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NOVEL HYPERACTIVE GLUCOCORTICOID RECEPTOR ISOFORM IDENTIFIED WITHIN A HUMAN POPULATION

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ABSTRACT—Glucocorticoids serve as important therapeutic agents in diseases of inflammation, but clinical use, especially in advanced septic shock, remains controversial because of the unpredictable response. Prior studies correlate human glucocorticoid receptor (hGR) isoforms with a decreased response to steroid therapy. Further analysis of additional hGR isoforms may improve the understanding of the steroid response. Ninety-seven human volunteers' blood samples were surveyed for hGR isoforms. An isoform matching National Center for Biotechnology Informatics (NCBI) hGRα (hGR NCBI) served as a reference. Two isoforms were of particular interest—one isoform had three nonsynonymous single-nucleotide polymorphisms (SNPs) (hGR NS-1), and the second had a single-nucleotide deletion (hGR DL-1) resulting in a truncated protein. Transactivation potentials were measured using a luciferase reporter assay. Human glucocorticoid receptor NS-1 had activity more than twice of hGR NCBI, whereas hGR DL-1 demonstrated less than 10% of the activity of hGR NCBI. Cotransfection of two isoforms revealed that the presence of hGR NS-1 increased transactivation potential, whereas hGR DL-1 decreased activity. Synthetic constructs isolating individual and paired SNPs of hGR NS-1 were created to identify the SNP responsible for hyperactivity. Transactivation studies revealed a SNP within the ligand-binding domain exerted the greatest influence over hyperactivity. In evaluating the response to hydrocortisone, hGR NCBI and hGR NS-1 displayed an increased dose-dependent response, but hGR NS-1 had a response more than twice hGR NCBI. Characterization of the novel hyperactive hGR NS-1 provides insight into a possible mechanism underlying the unpredictable response to steroid treatment.

KEYWORDS—Human glucocorticoid receptor, inflammation, stress response, steroids, adrenal insufficiency, sepsis

INTRODUCTION

Production of cortisol, the human endogenous glucocorticoid, is initiated by numerous stress signals. The brain recognizes stressors such as burn injury, trauma, and sepsis, which triggers the hypothalamic-pituitary-adrenal axis to produce cortisol. Cortisol then binds to the glucocorticoid receptor (GR) and alters the transcription of certain inflammatory mediators (1, 2). Similar stressors would be expected to produce comparable inflammatory responses. However, the stress response can be widely variable among different individuals even with the same inciting event—two siblings of similar age suffering the same total body surface area burn can have dramatically different outcomes. In fact, people who lack the ability to release or respond to cortisol in times of stress will rapidly die during extreme states of stress such as sepsis (3). Early studies failed to demonstrate any efficacy for treating septic patients with exogenous steroids, and the most recent evidence remains conflicted whether certain patients experiencing a massive inflammatory response from sepsis or trauma will benefit from exogenous steroids (4–8).

In addition to septic patients, those with proinflammatory conditions experience a variable response to steroid therapy. Some patients improve after receiving a standard dose of steroids in diseases such as asthma, inflammatory bowel disease, and rheumatoid arthritis, whereas others require much higher doses to achieve a similar effect or are simply resistant (9–14). Because glucocorticoid requires interaction with its receptor before an effect is seen, variations in GRs may contribute to stress and steroid responses. Previously, our laboratory demonstrated that varying lengths of CAG repeats found in the transactivation domain of the mouse GR between several mouse strains resulted in a difference in transactivation potential (15). These findings suggest that a simple polymorphism in the mouse GR transactivation domain significantly alters the activity of the receptor.

In humans, the GR gene resides on chromosome 5q31–32 (16). The gene consists of nine exons, of which exons 2 through 9 are coding sequences. The resultant human glucocorticoid receptor α (hGRα) protein is of 777 amino acids—the protein contains two transactivation domains, a DNA-binding domain, hinge region, and a ligand-binding domain (LBD) (17). Exon 9 can undergo alternative splicing leading to hGRα or hGRβ isoforms. The putative hGRβ protein is of 742 amino acids. The hGRα is the predominant active isoform in humans, whereas the hGRβ isoform has been identified as functionally deficient and implicated as a competitive inhibitor of the hGRα (18). In examining cell lines and pathologic tissue samples, other hGR splice variants and single-nucleotide polymorphisms (SNPs) have been identified and associated with human disease states refractory to glucocorticoid therapy (19–26). In this study, we identify novel hGR isoforms from human blood samples and characterize their activities

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TABLE 1. **Demographics of human subject study population (n = 97)**

Characteristics	
Age, mean (SD), y	40.2 (10.9)
Male-female ratio, n	27:70
Ethnicity, n	
White	51
African American	8
Hispanic	10
Asian	21
Other	7

to further understand the role of hGR isoforms in explaining the variable response to steroid therapy.

MATERIALS AND METHODS

Study population

This study was approved by the institutional review board of the University of California, Davis. Subjects with a history of major illness (diabetes mellitus, hypertension, chronic obstructive pulmonary disease, inflammatory bowel disease, autoimmune disease, and cancer), pregnant women, and individuals taking exogenous steroids were excluded from the study. The study cohort involved 97 volunteer human subjects—70 females and 27 males. Subject ages ranged from 20 to 67 years. About half of the subjects were white, one fourth were Asian, and the remaining were African American, Hispanic, or other (Table 1).

Identification and nomenclature of hGR isoforms

Total RNA was isolated from buffy coat samples using the RNeasy Miniprep Kit (Qiagen, Valencia, Calif). Reverse transcriptase–polymerase chain reaction using the Sensiscript RT Kit (Qiagen) was performed to amplify the hGR coding sequence in two sections (exons 2–3, forward 5′-tcactgatggactc caaag-3′, reverse 5′-aagcttcatcagagcacacc-3′; exon 3–9α, forward 5′-ccagcat gagaccagatgta-3′, reverse 5′-ttaaggcagtcacttttgatgaaac-3′). The fragments were cloned into pGEM-T Easy vector (Promega, Madison, Wis) and sequenced at MCLAB (South San Francisco, Calif). Polymorphisms were identified by comparison to the hGRα reference sequence (NM_001018077) from the National Center for Biotechnology Informatics (NCBI). The coding sequence fragments were then cut with restriction enzymes for recombination into a full-length coding sequence and cloned into a pcDNA4-HisMax vector (Invitrogen, Carlsbad, Calif) for functional analysis.

The isoforms have been named based on structure. The hGR NCBI designation represents an isoform derived from a human subject that matches the NCBI reference sequence for hGRα. The designation hGR NS-1 represents the three nonsynonymous (NS) SNPs in the isoform, and the name hGR DL-1 refers to a base pair deletion (DL) and resultant truncated protein. The number after the aforementioned abbreviations represents the order in which our laboratory has functionally evaluated the isoform (e.g., hGR NS-1 is the first NS isoform to be studied).

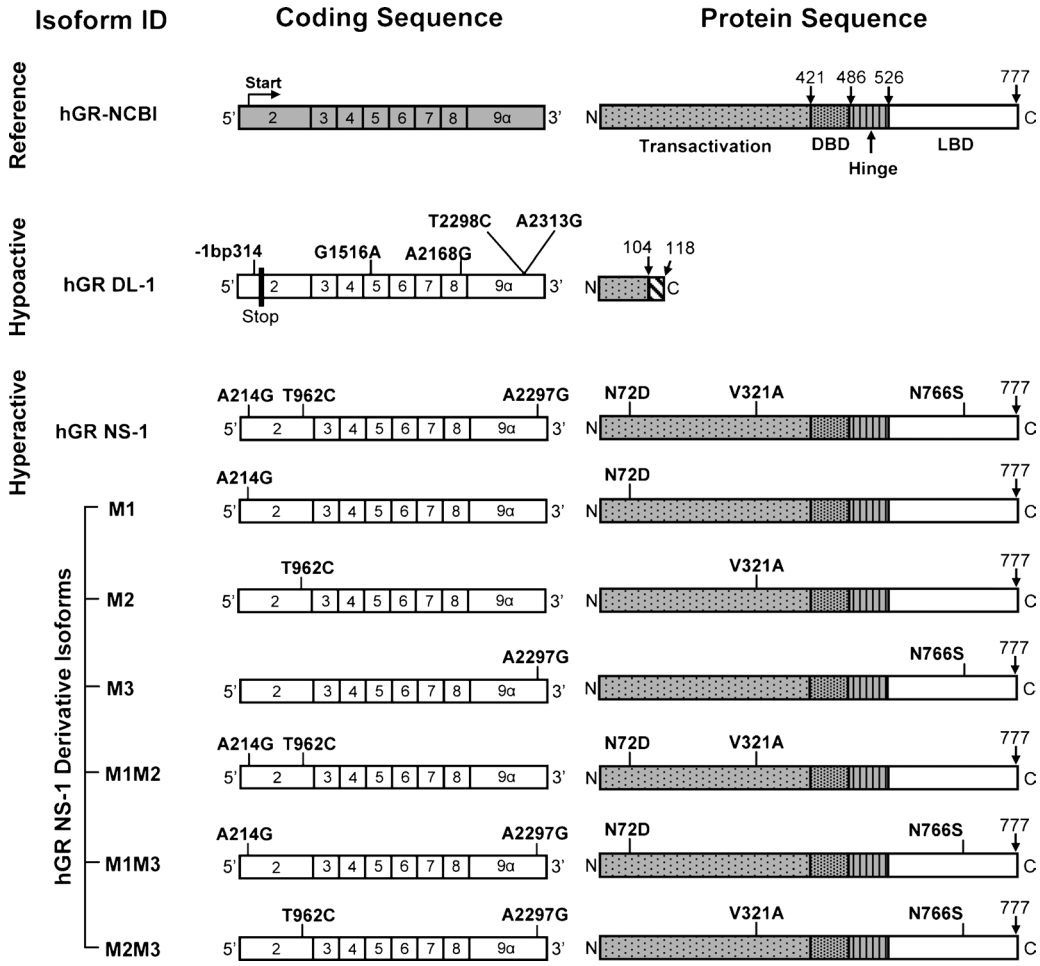

FIG. 1. **Coding sequence and protein structure of hGR isoforms**. The first column lists the identification label for each isoform (NCBI, reference hGRα sequence; DL, nucleotide deletion; NS, nonsynonymous change). The second column depicts the coding sequence for each hGR isoform. Single-nucleotide polymorphisms are shown above the respective hGR isoforms with the following SNP identifications: reference nucleotide, location in the coding sequence, new polymorphic nucleotide (i.e., A214G). Deletions are denoted with a minus sign, number of nucleotides deleted, and the nucleotide location of the 5′ end of the deletion (i.e., −1bp314). For hGR DL-1, the thick vertical bar denotes early termination. The third column shows the predicted protein sequences. Amino acid changes are labeled above the respective protein sequence using a single-letter amino acid code. The numbers above the hGR NCBI protein denote the domain boundaries. Diagonal shaded areas in the C-terminus of the protein sequence denote the sequence changes from hGR NCBI.

Construction of derivative isoforms of hGR NS-1

Human glucocorticoid receptor NS-1 contains three SNPs–A214G, T962C, and A2297G, from which six derivative hGR isoform constructs were created consisting of individual and paired SNPs (Fig. 1). These isoform constructs were created using a different reverse primer (5′-ccaaggactctcattcgtctc-3′) for exons 2–3 and a different forward primer (5′-tctgtccaaagcagtttcac-3′) for exons 3–9α.

Measurement of transactivation potentials of hGR isoforms

TSA201 cells (a HEK 293 cell subclone stably transfected with SV40 large T antigen) were transfected with either plasmids containing individual hGR isoforms (hGR NCBI, hGR NS-1, hGR DL-1, and hGR NS-1 derivatives) or plasmids of two separate hGR isoforms (for cotransfection experiments). For each transfection experiment, 20,000 cells were seeded in 100 μL of antibiotic-free Dulbecco's modified Eagle medium (Invitrogen) with fetal bovine serum (JR Scientific, Woodland, Calif) media in a 96-well plate. The plates were incubated at 37°C in 5% CO_2 atmosphere. Twenty-four hours after seeding, the cells were transfected with the appropriate hGR isoforms and a glucocorticoid response element (GRE)–luciferase reporter plasmid (PathDetect GRE Cis-Reporter Plasmid; Agilent Technologies, La Jolla, Calif) using Fugene 6 or Fugene HD (Roche, Indianapolis, Ind) per the manufacturer's protocol. Transcriptional activity was assessed 24 h after incubation using the Luciferase Assay Kit (Agilent Technologies). The luciferase activity was measured on the Perkin-Elmer MicroBeta TriLux (1 detector; Perkin-Elmer, Waltham, Mass).

For all hydrocortisone treatment experiments, cells transfected for 24 h with hGR NS-1 or hGR NCBI were treated with graded concentrations of hydrocortisone (0.000001–1 μM) in vehicle (0.9% saline) or vehicle alone for 24 h. Luciferase activity was assessed as described above.

Western blot analysis of hGR isoforms

TSA 201 cells transfected with recombinant hGR isoforms were lysed in ice-cold mild lysis buffer containing 1% Igepal CA-630, 0.15 M NaCl, 0.01 M $NaPO_4$ (pH 7.2), 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate, and 1 μg/mL aprotinin. Extracted protein was run on a 10% BioRad Criterion gel (Hercules, Calif). Separated proteins were transferred to a polyvinylidene fluoride Hybond-P membrane (GE Healthcare, Piscataway, NJ). Membranes were blocked with 5% nonfat dry milk, washed, and incubated overnight with 1:5,000 anti–HisG–horseradish peroxidase antibody (Invitrogen) at 4°C. The protein signal was visualized via chemiluminescence using the ECL Plus Western Blot Detection System (GE Healthcare).

Statistical analysis

All experiment samples were run in triplicate, and each experiment repeated more than three times. After multiple experiments confirmed the obtained data, a representative experiment was chosen for analysis. These results were compared by one-way ANOVA, and significance was confirmed with Tukey post hoc test.

RESULTS

Novel hGR isoforms and measured transactivation potentials

A multitude of SNPs were identified in our study population, and two novel isoforms (hGR NS-1 and hGR DL-1) were isolated from two separate blood samples and selected for further functional analysis. Interestingly, a majority of the blood samples revealed previously unreported isoforms; however, our small sample size precluded an adequate frequency analysis.

The early termination hGR isoform analyzed in this study, hGR DL-1, has 4 SNPs (G1516A, A2168G, T2298C, A2313G) and a base pair deletion at position 314, which leads to amino acid changes between positions 104 to 118 and an early termination at 118 (Fig. 1). This isoform lacks most of exon 2 and all of exons 3 through 9, which encode the transactivation domains, DNA-binding domain, hinge region, and LBD. *In vitro* functional assays of this truncated isoform had a greater than 90% decrease in transactivation potential when compared with hGR NCBI ($P < 0.01$) (Fig. 2A). The Western blot of hGR DL-1 confirmed the expression of a truncated protein (Fig. 2B).

Among the isoforms with SNPs, hGR NS-1 was identified as a unique isoform with consistently higher activity compared with hGR NCBI. This isoform contains three NS SNPs (A214G, T962C, and A2297G) corresponding to three amino acid changes at position 72 (asparagine to aspartic acid), position 321 (valine to alanine), and position 766 (asparagine to serine) (Fig. 1). The transactivation potential of hGR NS-1 was more than twice that of hGR NCBI ($P < 0.01$) (Fig. 2A). The protein expression of this isoform was also confirmed via Western blot (Fig. 2B).

The decreased activity of hGR DL-1 is partially counteracted by hGR NS-1

To simulate a heterozygous state, where two alleles are present in a single subject, isoforms hGR NS-1, hGR DL-1, and hGR NCBI were analyzed after cotransfection (Fig. 3). The cotransfection of hGR NCBI and the hyperactive hGR NS-1 caused an increase in transactivation potential when compared with hGR NCBI alone, but not to a statistically significant level. When hGR NCBI was cotransfected with the hypoactive hGR DL-1, transactivation potential was decreased greater than three times when compared with hGR NCBI alone. Finally, the activity of hGR DL-1 cotransfected with hGR NS-1 increased the activity greater than 45 times of hGR DL-1 alone; however, this cotransfection achieved only less than half of the activity as hGR NS-1 alone. Overall, the presence of hGR DL-1 decreased transcriptional activity, whereas hGR NS-1 tended to increase activity.

The SNP at position M3 exerts the greatest influence on hGR NS-1 hyperactivity

To define the contributions made by the three SNPs in hGR NS-1, each SNP was isolated into individual and paired constructs. The labels M1, M2, and M3 were used to denote the three SNPs in hGR NS-1 at positions 214, 962, and 2,297, respectively (Fig. 1). The transcriptional activities of the engineered hGR NS-1 "derivative isoforms" were all significantly lower than that of hGR NS-1. Interestingly, the M1 and M2

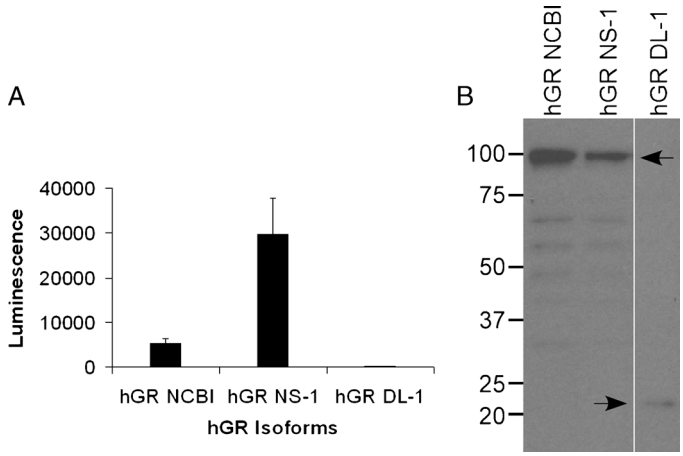

FIG. 2. **Transactivation potentials and Western blot of hGR isoforms**. A, Human glucocorticoid receptor NS-1 has transactivation potential greater than twice that of hGR NCBI. Human glucocorticoid receptor DL-1 demonstrates transactivation potential less than 10% that of hGR NCBI ($P < 0.01$). B, Western blot confirms expected protein size for each isoform (black arrows).

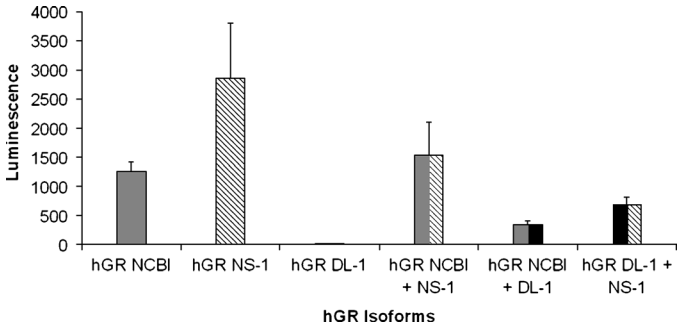

FIG. 3. **Transactivation potentials of cotransfected hGR isoforms**. Cotransfection of hGR NS-1 with hGR NCBI increased transactivation compared with hGR NCBI alone, but not to a significant level. Human glucocorticoid receptor DL-1 cotransfection with hGR NCBI reduced activity compared with hGR NCBI alone ($P < 0.01$). When isoforms hGR NS-1 and DL-1 were cotransfected, activity was increased when compared with hGR DL-1 alone ($P < 0.01$), but the presence of hGR DL-1 resulted in overall decreased activity when compared with hGR NS-1 alone ($P < 0.01$).

derivative isoforms had 63% of the activity level of M3, although only M2 compared with M3 showed a statistically significant difference. When the SNPs were combined in pairs, M1M2 had activity three times lower than M1M3, and also three times lower than M2M3 (Fig. 4). Comparison of the transactivation potentials of M3 with M1M3 and M2M3 revealed no statistical significance.

Effects of hydrocortisone treatment on hGR NCBI and hGR NS-1

Hydrocortisone treatment enhanced the transactivation potential of hGR NCBI at steroid concentrations ranging from 0.000001 to 1 μM, but statistical significance was shown only at concentrations of 0.001 and 1 μM. Hydrocortisone treatment also increased the transactivation potential of hGR NS-1 at concentrations ranging from 0.000001 to 0.001 μM, although statistical significance was demonstrated only at 0.001 μM (Fig. 5). Interestingly, administration of a hydrocortisone dose of 1 μM resulted in a significant decrease in activity of hGR NS-1. The peak response for both hGR NCBI and hGR NS-1 occurred at a hydrocortisone concentration of 0.001 μM. Isoform hGR NS-1 without hydrocortisone consistently demonstrated greater activity even when compared with hGR NCBI stimulated with hydrocortisone (Fig. 5).

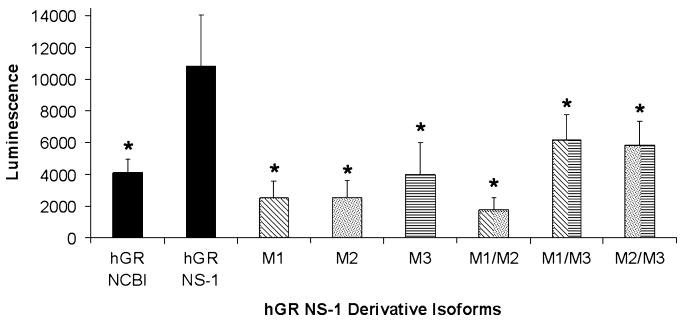

FIG. 4. **Comparison of transactivation potential of the hyperactive isoform (hGR NS-1) and its derivative isoforms**. Transactivation potentials for all the individual derivative isoforms (M1, M2, M3) and paired isoforms (M1M2, M1M3, M2M3) were all significantly lower when compared with hGR NS-1 (*$P < 0.01$). M1 and M2 derivative isoforms had activity levels trending lower than M3, although only M2 compared with M3 showed a statistically significant difference ($P < 0.01$). When the SNPs were combined in pairs, M1M2 had a statistically significant lower activity level compared with M1M3 ($P < 0.01$) and M2M3 ($P < 0.05$). Comparison of the transactivation potentials of M3 with M1M3 and M2M3 revealed no statistical significance.

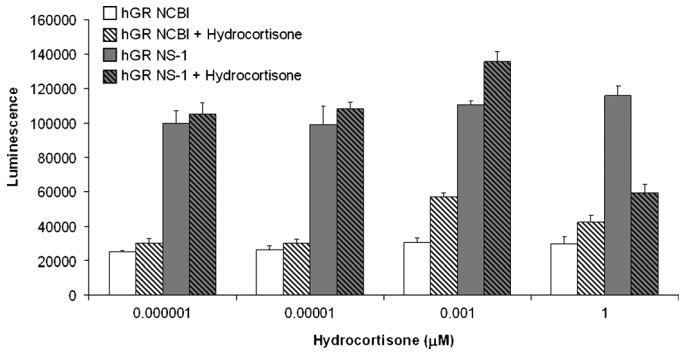

FIG. 5. **Transactivation potentials of hGR NCBI and hGR NS-1 with and without exogenous hydrocortisone stimulation**. Equivalent dilutions of vehicle (0.9% saline) were used as control. Human glucocorticoid receptor NS-1 consistently maintains hyperactivity above hGR NCBI with and without hydrocortisone (hGR NS-1 vs. hGR NCBI, $P < 0.01$; hGR NS-1 vs. hGR NCBI + hydrocortisone, $P < 0.01$). Human glucocorticoid receptor NCBI has a significant increase in activity with administration of hydrocortisone doses of 0.001 and 1 μM ($P < 0.01$). Human glucocorticoid receptor NS-1 isoform's activity is significantly increased above control with administration of hydrocortisone at a dose of 0.001 μM ($P < 0.01$). A significant decrease in activity of hGR NS-1 occurs after administration of a hydrocortisone dose of 1 μM ($P < 0.01$).

DISCUSSION

Previous studies have shown that GR isoforms may contribute to an impaired ability to respond to steroid therapy in certain diseases of inflammation such as asthma, inflammatory bowel disease, leukemia, and sepsis (12–14, 24, 27–34). In contrast to surveying diseased tissues or cell lines, we surveyed blood samples obtained from a volunteer group of humans screened for major illnesses or steroid use to further analyze the role of hGR isoforms in the variable response to steroids. As we investigated the functional activities of the various SNPs, we found some isoforms decreased activity. Even more interesting, however, we identified a novel hyperactive GR isoform with three NS SNPs—hGR NS-1. In the absence of hydrocortisone, this novel isoform demonstrates a transactivation potential twice that of hGR NCBI. When exposed to hydrocortisone, hGR NS-1 still displays peak activity two times greater than hGR NCBI at a dose of 0.001 μM. Another impressive characteristic is that hGR NS-1 without any hydrocortisone has an activity twice as high as hGR NCBI with hydrocortisone at all concentrations.

When examining the specific SNPs responsible, hyperactivity requires all three NS SNPs but is strongly influenced by the SNP change A2297G (N766S) in the LBD. The other two SNPs, A214G (N72D) and T962C (V321A), are located just outside the transactivation domain and did not influence the hyperactivity individually or when paired with the SNP A2297G. The impressive influence these SNPs have all together on transactivation potential suggests a change in the secondary and/or tertiary protein structure may be occurring and possibly altering the interaction with associated chaperone and target molecules.

As opposed to the hyperactive response found in hGR NS-1, isoform hGR DL-1 confirms truncation of the hGR results in

decreased activity. A previous study suggested that truncation of the hGR isoform is related to a pathologic state—this isoform, termed hGR-P, has been identified in cell lines of multiple myeloma patients resistant to glucocorticoid therapy (21, 22). Identification of hGR DL-1 in a population without major disease suggests that truncation may also serve as a normal mechanism for hGR regulation and response. More likely, this truncated isoform does not exist in isolation, but rather in a heterozygous state with other isoforms. Thus, the significance of the overall activity may depend on the interaction between different isoforms. Our cotransfection studies, which were designed to recreate a heterozygous state, show simultaneous expression of different isoforms contributes to overall hGR activity—cotransfection with the hyperactive hGR NS-1 tended to elevate activity, but the full hyperactive response seen with hGR NS-1 alone was always decreased when combined with another, less active, isoform. Likewise, hGR activity was decreased when cotransfected with the hypoactive hGR DL-1 isoform. The isoforms hGR NS-1 and hGR DL-1 may possibly interact with other isoforms and therefore contribute to the overall hGR response via a combined, rather than individual, mechanism.

This mechanism of interaction between different isoforms has been previously proposed regarding the alternative splice variant isoform hGRβ, which acts by blocking the transcriptional activity of the hGRα. The main difference between the two isoforms resides in the LBD in the C-terminus, and therefore, hGRβ cannot form an active ligand-binding pocket (35). This unique structure can, however, bind to the GREs within the nucleus, and remain functionally unresponsive to glucocorticoids. In addition to acting as a competitive inhibitor for the GRE target sites, other studies suggest the inhibitory effect of hGRβ occurs because it forms a heterodimer with hGRα to block activity (36, 37). Thus, the presence and interaction between two expressed isoforms play an important role in the overall response to steroids. In fact, a decreased ratio of hGRα levels compared with other known isoform levels such as hGRβ, hGRγ, hGR-A, and hGR-P has been found to correlate with steroid therapy resistance in multiple myeloma, asthma, inflammatory bowel disease, Cushing syndrome, leukemia, and sepsis (12, 13, 23, 28–31).

In contrast to other studies that suggest variations in the hGR isoform result in steroid resistance, the novel isoform hGR NS-1 identified in this study suggests that an optimal dose range exists for peak transactivation and may help to explain why certain patients have a more pronounced response to steroids. Further studies may reveal hGR isoforms function as an important contributing factor to the variable re sponse to steroid therapy. Eventually, tailoring of steroid therapy may be directed based on a patient's hGR isoform.

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