SHOCK, Vol. 38, No. 1, pp. 11–17, 2012

ENHANCED STEROID RESPONSE OF A HUMAN GLUCOCORTICOID RECEPTOR SPLICE VARIANT

Aaron C. Baker, Tajia L. Green, Victoria W. Chew, Kelly Tung, Amir Amini, Debora Lim, Kiho Cho, and David G. Greenhalgh

Shriners Hospitals for Children, Northern California, Sacramento, California

Received 2 Mar 2012; first review completed 15 Mar 2012; accepted in final form 28 Mar 2012

ABSTRACT—Glucocorticoids remain a recommended therapy in advanced septic shock despite the often unpredictable response, and our understanding of the mechanisms regulating the steroid and stress response remains limited. Since the initial sequencing of the human glucocorticoid receptor α and β gene (hGRα and hGRβ), only three additional splice variants have been identified—all of which have been postulated to contribute to steroid resistance. During a survey of 97 healthy humans' blood, we identified two novel hGR splice isoforms (hGR-S1 and hGR-S1(−349A) retaining intron H between exons 8 and 9. Human GR-S1(−349A) contained a base deletion causing an early termination and a truncated protein of 118 amino acids, whereas hGR-S1 had an early termination occurring within intron H and resulted in a 745–amino acid protein. Both isoforms had decreased transactivation potentials compared with hGRα when tested in the absence of exogenous steroids. However, after treating with exogenous steroids, dose-response studies showed hGR-S1(−349A) had a substantial augmentation in activity at higher concentrations of hydrocortisone and methylprednisolone when compared with hGRα, whereas hGR-S1 did not. Removal of the 3′ untranslated region (3′UTR) of the hGR-S1(−349A) mRNA sequence resulted in a loss of the augmented response. The isoform hGR-S1(−349A) augments the response to steroids, and this significant response appears to be critically regulated by the 3′UTR. The identification and evaluation of these unique hGR isoforms helps further the understanding of the complex genetic regulation of the stress and steroid response.

KEYWORDS—Glucocorticoid receptor, steroid response, alternative splicing, human glucocorticoid receptor, hGR, hGR-S1, hGR-S1(−349A)

Downloaded from http://journals.lww.com/shockjournal by BhDMf5ePHKav1zEoum1tQfN4a+kJLhEZgbsIHo4XMi0hCywCX1AWnYQp/IlQrHD3i3D0OdRyi7TvSFl4Cf3VC1y0abggQZXdgGj2MwlZLeI= on 01/21/2021

INTRODUCTION

Glucocorticoids function as critical components to the stress response via the hypothalamic-pituitary-adrenal axis and as pharmacological agents. Glucocorticoids act by binding to the glucocorticoid receptor (GR), which then dimerizes, localizes to the nucleus, and directly binds to the promoter regions of target genes to alter the inflammatory response (1, 2). Some clinical studies have identified a beneficial outcome after administering steroids to patients in advanced septic shock, whereas other studies have not (3–6). Despite the often unpredictable and variable anti-inflammatory response, steroids still remain a recommended therapy not only in the setting of advanced septic shock unresponsive to intravenous fluid and vasopressor support, but also for a cadre of other inflammatory diseases (7–9).

Despite the clinical and physiologic importance of glucocorticoids, our understanding of the mechanisms regulating the steroid and stress response remains limited. Alterations occurring at different steps of human GR (hGR) transcription and translation including alternative splicing, single-nucleotide polymorphisms, alternative translation initiation, and posttranslational modifications have all been postulated as contributors to the variable steroid response (10, 11). In the human, the GR gene resides on chromosome 5q31-32 (12, 13) (Fig. 1A). The gene consists of nine exons; however, only exons 2 through 9 undergo translation with a putative hGRα protein 777 amino acids in length. The protein includes two transactivation domains, a DNA-binding domain and a ligand-binding domain (14). In addition, hGR undergoes alternative splicing of exon 9 to produce either the hGRα or hGRβ isoform. Human GRα is the predominant bioactive isoform, whereas the hGRβ isoform is functionally deficient and implicated as a competitive inhibitor of hGRα (15).

In addition to hGRα and hGRβ, three other splice variants, hGRγ, hGR-P, and hGR-A, have been identified to date (Fig. 1A) (12, 16–18). Studies examining alternative splicing of the hGR have suggested the resultant isoforms may influence the response to steroids. Human GRγ retains three nucleotides from the intron separating exons 3 and 4. This isoform not only demonstrated variability in levels of expression across different human tissues, but also exhibited about half the transcriptional activity of hGRα (19). Expression of this isoform has been associated with steroid resistance in small cell lung cancer cells and in childhood acute lymphoblastic leukemia (18, 20). The other splice variants, hGR-P and hGR-A, were identified in a glucocorticoid-resistant multiple myeloma cell line (17, 21, 22). Human GR-P retains intron G between exons 7 and 8, whereas hGR-A is missing exons 5, 6, and 7. Whereas most of the initial studies of these hGR splice variants focused on correlating steroid resistance with the presence of specific hGR splice isoforms, more recent studies have suggested that the splice variants may not necessarily be pathologic and may even augment a response to steroids (23–26).

Regardless of whether the hGR isoforms formed by splicing results in steroid resistance or not, alternative splicing in general serves as an important step in diversifying gene expression and, in relation to the hGR, appears to impact the functional response to steroids (27, 28). In the current study, we report two novel splice isoforms, which despite significant

Address reprint requests to David G. Greenhalgh, MD, 2425 Stockton Blvd, Sacramento, CA 95817. E-mail: david.greenhalgh@ucdmc.ucdavis.edu.

Financial support/grant was received from Shriners Hospitals for Children (grant 8660).

DOI: 10.1097/SHK.0b013e318257c0c0

Copyright © 2012 by the Shock Society

Copyright © 2012 by the Shock Society. Unauthorized reproduction of this article is prohibited.

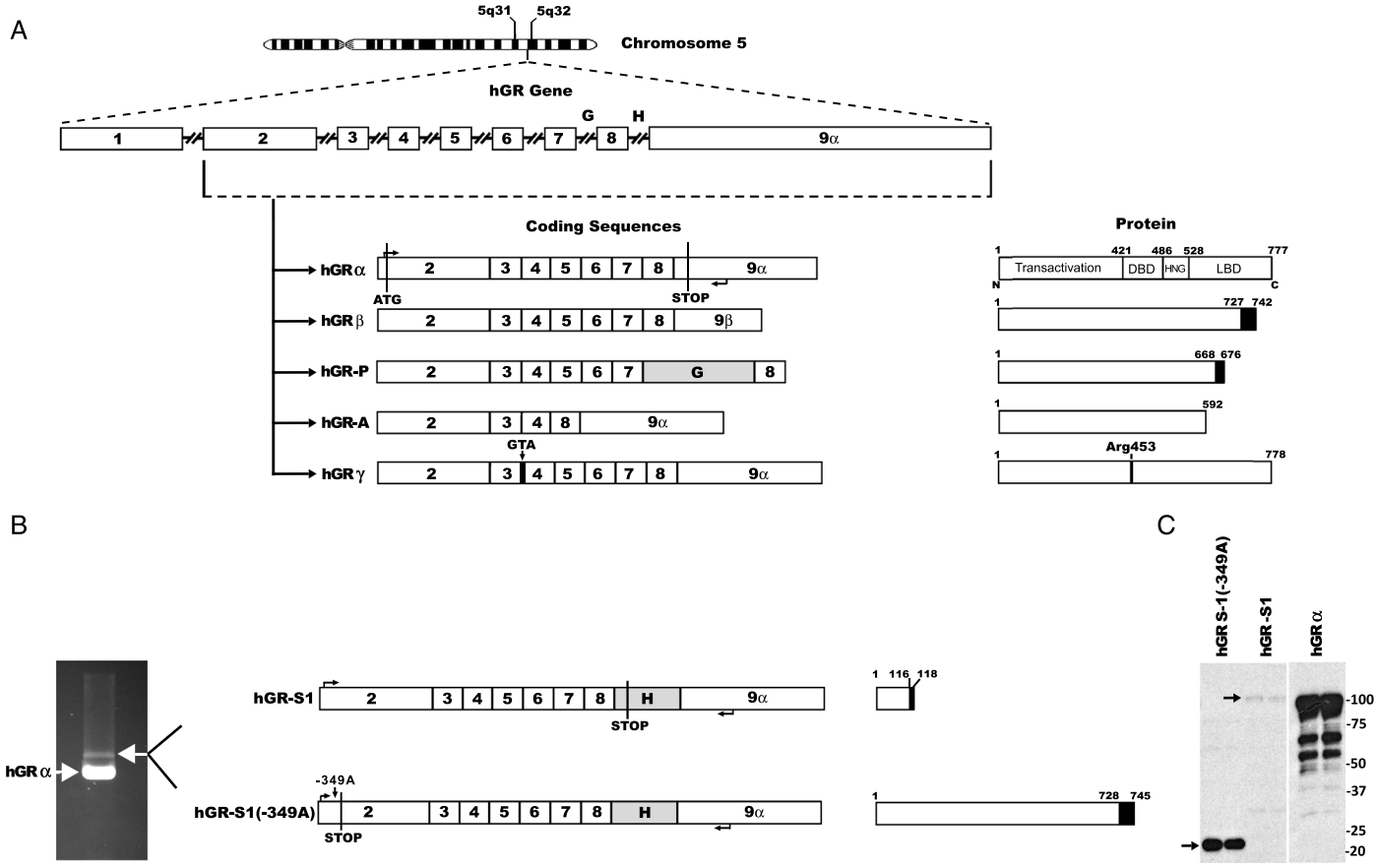

FIG. 1. **Coding sequences and protein structures of hGR splice variants**. A, The hGR gene resides on chromosome 5q31-32 (NCBI Ideogram). The gene consists of nine exons (1–9) and eight introns (A-H). The coding sequences for hGRα and the four splice variants of hGR identified to date hGRβ, hGR-P, hGR-A, and hGRγ are shown (exons not to scale). The location of the start codon, stop codon (vertical lines), and location of our forward and reverse primers (arrows) are shown for hGRα. The putative protein structure is shown adjacent to the coding sequence. Amino acid lengths are noted. Boundaries for the transactivation, DNA-binding domain (DBD), hinge (HNG) and ligand-binding domain (LBD) are denoted on the hGRα protein structure. Protein sequences differing from their respective position in hGRα are shown in black. B, Two additional novel splice isoforms, hGR-S1 and hGR-S1(−349A), were isolated from a larger band during a PCR screen for the hGR. Both novel variants retained intron H. Human GR-S1 did not contain any SNPs or base deletions, but had a premature stop codon occurring within intron H. The resultant protein was 745 amino acids in length, with the last 18 amino acids differing from the respective amino acids found in the hGRα protein. Human GR-S1(−349A) contained a base deletion at position 349 causing a frameshift and early stop—the resultant protein was 118 amino acids, with the last two amino acids differing from their respective amino acids in hGRα. C, The Western blot confirmed the protein expression of hGR-S1, hGR-S1(−349A), and hGRα.

changes in the hGR structure had surprising functional characterizations. An improved understanding of these hGR splice isoforms may further elucidate the mechanisms involved in steroid and stress response.

MATERIALS AND METHODS

Study population

All protocols involving collection, processing, and analysis of human blood samples in this study were approved by the institutional review board of the University of California, Davis. Written informed consent was obtained from all participants in the study. 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 unique hGR isoforms evaluated in this study were identified from a mixed cohort of 97 healthy volunteer human subjects (70 women and 27 men; age range, 20–67). About half the subjects were white, one-fourth Asian, and the remaining African American, Hispanic, or other.

Nomenclature of novel hGR isoforms

The isoforms have been named based on coding sequence. The hGRα designation represents an isoform derived from a human subject that matches the National Center for Biotechnology Informatics (NCBI) reference sequence for hGRα (NM_001018077). The designation "hGR S" denotes the novel hGR alternative *s*plice variant. The number after the aforementioned abbreviation represents the order in which our laboratory has identified the isoform (e.g., hGR-S1 is the first splice isoform to be identified). When additional alterations of the hGR sequence occur, it is denoted after the splice designation. For example, if a base pair deletion occurred in conjunction with hGR-S1, as will be reported in this article, then the designation would be hGR-S1 (minus base pair position–base pair deleted) or hGR-S1(−349A).

Identification and cloning of hGR isoforms

Total RNA was isolated from buffy coat samples, lysed using QIAzol reagent, and RNA was extracted using the RNeasy Lipid Miniprep Kit with a modified protocol (Qiagen, Valencia, Calif). Reverse transcriptase–polymerase chain reaction (RT-PCR) using the QuantiTect RT Kit and Taq DNA Polymerase Kit (Qiagen) was performed to amplify the hGR coding sequence (forward primer 5′-TTCACTGATGGACTCCAAAGAATCATTAAC-3′; reverse primer 5′-GGTGCCATCCTTCTTTGACTGTG-3′). Human GRα was isolated and extracted from the expected band size of ~2.6 kb, whereas hGR-S1(JN797824) and hGR-S1(−349A) (JN797823) were isolated and extracted from a larger band of ~3.1 kb from the gel using the QIAquick Gel Extraction Kit (Qiagen). The PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, Wis) and sequenced at Functional Biosciences (Madison, Wis). Polymorphisms were identified by comparison to the hGRα reference sequences (NM_001018077 and NG_009062) from the NCBI. The coding sequences were then cut with the restriction enzyme Not*I* and subcloned into a pcDNA4-HisMax vector (Invitrogen, Carlsbad, Calif) for functional analysis.

A second hGRα isoform, which has a 3′ untranslated region (3′UTR) 294 base pairs shorter than the previously mentioned hGRα, was also identified and isolated as described in a previous study by combining the exons 2 to 3 fragment with the exons 3 to 9 fragment using restriction enzymes

Copyright © 2012 by the Shock Society. Unauthorized reproduction of this article is prohibited.

(29). In brief, RT-PCR using the Sensiscript RT (Qiagen) was performed to amplify the hGRα coding sequence in two sections (exon 2–3: forward primer 5′-TCACTGATGGACTCCAAAG-3′, reverse primer 5′-GTTTCATCAAAAGT GACTGCCTTAA-3′; exon 2–9α: forward primer 5′-CCAGCATGAGACCAGATGTA-3′, reverse primer 5′-TTAAGGCAGTCACTTTTGATGAAAC-3′). The fragments were cloned into pGEM-T Easy vector (Promega) and sequenced at MCLAB (South San Francisco, Calif). 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). This hGRα isoform was used only for a reference in the experiments represented by B. Both hGRα isoforms resulted in the same putative protein structure and did not have any significant difference in activities when stimulated with steroids (data not shown).

Creation of hGR-S1(−349A/−3′UTR) deletion clone

Polymerase chain reaction was performed using 100 ng of hGR-S1(−349A) plasmid DNA and specific forward and reverse primers (forward primer 5′-ACAGTGGCGGCCGCGGGAATTGCATTTTCA-3′; reverse primer 5′-ACTCCTCGAGTCATTAAGTCTGTTCCC-3′). The reverse primer was designed such that a second stop codon was inserted immediately after the original stop codon to ensure termination during isoform translation. In addition, the primers were designed to incorporate restriction enzyme digestion sites, which allowed direct cloning into the pcDNA4-HisMax vector (Invitrogen). The new clone was confirmed by sequencing at Functional Biosciences.

Measurement of transactivation potentials of hGR isoforms

Cells, 2×10^4 tsA201 (an HEK 293 cell subclone stably transfected with SV40 large T-antigen given), were obtained from Dr. Daniel Feldman at Shriners Hospitals for Children (29). The cells were seeded in 100 μL of antibiotic-free Dulbecco modified eagle medium (Invitrogen) with 10% fetal bovine serum (JR Scientific, Woodland, Calif) in a 96 well plate. The plates were incubated at 37°C in 5% CO_2 atmosphere overnight. The cells were transfected using 50 ng of the respective plasmid containing the hGR isoform [hGRα, hGR-S1, hGR-S1(−349A), or hGR S1(−349A/−3′UTR)] 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 steroid treatment experiments, 1.5×10^4 tsA201 cells were seeded, incubated, and transfected in the same fashion as described above. Twenty-four hours after transfection, the cells were treated with graded concentrations of hydrocortisone (0.000001–1000 μM) or methylprednisolone (MPS) (0.000001–1 μM) in vehicle (0.9% saline) or vehicle alone for 24 h. Pharmaceutical-grade MPS sodium succinate (Solu-Medrol; Pfizer Inc, New York, NY) and hydrocortisone sodium succinate (Solu-Cortef) (Pfizer) were used for all experiments. Luciferase activity was assessed as described above.

Western blot analysis of hGR isoforms

In a similar manner described above, tsA201 cells were transfected using Fugene 6 with 1 μg of recombinant hGR DNA [hGRα, hGR-S1, hGR-S1(−349A), or hGR S1(−349A/−3′UTR)] and collected 1 day after transfection. Western blot detection was carried out in a manner previously described (30). In brief, cells 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. The supernatant was collected and run on a 4% to 20% BioRad Criterion gel (Hercules, Calif). Separated proteins were transferred to a PVDF 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-HRP antibody (Invitrogen) at 4°C. The protein signal was visualized via chemiluminescence using the ECL Plus Western Blot Detection System (GE Healthcare).

Survey of additional humans for alternative splicing of intron H

To screen for the presence of a retained intron H in other human subjects, total RNA was isolated from an additional eight humans' buffy coat using the RNeasy Lipid Miniprep Kit (Qiagen) after lysing the samples using TRIZOL reagent (Invitrogen). The RNA underwent RT-PCR as described above. A forward primer binding within exon 8 and a reverse primer binding within intron H were used (forward primer 5′-TCCTAAGGACGGTCTGAAGAGCC-3′; reverse primer 5′-GGTCAGTGGGAACATCTCA-3′). A selected sample was chosen for cloning and sequence confirmation at Functional Biosciences. Each sample was also run with primers for β-actin (forward primer 5′-CCAACTGGGACGA CATGGAG-3′; reverse primer 5′-GTAGATGGGCACAGTGTGGG-3′) as a control.

Including the initial human subject, retention of intron H was identified in a total of nine human subjects in our study. Eight subjects were women, and one subject was a man. The ages ranged from 34 to 55 years and, all subjects were white.

Statistical analysis

All experiment samples were run in triplicate, and each experiment repeated at least three times except for the experiments evaluating hGRα, hGR-S1, and hGR-S1(−349A) with MPS where two experiments were done in triplicate. Statistical analysis confirmed the obtained data for each experiment using a one-way analysis of variance test, and significance was confirmed with Tukey honestly significant difference post hoc test. A representative experiment was chosen for figure presentation.

RESULTS

Identification of novel splice variants hGR-S1 and hGR-S1(−349A)

The hGR splice variants, hGR-S1 and hGR-S1(−349A), were identified during PCR screening for hGR from a single subject as a larger ~3.1-kb band (compared with the hGRα ~2.6-kb band) (Fig. 1B). Sequencing revealed both isoforms retained intron H between exons 8 and 9 due to alternative splicing. However, hGR-S1(−349A) contained a single-nucleotide deletion at position 349, which resulted in a frameshift, premature stop codon, and a putative protein of 118 amino acids in length. As a result of the frameshift, the last two amino acids at the C-terminus differed from the respective amino acids found in the hGRα protein sequence. Human GR-S1, on the other hand, had a nucleotide sequence that matched hGRα except for the retention of intron H. A stop codon occurred within intron H, which resulted in a putative 745–amino acid protein. The last 18 amino acids of hGR-S1 differed from the respective amino acids in hGRα. The protein products of hGR-S1 and hGR-S1(−349A) were confirmed by Western blot (Fig. 1C).

hGR-S1 and hGR-S1(−349A) had decreased transactivation potential compared with hGRα in the absence of exogenous steroid

To characterize the baseline response of hGR-S1 and hGR-S1(−349A) in comparison to hGRα, the transactivation potentials were measured using a luciferase assay after the respective isoform was transfected into tsA201 cells. Luciferase assays showed both isoforms hGR-S1 and hGR-S1(−349A) had a significantly lower transactivation potential in comparison to hGRα (Fig. 2). Human GR-S1(−349A) had a transactivation potential less than 1% of hGRα, whereas hGR-S1 had less than 10% the activity of hGRα. When comparing hGR-S1 to hGR-S1(−349A), the transactivation potential of hGR-S1 was significantly greater than the transactivation potential for hGR-S1(−349A).

Augmented transactivation potential of hGR-S1(−349A), but not hGR-S1, compared with hGRα occurred at higher concentrations of hydrocortisone and MPS

To further functionally characterize the isoforms hGR-S1 and hGR-S1(−349A), graded doses of exogenous hydrocortisone and MPS were used to stimulate tsA201 cells after transfection

Copyright © 2012 by the Shock Society. Unauthorized reproduction of this article is prohibited.

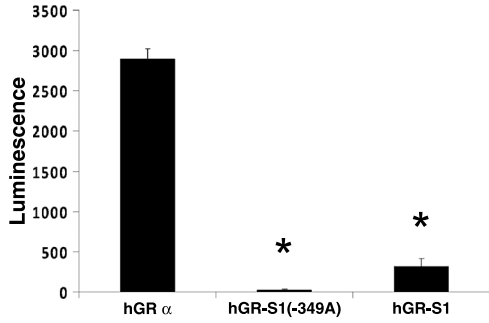

FIG. 2. **Transactivation potentials of novel hGR splice variants without exogenous steroid stimulation**. Human GR-S1 and hGR-S1(−349A) had a transactivation potential significantly lower than hGRα in the absence of exogenous steroids (*$P < 0.01$). Human GR-S1(−349A) had less than 1% of the activity of hGRα, whereas hGR-S1 had less than 10% the activity of hGRα.

of the respective isoforms. The isoform hGRα served as a reference for comparison.

At the lower concentration range of hydrocortisone (0.0001–0.01 μM), hGRα had greater activity than hGR-S1(−349A) (Fig. 3A). Human GRα had a significant response to hydrocortisone starting at 0.001 μM, which then peaked at 0.01 μM. At the higher concentration range (0.1–1,000 μM), hGRα had a steady decline in its activity. Human GR-S1(−349A), however, had negligible transactivation when compared with hGRα at the lower concentrations of hydrocortisone, but then had greatly increased activity at the higher concentration ranges, surpassing the activity of hGRα. Starting at 0.1 μM, hGR-S1(−349A) not only started to have a significant response to hydrocortisone but also had a response more than 1.5 times greater than hGRα. Furthermore, the activity of hGR-S1(−349A) increased to more than 10 times the activity of hGRα at 1 μM, which was also the peak response for hGR-S1(−349A). This augmentation in response, in comparison to hGRα, is maintained up to a hydrocortisone concentration of 1,000 μM. In contrast, whereas hGR-S1 was able to respond to hydrocortisone concentrations ranging from 0.01 to 1,000 μM, hGR-S1 did not display an augmented response to hydrocortisone at any concentration when compared with hGRα (Fig. 3B). The peak response for hGR-S1 occurred at 0.01 μM, but the peak did not surpass the peak activity of hGRα at the same concentration.

As seen with the hydrocortisone experiment, a similar pattern of response was identified with hGRα and hGR-S1(−349A) when stimulated with MPS (Fig. 4, A and B). Human GRα had a higher activity level at the lower concentration ranges (0.000001 and 0.0001 μM), which then steadily declined at the higher concentrations (0.01 and 1 μM) (Fig. 4A). The peak response of hGRα to MPS occurred at 0.0001 μM, which was more than 100-fold lower than the peak response concentration seen for hGRα with hydrocortisone (Fig. 4A). As seen with the hydrocortisone experiments, hGR-S1(−349A) had an augmentation in response at the higher concentrations of MPS in comparison to hGRα. An initial response occurred at 0.01 μM, which then increased more than seven times higher than hGRα at 1 μM. Similar to hydrocortisone stimulation, the peak response of hGR-S1(−349A) to MPS occurred at 1 μM. When assessing the transactivation potential of hGR-S1 with MPS, it again did not reveal an augmented response at the higher concentrations, but did have a peak response at 0.01 μM (Fig. 4B).

Removal of the 3′ untranslated region (3′UTR) from hGR-S1(−349A) resulted in loss of the augmented response to steroids

One unique component of the hGR-S1(−349A) mRNA sequence, in comparison to the hGR-S1 sequence, is the early single-nucleotide deletion, frameshift, and premature termination, which results in a longer 3′UTR containing the retained intron H (Fig. 5A). To further explore a possible mechanism contributing to the ability of hGR-S1(−349A) to augment the response to steroids, we decided to evaluate the role of the 3′UTR. A deletion clone that matched the coding sequence of hGR-S1(−349A), but removed the 3′UTR, was created (Fig. 5A). This clone was termed hGR-S1(−349A/−3′UTR), and the coding sequence resulted in the same 118–amino acid protein as hGR-S1(−349A), which was confirmed by Western blot (Fig. 5A).

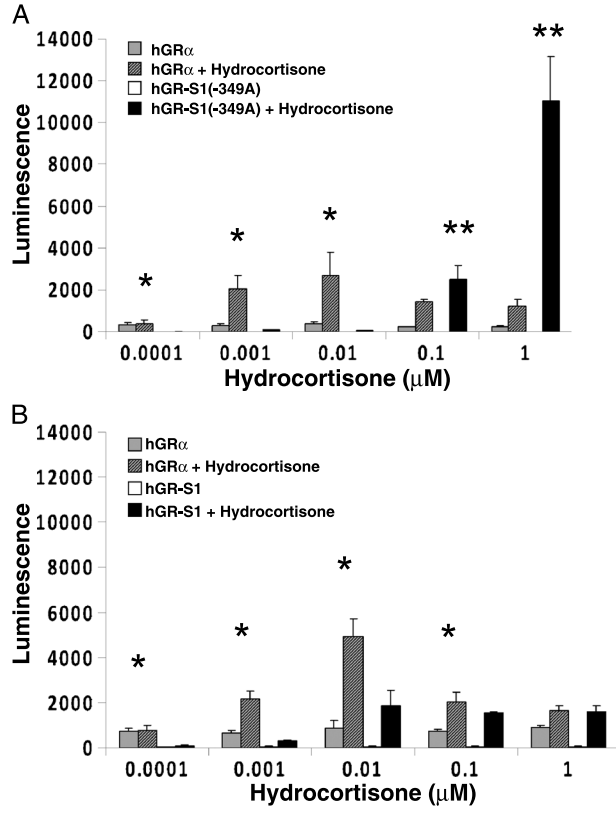

FIG. 3. **Transactivation potentials of hGR splice variants hGR-S1 and hGR-S1(−349A) with exogenous hydrocortisone**. A, When stimulated with hydrocortisone, hGRα demonstrated a significantly greater response when compared with hGR-S1(−349A) at the lower concentration ranges (0.0001–0.01 μM) (*$P < 0.01$). A peak response for hGRα occurred at 0.01 μM, which then had a steady decrease in response as the concentration increased. At the lower concentrations, hGR-S1(−349A) had negligible activity, but then had an augmented response to hydrocortisone when compared with hGRα at the higher concentrations (0.1–1000 μM) (**$P < 0.01$). A peak response for hGR-S1(−349A) occurred at 1 μM. B, Again, hGRα demonstrated a significantly greater response than hGR-S1 at the lower concentrations of hydrocortisone (0.0001–0.1 μM) (* $P < 0.01$). At the higher concentration ranges (0.1–1000 μM), hGR-S1 did not have a significant increase in response like hGR-S1(−349A). Equivalent dilutions of vehicle (0.9% saline) were used as controls. Human GR-S1 and hGR-S1(−349A) vehicle experiments had negligible transactivation potentials and are not easily visualized on this scale.

Copyright © 2012 by the Shock Society. Unauthorized reproduction of this article is prohibited.

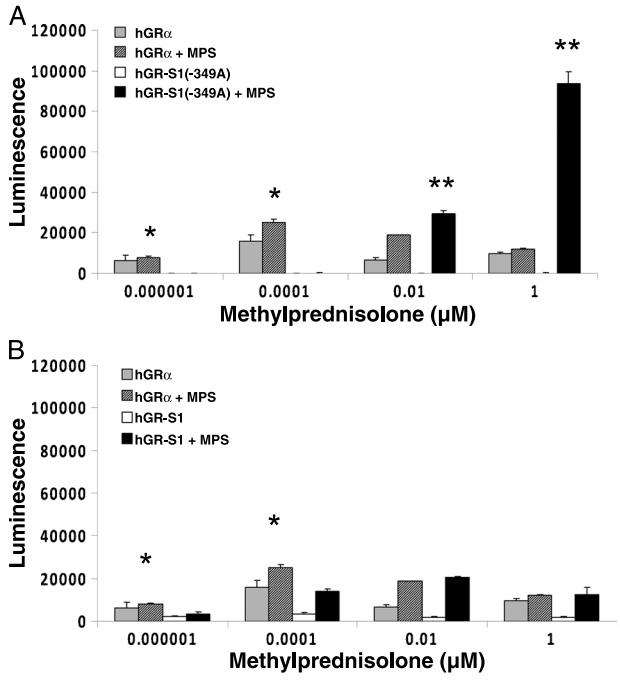

FIG. 4. **Transactivation potentials of hGR splice variants hGR-S1 and hGR-S1(−349A) with exogenous MPS**. A, When stimulated with MPS, hGRα demonstrated a significantly greater response than hGR-S1(−349A) at the lower concentrations (0.000001 and 0.0001 μM) (*$P < 0.01$). A peak response of hGRα was seen at 0.0001 μM. Human GR-S1(−349A) had a negligible transactivation potential at the lower concentrations, but then had a significant augmentation in the response compared with hGRα at the higher concentrations (0.01 and 1 μM) of MPS (**$P < 0.01$). The peak response for hGR-S1(−349A) occurred at 1 μM. B, Human GRα had a significantly greater response than hGR-S1 when stimulated with MPS at the lower concentrations, but hGR-S1 did not have an augmented response at the higher concentrations as seen with hGR-S1(−349A) (*$P < 0.01$). Equivalent dilutions of vehicle (0.9% saline) were used as controls. Human GR-S1 and hGR-S1(−349A) vehicle experiments had negligible transactivation potentials and are not easily visualized on this scale.

As before, hGR-S1(−349A) displayed a similar response to hydrocortisone with a significantly greater response occurring at the higher doses (0.1 and 1 μM) of both hydrocortisone and MPS (Fig. 5, B and C). More interestingly, the augmented response was lost at the higher concentrations of both hydrocortisone and MPS when isoform hGR-S1(−349A/−3′UTR) was transfected. The response of hGR-S1(−349A/−3′UTR) had a transactivation potential significantly lower than hGR-S1(−349A) at all concentrations of hydrocortisone treatment—at the higher concentrations, the activity of hGR-S1(−349A/−3′UTR) had 7% of the activity of hGR-S1(−349A) at 0.1 μM and 25% of the activity at 1 μM. In the presence of MPS, hGR-S1(−349A/−3′UTR) also had a lower activity at all concentrations when compared with hGR-S1(−349A). Specifically, at the higher concentrations of MPS, hGR-S1(−349A/−3′UTR) had less than 3% of the activity of hGR-S1(−349A) at 0.1 μM and less than 7% of the activity at 1 μM.

Alternative splicing and retention of intron H occur in other human subjects

To determine if alternative splicing and retention of intron H occurred in other subjects, a survey utilizing primers specifically designed to identify intron H was performed in

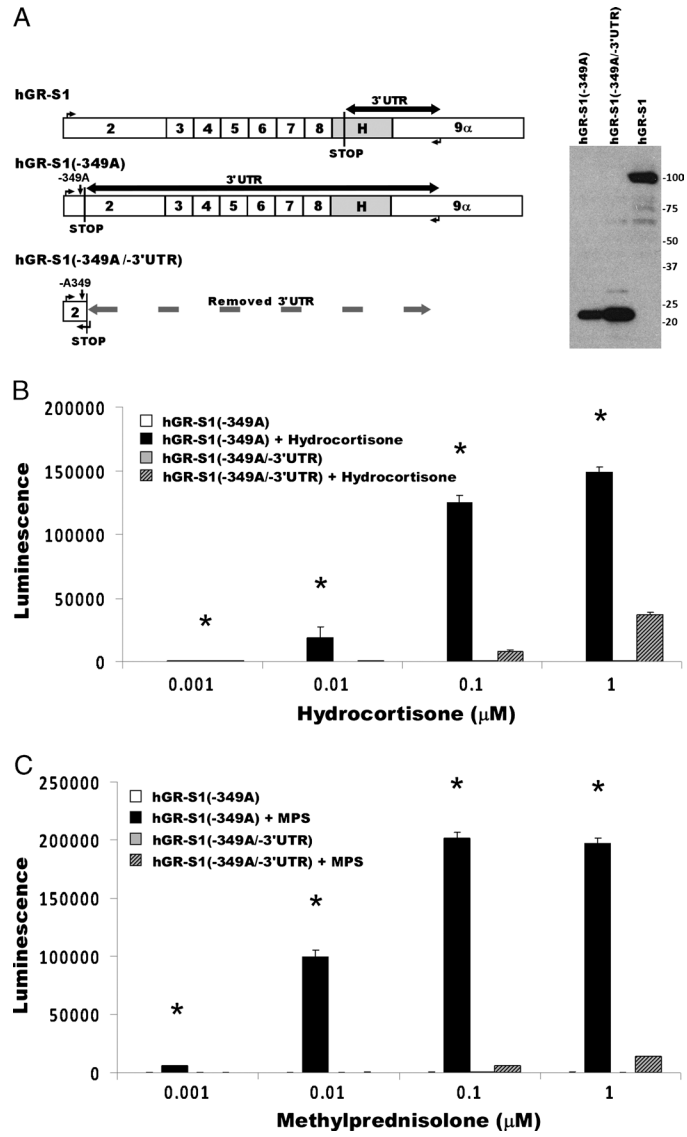

FIG. 5. **Removal of the 3′ untranslated region (3′UTR) results in a loss of the augmented transactivation potential in hGR-S1(−349A)**. A, Line diagrams depicts the 3′UTR of hGR-S1, hGR-S1(−349A), and hGR-S1(−349A/−3′UTR). Human GR-S1 contains an early stop codon within the retained intron. The 3′UTR beyond the stop codon is labeled. Human GR-S1(−349A) contains a base deletion at position 349, early stop codon, and 3′UTR containing intron H. The boundary of the 3′UTR for hGR-S1 and hGR-S1(−349A) is defined by the location of the reverse primer shown on exon 9α that was used to isolate this sequence (small black arrows). The coding sequence for hGR-S1(−349A/−3′UTR) contains the same base deletion and stop codon as hGR-S1(−349A), but had the 3′UTR removed. Both hGR-S1(−349A) and hGR-S1(−349A/−3′UTR) translate into the same protein sequence. Western blot confirmed protein expression. B, Human GR-S1(−349A/−3′UTR) did not show an augmented response at the higher concentrations (0.1 and 1 μM) of hydrocortisone. Human GR-S1(−349A) had a significantly greater response to hydrocortisone at all concentrations compared with hGR-S1(−349A/−3′UTR) and also had the expected increase in response at the higher concentrations (*$P < 0.01$). C, Human GR-S1(−349A/−3′UTR) did not have an augmented response at the higher concentrations (0.1 and 1 μM) of MPS. Human GR-S1(−349A) had a significantly greater response to MPS at all concentrations when compared with hGR-S1(−349A/−3′UTR) and also had the expected augmentation in response at the higher concentrations (*$P < 0.01$). Equivalent dilutions of vehicle (0.9% saline) were used as controls. Human GR-S1(−349A) and hGR-S1(−349A/−3′UTR) vehicle experiments shown had negligible transactivation potentials and are not easily visualized.

Copyright © 2012 by the Shock Society. Unauthorized reproduction of this article is prohibited.

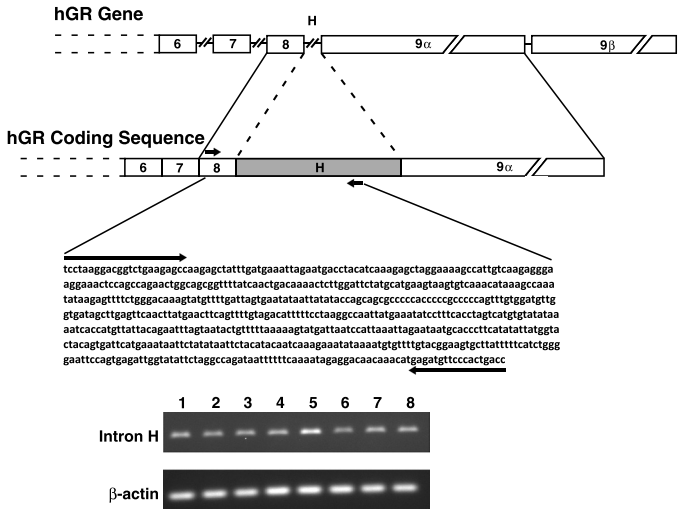

FIG. 6. **Alternative splicing of intron H is observed in eight additional human subjects**. To verify the retention of intron H in additional subjects, a forward primer and a reverse primer specific for intron H (black arrows) were used to perform a PCR screen on eight additional humans. Intron H was identified in the coding sequences of all eight subjects. A negative reverse transcription (−RT) control performed for seven samples confirmed there was no genomic DNA contamination (not shown). A positive control using genomic DNA confirmed the ability of the primer set to isolate the intron H sequence (not shown). Reverse transcriptase–polymerase chain reaction amplification of β-actin was performed as an internal control.

an additional eight subjects (Fig. 6). The PCR screen demonstrated that the retained intron H was present in the coding sequences of all eight subjects. A negative reverse transcription control confirmed there was no genomic DNA contamination.

DISCUSSION

Alternative splicing serves as an important mechanism in altering the hGR's response to steroids. To date, three additional hGR splice variants (hGRγ, A, and P) have been reported since the initial report of hGRα and hGRβ over 20 years ago (12, 17, 18). In this current study, we contribute to the known catalog of hGR splice variants and show important functional characterizations of the two novel isoforms retaining intron H termed hGR-S1 and hGR-S1(−349A). Although both isoforms have a decreased transactivation potential compared with hGRα without exogenous steroids, hGR-S1(−349A), the splice variant containing a base deletion and early termination, had an increased response to exogenous hydrocortisone and MPS. Furthermore, the augmented response occurred in a concentration-dependent fashion, which happened at a higher concentration range than seen with hGRα.

Clinically, patients with similar stressors (burn, trauma, sepsis, etc) often have different outcomes. These differences may be related to their ability to respond to stress and steroids, and our findings suggest alternative splicing of the hGR impacts the steroid response. Although our experiments were *in vitro*, the lower steroid dose range we evaluated encompassed the physiologic range of plasma cortisol concentrations in healthy controls (0.09–0.4 μM), which happened to coincide with the optimal activity range of hGRα. More interestingly, the plasma cortisol levels for patients in septic shock can be around 1 μM, which is also the concentration a peak response was seen for hGR-S1(−349A) (3, 31). Potentially, a patient who can express hGR-S1(−349A) may be able to mount an augmented response to higher doses of steroid and thus have a better outcome than a patient who does not express this isoform. Therefore, the presence or absence of certain splice variant isoforms, such as hGR-S1 and hGR-S1(−349A), may play a role in the variable response to stress and steroids between individuals.

Initial studies examined hGR splice variants isolated from cancer cell lines resistant to steroids, and thus proposed hGR alternative splicing was a contributor to glucocorticoid resistance in these diseases (17, 18, 20, 21). Further studies eventually suggested that many of these alternative splice isoforms were not specific for a pathologic state and even may be part of a normal mechanism in steroid and stress response (23, 25, 26). For example, the hGR-P splice variant, which retains intron G and one of the most commonly studied, was initially thought to contribute to steroid resistance in multiple myeloma (21). More recent data show hGR-P not only exists in healthy human controls, but also augments the response of hGRα in a cell- and concentration-specific manner (26). In fact, alterations in the steroid response may be more associated with a change in the relative levels of multiple isoforms rather than the presence or absence of a specific isoform (22). In our current study, we show not only additional alternative splice isoforms exist, but also how these isoforms may be important contributors to the stress and steroid response in a dose-dependent manner.

Another unique finding in this study is the truncated hGR-S1(−349A) protein does not have a response in the absence of steroid but shows an augmented response in the presence of steroid. The locations of the ligand-binding domain, nuclear localization signal, DNA-binding domain, and chaperone binding sites found in the hGRα protein do not exist in the hGR-S1(−349A) protein. Although truncation of the critical components of specific protein structures would be expected to result in an overall loss of function, this alteration of the hGR-S1(−349A) protein does not seem to impede the steroid response. However, the exact mechanism that allows the hGR-S1(−349A) protein to maintain function remains unclear. One possible explanation is that hGR-S1(−349A) is acting as a nuclear transcription factor, given its small size and ability to easily localize to the nucleus. Our current experimental model contains endogenous hGR, but we have confirmed it does not have a significant response to steroid stimulation. If hGR-S1(−349A) protein is acting as a transcription factor, then it may potentially be augmenting the activity of the endogenous hGR in the presence of steroid. However, whether the protein functions as a transcription factor or exerts its influence via some other method by localizing to unique nuclear targets faster due to a lack of a chaperone protein binding site, interacting directly with hGRα or other hGR isoforms or even engaging in a separate pathway, will require further investigation.

One mechanistic insight found in our study is the critical contribution of the 3′UTR in augmenting the response to hydrocortisone and MPS for hGR-S1(−349A). Although this section of the mRNA sequence does not translate into protein, the role of the 3′UTR has been previously identified as an

Copyright © 2012 by the Shock Society. Unauthorized reproduction of this article is prohibited.

important component controlling mRNA processing including mRNA stability, nuclear and cytoplasmic localization, and translational efficiency, and its alteration has been implicated in some diseases (32, 33). In fact, increased presence of the repeat element motifs AUUUA within the 3′UTR has been found to destabilize hGRα mRNA and decrease hGRα protein expression (34). The 3′UTR of hGR-S1(−349A) contains only a single AUUUA motif, which may, in part, account for greater stability of the isoform, but other unique components found in this specific 3′UTR such as the intron H may also potentially contribute to isoform stability.

A limitation of this current study is that the frequency of this novel splice isoform was not evaluated in all 97 human subjects. Our initial screening primers were not specific for the intron H retention. We did perform a repeat survey specific for intron H on eight human subjects available for blood redraws and did identify this splice pattern occurred in each subject. More important to this report are the identification and functional evaluation of this novel hGR splice isoform because it offers additional insight to glucocorticoid regulation.

Additional investigation of hGR splice variants will hopefully continue to improve our understanding of the regulation of the steroid and stress response. These insights may demonstrate determinants of the variable steroid and stress response are not simply influenced by the genotype of an individual, but also an individual's alternative splice patterns. The novel isoforms reported in this study will be important to survey in future clinical studies evaluating hGR function, especially in comparison to the other known isoforms. Even more interesting is the possibility that alternative splicing of the hGR gene causing a specific change to an untranslated region of the mRNA may play a role as a critical regulator of the steroid response. Further studies will hopefully help elucidate a more precise role of the 3′UTR's contribution to the hGR's function. Ongoing evaluation of hGR isoform variation in septic shock or inflammatory states will be important to further define the role of hGR alternative splicing and help determine whether these isoforms serve a physiologic, or even protective, role or a pathologic one.

REFERENCES

1. Charmandari E, Kino T, Chrousos GP: Glucocorticoids and their actions: an introduction. *Ann N Y Acad Sci* 1024:1–8, 2004.
2. Zhou J, Cidlowski JA: The human glucocorticoid receptor: one gene, multiple proteins and diverse responses. *Steroids* 70:407–417, 2005.
3. Annane D, Sebille V, Charpentier C, Bollaert PE, Francois B, et al.: Effect of treatment with low doses of hydrocortisone and fludrocortisone on mortality in patients with septic shock. *JAMA* 288:862–871, 2002.
4. Bernard GR, Luce JM, Sprung CL, Rinaldo JE, Tate RM, et al.: High-dose corticosteroids in patients with the adult respiratory distress syndrome. *N Engl J Med* 317:1565–1570, 1987.
5. Cronin L, Cook DJ, Carlet J, Heyland DK, King D, et al.: Corticosteroid treatment for sepsis: a critical appraisal and meta-analysis of the literature. *Crit Care Med* 23:1430–1439, 1995.
6. Sprung CL, Annane D, Keh D, Moreno R, Singer M, et al.: Hydrocortisone therapy for patients with septic shock. *N Engl J Med* 358:111–124, 2008.
7. Barnes PJ, Greening AP, Crompton GK: Glucocorticoid resistance in asthma. *Am J Respir Crit Care Med* 152:S125–S140, 1995.
8. Faubion WA Jr, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Sandborn WJ: The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. *Gastroenterology* 121:255–260, 2001.
9. Dellinger RP, Levy MM, Carlet JM, Bion J, Parker MM, et al.: Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008. *Crit Care Med* 36:296–327, 2008.
10. Revollo JR, Cidlowski JA: Mechanisms generating diversity in glucocorticoid receptor signaling. *Ann N Y Acad Sci* 1179:167–178, 2009.
11. Tung K, Baker AC, Amini A, Green TL, Chew VW, et al.: Novel hyperactive glucocorticoid receptor isoform identified within a human population. *Shock* 36:339–344, 2011.
12. Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, et al.: Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature* 318:635–641, 1985.
13. Encio IJ, Detera-Wadleigh SD: The genomic structure of the human glucocorticoid receptor. *J Biol Chem* 266:7182–7188, 1991.
14. Lu NZ, Cidlowski JA: The origin and functions of multiple human glucocorticoid receptor isoforms. *Ann N Y Acad Sci* 1024:102–123, 2004.
15. Oakley RH, Jewell CM, Yudt MR, Bofetiado DM, Cidlowski JA: The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and mechanisms of action. *J Biol Chem* 274:27857–27866, 1999.
16. Manenschijn L, van den Akker EL, Lamberts SW, van Rossum EF: Clinical features associated with glucocorticoid receptor polymorphisms. An overview. *Ann N Y Acad Sci* 1179:179–198, 2009.
17. Moalli PA, Pillay S, Krett NL, Rosen ST: Alternatively spliced glucocorticoid receptor messenger RNAs in glucocorticoid-resistant human multiple myeloma cells. *Cancer Res* 53:3877–3879, 1993.
18. Ray DW, Davis JR, White A, Clark AJ: Glucocorticoid receptor structure and function in glucocorticoid-resistant small cell lung carcinoma cells. *Cancer Res* 56:3276–3280, 1996.
19. Rivers C, Levy A, Hancock J, Lightman S, Norman M: Insertion of an amino acid in the DNA-binding domain of the glucocorticoid receptor as a result of alternative splicing. *J Clin Endocrinol Metab* 84:4283–4286, 1999.
20. Beger C, Gerdes K, Lauten M, Tissing WJ, Fernandez-Munoz I, et al.: Expression and structural analysis of glucocorticoid receptor isoform gamma in human leukaemia cells using an isoform-specific real-time polymerase chain reaction approach. *Br J Haematol* 122:245–252, 2003.
21. Krett NL, Pillay S, Moalli PA, Greipp PR, Rosen ST: A variant glucocorticoid receptor messenger RNA is expressed in multiple myeloma patients. *Cancer Res* 55:2727–2729, 1995.
22. Sanchez-Vega B, Krett N, Rosen ST, Gandhi V: Glucocorticoid receptor transcriptional isoforms and resistance in multiple myeloma cells. *Mol Cancer Ther* 5:3062–3070, 2006.
23. Chen Y, Burckart GJ, Shah T, Pravica V, Hutchinson IV: A novel method for monitoring glucocorticoid-induced changes of the glucocorticoid receptor in kidney transplant recipients. *Transpl Immunol* 20:249–252, 2009.
24. Geng CD, Pedersen KB, Nunez BS, Vedeckis WV: Human glucocorticoid receptor alpha transcript splice variants with exon 2 deletions: evidence for tissue- and cell type-specific functions. *Biochemistry* 44:7395–7405, 2005.
25. van den Akker EL, Koper JW, Joosten K, de Jong FH, Hazelzet JA, et al.: Glucocorticoid receptor mRNA levels are selectively decreased in neutrophils of children with sepsis. *Intensive Care Med* 35:1247–1254, 2009.
26. de Lange P, Segeren CM, Koper JW, Wiemer E, Sonneveld P, et al.: Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor–mediated effects in transfected cells. *Cancer Res* 61:3937–3941, 2001.
27. Keren H, Lev-Maor G, Ast G: Alternative splicing and evolution: diversification, exon definition and function. *Nat Rev Genet* 11:345–355, 2010.
28. Wang ET, Sandberg R, Luo S, Khrebtukova I, Zhang L, et al.. Alternative isoform regulation in human tissue transcriptomes. *Nature* 456:470–476, 2008.
29. Yee KS, Cho K, Green T, Chandler J, Greenhalgh DG: The effect of CAG repeat length polymorphism in the murine glucocorticoid receptor on transactivation potential. *Exp Mol Pathol* 84:200–205, 2008.
30. Phan HH, Cho K, Sainz-Lyon KS, Shin S, Greenhalgh DG: CD14-dependent modulation of NF-kappaB alternative splicing in the lung after burn injury. *Gene* 371:121–129, 2006.
31. Goodman LS, Brunton LL, Chabner B, Knollmann BC: Goodman & Gilman's pharmacological basis of therapeutics. New York: McGraw-Hill, 2011.
32. Conne B, Stutz A, Vassalli JD: The 3′ untranslated region of messenger RNA: a molecular 'hotspot' for pathology? *Nat Med* 6:637–641, 2000.
33. Andreassi C, Riccio A: To localize or not to localize: mRNA fate is in 3′UTR ends. *Trends Cell Biol* 19:465–474, 2009.
34. Schaaf MJ, Cidlowski JA: AUUUA motifs in the 3′UTR of human glucocorticoid receptor alpha and beta mRNA destabilize mRNA and decrease receptor protein expression. *Steroids* 67:627–636, 2002.

Copyright © 2012 by the Shock Society. Unauthorized reproduction of this article is prohibited.