μ M Dex long-term and 100 μ M Dex short-term treatment resulted in reduced GR protein and in its increased phosphorylation after 24 h. Detection of GAPDH served as a loading control (B).

Interestingly, maximal induction of FKBP51 and PST1 mRNA in response to short-term treatment (3 or 5 days) occurred despite the fact that the amount of the total GR protein was already reduced (Figs. 2, 3, and 5). This suggests the action of additional regulatory mechanisms. One candidate mechanism is the transient phosphorylation of the mouse GR at Ser220 (corresponds to human Ser211), which is thought to be related to activation of the GR (11). Phosphorylation at Ser220 peaked after 2 h of either treatment before it returned to baseline levels thereafter. While this transient phosphorylation alone is probably not sufficient to explain the continuous accumulation of FKBP51 and PST1 mRNA seen in the short-term treatment, it may well facilitate other regulatory events, e.g., the phosphorylation of the GR at other sites (11).

Taken together, the regulation of the four studied GC-responsive genes in podocytes was quite diverse, suggesting that podocytes possess specific and differentially regulated mechanisms for each of these genes. Moreover, the podocyte GR appears to be subject to opposing (positive and negative) regulatory mechanisms in response to GC treatment, involving both downregulation and phosphorylation. These responses were also verified in isolated glomeruli, suggesting that these mechanisms likely operate in vivo as well.

The large-molecular-mass immunophilins FKBP51 and FKBP52 are known to be involved in GR-mediated signaling, although their roles are not completely understood. They enter into GR complexes by directly binding to HSP90 in the cytoplasm, with both proteins competing for the same binding site. FKBP52 generally enhances the transcriptional activity of GR in both yeast and mammalian systems and is apparently a modulatory factor for a subset of GC-responsive genes (3, 25, 24, 41). This may be the result of FKBP52's ability to serve as a central linker to dynein motor complexes, thus enabling translocation of the GR complex into the nucleus (7). Despite their similarity, FKBP51 has very different functions in GR-mediated signaling than FKBP52. In the absence of the hormone, FKBP51 binds the GR complex and keeps it in an inactive form. Ligand binding to the GR induces switching of FKBP51 for FKBP52 in the GR complex and results in activation of the complex. Additionally, FKBP51 regulates the nuclear transport of the dominant-negative GR splice form β , which may contribute to decreased GR sensitivity (45). Thus induction of FKBP51 as shown in this study identifies another negative feedback mechanism in podocyte GR signaling, in addition to the downregulation of the GR itself. Interestingly, both negative feedback mechanisms were activated to a similar extent by both 1 μ M Dex long-term treatment and 100 μ M Dex short-term treatment. Of particular note, in New World primates elevated expression of the negative regulator FKBP51 has been associated with GC resistance (39). In addition, an intronic single nucleotide polymorphism in the human *FKBP5* gene has also been associated with increased protein expression, suggesting this might have relevance to some forms of human GC resistance (2). Downregulation of FKBP51 by siRNA in neurons has also been associated with increased baseline GR nuclear localization (indicating elevated GR activity), further suggesting that FKBP51 is a negative modulator of the cortisol-hypothalamic-pituitary-adrenal axis (32).

Pharmacological inactivation of such negative feedback mechanisms in podocytes may represent a novel more-targeted strategy to improve the clinical efficacy of GC therapy in

humans, as well as to overcome some forms of clinical steroid resistance in idiopathic NS. For example, the efficacy of GC in clinical practice could potentially be augmented by inhibition of degradation of the GR using proteasome inhibitors such as the FDA-approved drug bortezomib. Similarly, attenuation of either the expression or activity of FKBP51 may also be a useful strategy to improve the clinical efficacy of GC therapy. Of particular relevance to the clinical care of patients with GC resistance is that drugs such as FK506 (e.g., tacrolimus), which bind to FKBP51 and other "FKBPs" and inhibit their peptidyl-prolyl-isomerase activity, are already widely used for the treatment of patients with clinical GC resistance (38, 40). However, the effect of these drugs on GR signaling is not yet clear.

In summary, our data suggest that short-term, high-dose or long-term, low-dose GC treatment induces largely similar molecular responses in podocytes. These findings may thus provide a molecular rationale for the known clinical efficacy of intravenous pulse GC therapy in NS. In addition, the findings of induction of FKBP51 and downregulation of the GR in podocytes also represent negative feedback mechanisms that can potentially be exploited to improve clinical GC efficacy. Together, these findings demonstrate the presence and GC regulation of key molecular components of the GR signaling pathway in podocytes and identify novel opportunities for improving clinical GC efficacy in the treatment of NS.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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