Glucocorticoid receptor represses the Dex-mediated induction of human androgen response element-linked Luc activity

M. K. Jang[1], K. R. Chae[1], D. Y. Hwang[1], C. K. Kim[1], B. G. Kim[1], S. B. Shim[1], S. W. Jee[1], S. H. Lee[1], J. S. Shin[1], S. H. Lee[1], N. H. Chung[2], J. S. Cho[1], S. Y. Choi[3] and Y. K. Kim[1]

[1] *Division of Laboratory Animal Resources, National Institute of Toxicological Research, Korea Food and Drug Administration (KFDA), Seoul, Korea*
[2] *Graduate School of Life and Environmental Science, Korea University, Seoul 136-701, Korea*
[3] *National Institute of Toxicological Research, KFDA, Seoul 122-704, Korea*

Abstract. A human androgen response element (hARE), identified within intron 8 of the human sterol regulatory element-binding protein cleavage-activating protein, interacts with both glucocorticoid receptor (GR) and androgen receptors (AR). The aim of this study was to test the hypothesis that human GR (hGR) might modulate the expression of a hARE-linked reporter gene by dexamethasone (Dex). The hypothesis was tested by: a) co-transfecting HepG2 cells with a hGR and a luciferase (Luc)-reporter gene for performing *in vitro* investigations and b) by their co-injection into the tail vein of mice for *in vivo* investigation. *In vitro* co-transfected cells and the *in vivo* co-injected mice were then treated with Dex. Our results have led us to concluded that both transfection and injection of the hGR leads to a repression in the Dex-mediated induction of hARE-linked Luc activity both *in vitro* and *in vivo* settings. These findings suggest that this assay system allows screening of drug candidates affecting to a signal transduction pathway of the GR and AR and may help in the future discovery and analysis of novel and selection of GR and AR agonists.

Key words: Androgen response element — Dexamethasone — Glucocorticoid — Glucocorticoid receptor

Introduction

Glucocorticoids (GCs) are steroid hormones synthesized and secreted by the adrenal cortex under the regulatory influence of adrenocorticotrophic hormone. GCs are involved in the regulation of numerous physiological processes, including glucose, protein and fat metabolism and are among the most effective drugs used for immunosuppression and treating acute and chronic inflammatory diseases. Such effects of GCs are mediated by the translocation of the activated glucocorticoid receptor (GR) from cytosol to the nucleus (Htun et al. 1996).

The GR have three domains spanning the N- to C-terminus: i) N-terminal domain which is coded by exon 2 and contains an activation function (AF-1) segment involved in protein-protein interaction with various cofactors, ii) a DNA-binding domain (DBD), which is coded by exon 3 and 4 and is necessary for DNA binding and homodimerization, and iii) a C-terminal ligand-binding domain (LBD), which specifically binds GCs (Weinberger et al. 1985). There are two isoforms of the human GR (hGR): hGRα (777 amino acids) and hGRβ (742 amino acids) (Oakley et al. 1996; De Kloet et al. 1998; Yudt and Cidlowski 2001). Of the two isoforms, hGRα is the active form that can functionally bind GRs and LBD, but not hGRβ, due to a defective LBD (Weinberger et al. 1985; Wright et al. 1993; Oakley et al. 1996). hGRβ has an intact DBD identical to hGRα and thereby possesses potential dominant inhibitory effects on hGRα as suggested by competitive binding to the glucocorticoid response element (GRE) (Oakley et al. 1996).

The DBD in GR contains amino acids that contact specific bases in GRE to provide site specificity for GR-DNA binding. These amino acids are located in the first zinc finger where

Correspondence to: Yong K. Kim, Division of Laboratory Animal Resources, KFDA, National Institute of Toxicological Research, 5 Nokbun-dong Eunpyung-ku, Seoul 122-704, Republic of Korea
E-mail: kimyongkyu@hanmail.net

the P box, comprised of three amino acids, is responsible for responsive element discrimination (Luisi et al. 1991; Hard et al. 2005). In GREs, it consists of the palindromic GR-binding sequence to which a GCs-bound GR dimer can bind, but only one-half of the palindrome is sufficient to relay GC signaling, resulting in positive or negative effects on gene expression.

A human androgen response element (hARE, 5'-GGAA-GAaaaTGTTCT-3'), bearing resemblance to the 5'-TGT-TCT-3' consensus core-binding motif of a canonical ARE, was identified within intron 8 of the human sterol regulatory element-binding protein cleavage-activating protein (Heemers et al. 2004). After binding the GCs, the GRs dimerize, translocate to the nucleus, and thereafter function by binding to the consensus core motif of a canonical ARE (5'-TGT-TCT-3') (Verrijdt et al. 2003) and the GRE (Schoneveld et al. 1994). Although there is a report that hARE interacts with the both AR and GR on gel mobility shift assay, it has not been characterized whether hGRα modulates hARE-linked luciferase (Luc) reporter gene activity in the response to dexamethasone (Dex). The aim of this study was to test the hypothesis that hGRα might modulates the a hARE-linked Luc reporter gene in response to Dex. The hypothesis was tested by co-transfecting HepG2 cells with hGRα and a Luc reporter gene for performing *in vitro* investigations and by their co-injection into the tail vein of mice for *in vivo* investigation. *In vitro* co-transfected cells and the *in vivo* co-injected mice were then treated with Dex. Our results have led us to conclud that both transfection and injection of the hGRα leads to a repression in the Dex-mediated induction of hARE-linked Luc activity both *in vitro* and *in vivo* settings. These findings suggest that this assay system allows screening drug candidates influential to a signal transduction pathway of the GR or AR.

Materials and Methods

Gene constructions

hARE-linked Luc reporter gene

The phARE-tk/Luc was constructed using p2ETL, which was a gift from professor Carol K. Wrenn (University of Illinois, IL, USA) to Dr. Yhun Y. Sheen (Ewha Women University, Seoul, Korea). This plasmid contains two copies of ERE upstream of the thymidine kinase (tk) promoter linked to the Luc reporter gene. To replace two copies of ERE with hARE, two copies of hARE (small letter) and upstream of the tk promoter (capital letter) were used as a sense primer (5'-GCaag aggaa gaaaa tgtac ctctt aagag gaaaa tgtac ctctt CCAGC GTCTT GTCAT TGGCG A-3', corresponding to the nucleotides –95 to –116 of the tk promoter). Down stream of the Luc gene was used as an anti-sense primer (5'-CCTTT CAGCT CTGTG TTGCT C-3', corresponding to the nucleotides +1999 to +2020 of the Luc coding sequence). The resultant triple construct (hARE-tk-Luc) was then cloned into the EcoR1 site in the pGEM-T (phARE-tk/Luc) (Promega, WI, USA).

hGRβ expression vector

The pCMV/hGR*α* was constructed by inserting the hGR*α* (GeneBank accession No. NM_000176) down stream from the CMV promoter. The hGR*α* sequence was amplified by PCR with a full-length of the RNA isolated from the HepG2 cells. The primers used for amplification were the sense primer: 5-TCGAG AAGAG GAAGA AAATG TACCT CT-TAA GAGGA AGAAA ATGTA CCTCT TC-3' (corresponding to nucleotide 127 to 149 of the hGR*α*), and the antisense primer: 5'-GCTCA GCTAC CTGTG ATGCC GAA-3' (corresponding to nucleotide 2471 to 2449 of the hGR*α*). The amplified hGR*α* product was cloned into the pGEM-T (Promega) (pGEM-hGR*α*). The hGR*α* fragment was purified by digestion of pGEM-hGR*β* with PinA1 and Sac1 and then cloned into the PinA1 and Sac1-digested pEGFP-C1 that had been eleminated EGFP sequence (pCMV/hGR*α*). To verify whether or not the cloned hGR*α* sequence is identical with the known hGR*α* gene, sequence analyses were performed (Biotech, Korea), and the resulting sequences aligned with the NCBI (The National Center for Biotechnology Information) sequence database using the BLAST program.

DNA-PCR analysis

For DNA-PCR analysis, 10 pmol of these sense and antisense primers were added and the reaction mixtures subjected to 30 cycles of amplification. The amplifications were conducted using a Perkin–Elmer cycler programmed to the following cycles: 30 s at 94°C; 30 s at 62°C; 60 s at 72°C.

Gene transfection

For a transient transfection, HepG2 cells (4×10^5) were plated in 100 mm dishes in medium supplemented with 10% of fetal bovine serum. The cultures were maintained from 24 to 36 h in Dulbecco's Modified Eagle's medium (Gibco-BRL, MD, USA) containing 1% non-essential amino acids, 2 ml L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin. At 66 h, each dish of cells was washed with Opti-MEM and exposed to a mixture formed by combining 50 μg of Lipofectamine (Life Technologies Inc, California, USA) with 10 μg of phARE-tk/Luc and pCMV/hGR*α* for an experimental (Exp) group or with the 10 μg of phARE-tk/Luc and pCMV for a control (Cont) group. The lipofectamine-DNA mixture was then removed from the cells after 24 h of incubation, and the transfected cells were then exposed to Dex at different

concentrations over different time periods. Cells were washed in phosphate-buffered saline and lysed by adding a lysis buffer (25 mmol/l Tris-phosphate (pH 7.8), 2 mmol/l DTT, 2 mmol/l 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton^R X-100, 1.25 mg/ml lysozyme, 2.5 mg/ml BSA). Protein was then used for Luc activity.

Western blot analysis

Liver tissues obtained from the mice were solubilized with 1% Nonidet P-40 in 150 mmol/l NaCl, 10 mmol/l Tris HCl (pH 7.5), and 1 mmol/l EDTA supplemented with a protein inhibitor mixture (Roche, Mannheim, Germany) followed by centrifugation at 10,000 × *g* for 10 min at 4°C. Protein samples were run on 10% polyacrylamide gels, transferred to nitrocellulose membranes and were incubated with primary antibodies of anti-hGR (Abcam Inc., Cambridge, England) and anti-*β*-actin (Sigma, MI, USA). Each complex of antigen-antibody were visualized using biotylated secondary antibody (goat anti-rabbit)-conjugated HRP streptavidin (Histostain-Plus kit, Zymed, San Francisco, CA, USA).

Reporter gene assays

The Luc enzyme assay (Luciferase Assay System, Promega, WI, USA) was performed by mixing 80 *μ*l of each lysate with reaction buffer containing 20 *μ*l of the luciferin substrate (0.4 mg/ml). Light is produced by converting the chemical energy of luciferin oxidation through an electron transition forming the product molecule oxyluciferin whose intensity was measured for 10 s with a Monolight 2010B luminometer (Micro Lumat LB96V, Bertold Technologies, Germany). Firefly Luc catalyzes luciferin oxidation by using ATP·Mg^{2+} as a cosubstrate. The Luc enzyme assay system with the reporter gene was measured to normalize transcription efficiency in each experiment. Each plate was measured, in triplicate, for Luc activity. Here, no Luc activity was found in the cells transfected with a pGL3-Basic by treatment of Dex.

Mice and injection with constructs

ICR female mice (6 to 8-week-old) were obtained from the Division of Laboratory Animal Resources, National Institute of Toxicological Research, Korea Food and Drug Administration (KFDA). The mice used in this experiment were handled in an accredited animal facility in accordance with AAALAC International Animal Care Policies (Accredited Unit-KFDA No. 000936). All mice were housed in cages under controlled light cycle conditions (light on at 6:00 h and off at 18:00 h). Mice were fed a standard irradiated chow diet (Purina Inc., Korea) *ad libitum* and maintained pathogen-free. Injection of the tail vein with the construct was performed as described (Zhang et al. 2003). Briefly, phARE-tk/Luc (50 *μ*g) and pCMV/hGR*α* (50 *μ*g) for the Exp group or phARE-tk/Luc (50 *μ*g) and pCMV (50 *μ*g) for the Cont group were carefully injected into the tail vein of mice at a volume of 1 ml/10 g of body weight. Following tail vein injections, the Exp group was treated with 100 *μ*l of Dex at 100 mg/kg body weight through tail vein injection (Zhang et al. 2003). Cont group was treated with 100 *μ*l of 20% ethanol *per* body weight. Finally, liver tissue was removed from subset of group for Luc enzyme assay.

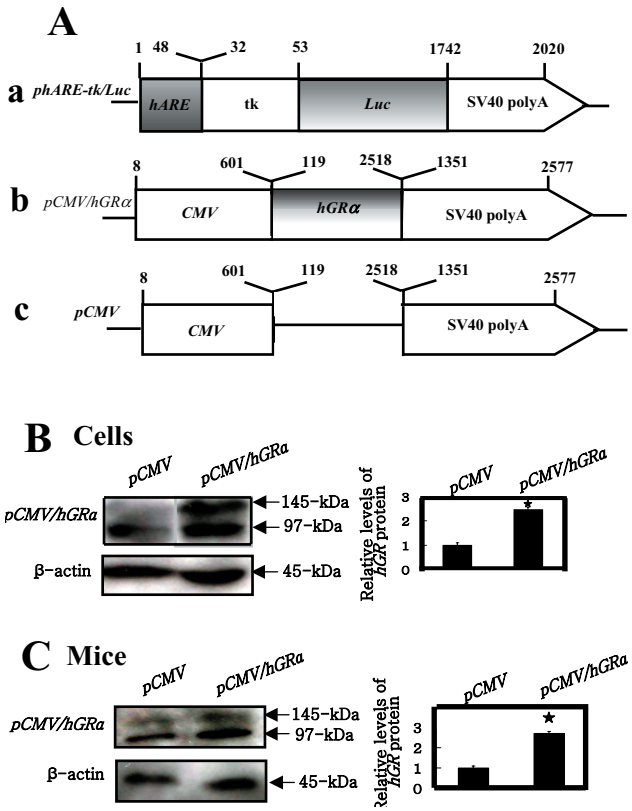

Figure 1. Constructions of vector. **A.** Constructions of hARE-linked Luc reporter gene and hGR*α* expression vector. a) hARE-linked Luc reporter gene (phARE-tk/Luc). Two copies of hARE sequences were inserted into upstream of tk promoter linked to the Luc reporter gene. b) hGR*α* expression vector (pCMV/hGR*α*). This plasmid contains the cDNA encoding the hGRα placed under the control of the CMV promoter. c) Control plasmid (pCMV). This plasmid lacks hGRα sequence. **B.** Confirmation of hGR*α* expression in cells. **C.** Expression of hGR*α* in injected mice. The protein (50 *μ*g) prepared from transfected cells and livers of injected mice was analyzed by western blotting using the anti-GR antibody. Quantification of bands in B and C are shown. All numbers indicated molecular weight of hGR*α* unidentified protein (145 kDa), and *β*-actin. The values are represented as a mean ± SD. * $p < 0.05$ *versus* control (pCMV).

Statistical analysis

Tests for significance between groups were performed using the One-way analysis of variance (SPSS for Windows, release 10.01, standard version – Chicago, IL, USA). All values are reported as the mean ± standard deviation(s). Statistical significance was accepted at $p < 0.05$.

Results

Expression of hGRα

Prior to testing the effects of Dex on hARE-linked Luc activity (Figure 1Aa), cells and mice were transiently transfected or injected with a pCMV/hGR*α* (Figure 1Ab) for an Exp group or a pCMV (Figure 1Ac) for a Cont group, respectively. These were then tested for the efficiency of both transfection and injection of constructs in a western blotting. A higher hGR*α* expression level (97 kD) was observed with an unidentified band (145 kDa) in cells (Figure 1B) and in mice (Figure 1C) of the Exp group than those in the Cont group. There were no changes in β-actin levels in cells and mice (Figure 1B and C). These results are important for subsequent analysis of the a hGR*α*-driven Luc activity in response to Dex.

hGRα-driven Luc activity in response to Dex in vitro

We tested a possible role for hGR*α* in modulating hARE-linked Luc activation. To test this, cells were co-transfected with a phARE-tk/Luc (Figure 1Aa) and with a pCMV/hGR*α* for a Exp group or with a phARE-tk/Luc and a pCMV for a Cont group, and were then treated with different doses of Dex (10 and 100 nmol/l) for 24 h. These doses were based on a recent report that cells transiently transfected with pGRE-tk/Luc activated the reporter gene to a peak induction value at 100 nmol/l of Dex (Ling et al. 2005). Protein was then prepared for measurement of the Luc activity. The induced levels of Luc activity were gradually repressed upon transfection of hGR*α* in the Exp group (Figure 2Aa).

To optimize the time of maximal induction of Luc activity, cells of the Cont and Exp groups were treated with at 100 nmol/l Dex at 12, 24, and 48 h, and the proteins were prepared for measurement of the Luc activity. During this time course, Luc activity gradually increased until 24 h in a time-dependent manner, and thereafter declined at 48 h (Figure 2Ab). Here, a lesser activity (<50%) was shown depending on hGR*α* transfection as compare to inducible level of Luc activity in the Exp group (Figure 2Ab). These results suggest that hGR*α* inhibits a Dex-mediated induction of Luc activity.

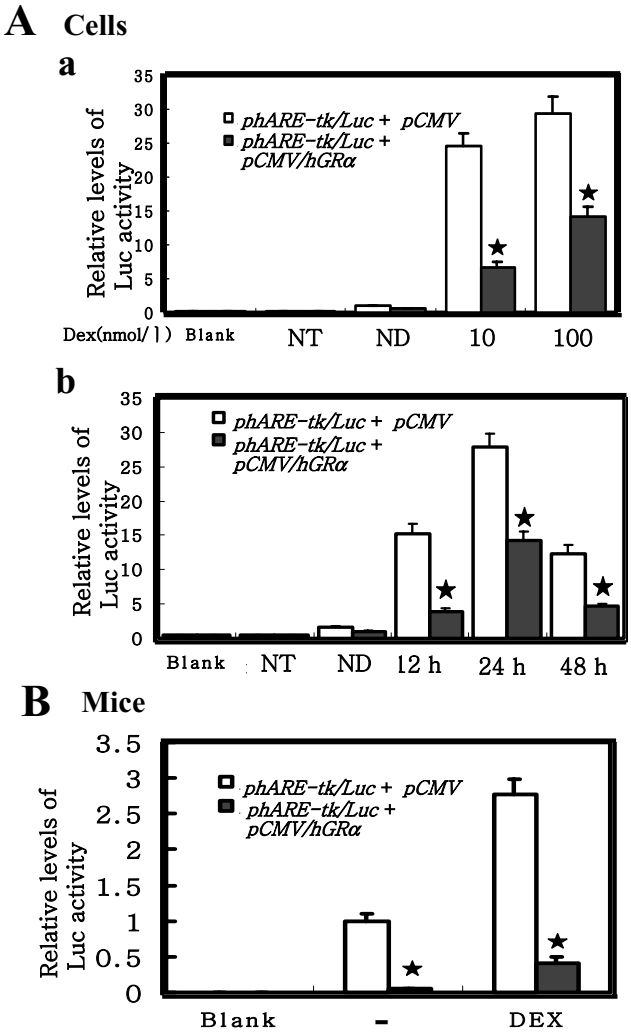

Figure 2. hGRα-driven Luc activity in response to Dex *in vitro* and *in vivo*. **A.** *In vitro* test (cells). a) Dose-effect of Dex on Luc activity. HepG2cells transfected with a phARE-tk/Luc and a pCMV for a Cont group or phARE-tk/Luc and pCMV/hGR*α* for an Exp group were exposed for 24 h at different doses of Dex. b) Time-course effect of Dex on Luc activity. Transfected cells were exposed at a 100 nmol/l Dex for different time course, and protein (50 μg) prepared from Cont and Exp groups was assayed for Luc activity in triplicates. **B.** *In vivo* test. Mice were injected with a phARE-tk/Luc and a pCMV for a Cont group or phARE-tk/Luc and pCMV/hGRα for an Exp group, and treated them with Dex. The protein (50 μg) prepared from livers of Cont and Exp groups was then assayed for Luc activity n triplicates. The value was defined as 1 (no Dex (ND) in cells or Cont in mice) for obtaining other relative value. The values are represented as a mean ± SD. * $p < 0.05$ *versus* Cont group. NT, non-transfection.

hGRα-driven Luc activity in response to Dex in vivo

Transfection of cells with hGR*α* led to a repression of the Dex-mediated induction of Luc activity (Figure 2A). To

Table 1. Comparison of sequences homology between hARE and GRE. Sequences of GRE (nGRE, GREs, and GRE1/2s) (Schoneveld et al. 1994) and hARE were compared to find the sequence homology. No homology was observed in this comparison.

hARE		GGAAGAaaaTGTACC
Consensus core motif of a canonical ARE		TGTTCT
Simple	GRE	CGGACAaaaTGTTCT
		GGCACAgtgTGGTCT
		GGGTGAgctTGTTCT
		ACATGAgtgTGTCCT
	GRE 1/2	TGTTCT
		TGTTCC
		TCTTCT
		TGTACA
		GGGACA
		TGTTCT
		TGTTTT
		TGTTCT
		GGTTAT
Composite	GRE	TGTACAggaTGTTCT
		GGACTTgttTGTTCT
		GACACCaccCCTCCC
		GCTCGTtccTTCTCT
		CACACAaaaTGTGCA
		AGCATAtgaAGTCCA
		AGAGCAgttTGTTCT
		AGAACTatcTGTTCC
		GGAACAtttTGTGCA
		CTGCCTctcTGTTCT
		CCAAGAatgTGTTCT
	nGRE	TTTTGTcaaTGGACA
		GGAAGGTCACGTCCA
		GGTATAaacAGTGCT
		CTGGAGCTTCGCCTC
		TGTCCT-n6-TGTCCT
		AGTGGTcctTGTCCT
		ACGTCAattTGATCT
		GGTACTtggTGTAAT

test whether this effect also occurred similarly *in vivo* in mice, phARE-tk/Luc and pCMV/GR*α* for the Exp group or phARE-tk/Luc and pCMV for the Cont group were co-injected into the tail vein of mice. At 13 h post-injection, both animal groups were treated with Dex for an additional 20 h and liver tissue was prepared for Luc enzyme assay. Injection of the hGR*α* caused a dramatic repression in Dex-induced hARE-linked Luc activity (Figure 2B), which suggests that hGR*α* injection led to a repression in Luc activity *in vivo* as was determined for *in vitro* transfected cells.

Comparison of sequence homology between hARE and GRE

Negative GRE (nGRE) direct negatively gene expression of target genes, and the binding of GR to the nGRE is required for repressing (Schoneveld at al. 1994). GREs and GRE1/2s, however, are involved in the activation of gene expression, although tethering GRE (tGRE) functions to both inhibit and activate gene expression (Schoneveld et al. 1994). Because the Dex-mediated induction of hARE-linked Luc activity was repressed by transfection or injection with hGR*α*, the sequence homology was established between hARE and GRE (nGRE and GRE1/2s). Sequence homology was not found in the comparison between hARE and GRE (Table 1) and between hARE and ARE within the various gene promoters (Verrijdt et al. 2003). Thus, it is possible that the hARE sequence is a novel negative binding site on the Dex ligand-GR complex in the cell nucleus which results in a repression of Luc activity.

Discussion

In this study, a novel hARE sequence was chosen to test the hypothesis that hGR*α* might modulate the Dex-mediated induction of its linked Luc activity *in vitro* (cells) and *in vivo* (mice). This approach (a two level investigations) provides critical insights, impacting a drug candidates influential to a signal transduction pathway of the GR or AR and a therapeutic strategy to treat patients with anti-inflammatory agents. In particular, new investigative approach is sought because GC therapy, as one of the most effective medications for treating acute and chronic inflammatory diseases (such as allergic and autoimmune diseases), is hampered by variations in individual patient responsiveness that is theorized to be due to GR-DNA interactions. Long-term therapy using GCs is limited by their metabolic side effects where these actions are mainly mediated by GR-DNA interaction either by activation or by the negative regulation of target gene expression.

GCs are activated by binding to a GR which forms a large heterodimeric complex composed of HSP 90, HSP 70, and immunophilins (Pratt and Toft 1997). Once bound, the complex then disassembles and the activated receptor with the GCs occupying a site in the LBD of the receptor translocates to the nucleus, where it interacts with regulatory elements on gluconeogenic target genes and enhancing their gene expression. This study, however, found that hGR*α* represses the Dex-mediated induction of hARE-linked Luc activity *in vitro* (cells) and *in vivo* (mice). The mechanism

of repression of hGRα-driven hARE-linked Luc activity can theoretically be divided into primary and secondary stages. In the primary stage, the effect of Dex occurs through a direct result of the decreased efficacy of GR to Dex. This interpretation is supported by a report that GC agonists significantly down-regulate the expression of the GR (Rosewicz et al. 1998; Marcel and Schaaf 2003), and the demonstration that an intragenic element within the LBD of the hGR is critical for the down-regulation event (Burnstein et al. 1994). This interpretation also supports the concept of Dex resistance and could, therefore, be a factor in the decreased efficacy of Dex used clinically. In the second stage, the Dex ligand-decreased GR interacts with the hARE region to repress the Luc reporter genes. nGRE also mediates the repression of target genes with the side effect of GCs mainly due to GR-DNA interactions (Dostert and Heinzel 2004). We were unable to find, however, any homology between hARE and nGRE or composite GREs (Table 1) and between hARE and ARE. Thus, it suggests that the hARE is another region responsible for negatively regulated target genes.

In conclusion, this paper provides the first experimental evidence that hGRα leads to the negatively repressed Dex-induced hARE-linked Luc activity through hGRα co-transfection or coinjection. Further, hARE and GR-driven trans-repression of Luc activity may help in the future discovery and analysis of novel and selective GR and AR agonists.

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