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Review

Recent advances in the molecular mechanisms causing primary generalized glucocorticoid resistance

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ABSTRACT

Primary Generalized Glucocorticoid Resistance is a rare condition characterized by generalized, partial, target tissue insensitivity to glucocorticoids owing to inactivating mutations, insertions or deletions in the human glucocorticoid receptor (hGR) gene *(NR3C1)*. Recent advances in molecular and structural biology have enabled us to elucidate the molecular mechanisms of action of the mutant receptors and to understand how certain conformational alterations of the defective hGRs result in generalized glucocorticoid resistance. Furthermore, our ever-increasing understanding of the molecular mechanisms of glucocorticoid action indicates that the glucocorticoid signaling pathway is a stochastic system that plays a fundamental role in maintaining both basal and stress-related homeostasis. In this review, we summarize the clinical manifestations and molecular pathogenesis of Primary Generalized Glucocorticoid Resistance, we present our recent findings from the functional characterization of three novel heterozygous point mutations in the *NR3C1* gene, and we discuss the diagnostic approach and therapeutic management of the condition. When the condition is suspected, we recommend sequencing analysis of the *NR3C1* gene as well as of other genes encoding proteins involved in the glucocorticoid signal transduction. The tremendous progress of next-generation sequencing will undoubtedly uncover novel hGR partners or cofactors.

Key words: Glucocorticoids, Glucocorticoid receptor, Glucocorticoid resistance, Glucocorticoid signaling, *NR3C1* mutations

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INTRODUCTION

Glucocorticoids (cortisol in humans, corticosterone in most rodents) are steroid hormones secreted by the adrenal cortex into the systemic circulation in an ultradian, circadian, and stress-related fashion under the control of the hypothalamic-pituitary-adrenal (HPA) axis.[1-4] These cholesterol-derived molecules

participate in the physiologic function of almost all organs and play a fundamental role in the stress response.[5] Glucocorticoids exert their pleiotropic effects through their cognate receptor, which belongs to the steroid receptor family of the nuclear receptor superfamily.[6] The glucocorticoid receptor functions as a ligand-activated transcription factor that influences the transcription rate of numerous genes through well-described genomic and less well understood non-genomic actions.[1,2,4-6] Since glucocorticoids contribute substantially to the steady state of the organism, it is generally accepted that glucocorticoid signaling is not merely a simplified signal transduction pathway but a complex homeostatic system that functions coordinately with other systems to help the organism cope with stressful stimuli.[2,5,7]

Homeostatic mechanisms, including the HPA axis, exert their effects in an inverted U-shaped dose-response curve[2,5,7] (Figure 1). Normal basal homeostasis or *eustasis* is achieved in the central, optimal range of the curve, whereas suboptimal effects may occur on either side of the curve and can lead to insufficient adaptation, a state that has been called *allostasis* or *cacostasis*.[2,5,7] The latter states of hypofunction or hyperfunction of the HPA axis may have short-term or long-term adverse consequences for the individual and can lead to a compromised sense of well-being and/or performance.[7-9] At the molecular level, any alterations in the glucocorticoid signal transduction are likely to result in impaired tissue sensitivity to glucocorticoids, which may present with clinical manifestations of *glucocorticoid resistance* or *glucocorticoid hypersensitivity* (Table 1).[10-14] One such allostatic condition is Primary Generalized Glucocorticoid Resistance.[11-18]

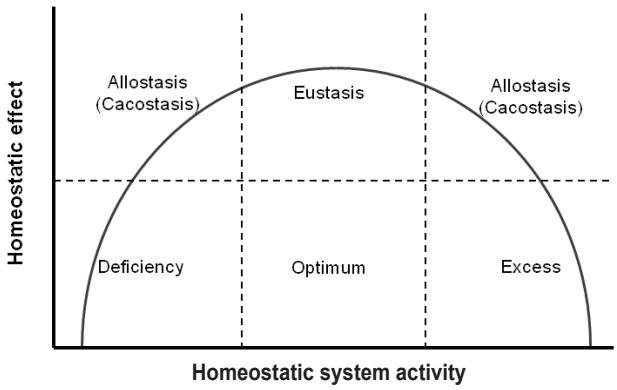

Figure 1. The inverted U-shaped dose-response curve. *Eustasis* is achieved in the middle range of the homeostatic system activity, whereas *allostasis* or *cacostasis* occurs when the homeostatic system activity is deficient or excessive.

PATHOPHYSIOLOGY AND CLINICAL MANIFESTATIONS

Primary Generalized Glucocorticoid Resistance is a rare familial or sporadic allostatic condition in which almost all organs have a different degree of insensitivity to glucocorticoids.[11-18] This decreased tissue responsiveness to glucocorticoids leads to compensatory activation of the HPA axis that causes hypersecretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland. The increased concentrations of ACTH cause adrenal cortex hypertrophy and activate the enzymatic biosynthetic pathway of cortisol, adrenal androgens [androstenedione, dehydroepiandrosterone (DHEA), and DHEA-sulfate (DHEAS)], and steroid precursors with mineralocorticoid activity (deoxycorticosterone and corticosterone).[11-18]

Patients with Primary Generalized Glucocorticoid

Table 1. Expected clinical manifestations in tissue-specific glucocorticoid excess or hypersensitivity and deficiency or resistance[11]

Target tissue	Glucocorticoid hypersensitivity = Glucocorticoid excess	Glucocorticoid resistance = Glucocorticoid deficiency
Central nervous system	Insomnia, anxiety, depression, defective cognition	Fatigue, somnolence, malaise, defective cognition
Liver	+ Gluconeogenesis, + lipogenesis	Hypoglycemia, resistance to diabetes mellitus
Fat	Accumulation of visceral fat (metabolic syndrome)	Loss of weight, resistance to weight gain
Blood vessels	Hypertension	Hypotension
Bone	Stunted growth, osteoporosis	
Inflammation/immunity	Immune suppression, anti-inflammation, vulnerability to certain infections and tumors	+ Inflammation, + autoimmunity, + allergy

Resistance may be asymptomatic or may present with clinical manifestations of mineralocorticoid and/or androgen excess. Therefore, hypertension and/or hypokalemic alkalosis can occur in patients with increased concentrations of steroid precursors with mineralocorticoid activity.[11-18] Adrenal androgen excess may cause ambiguous genitalia in karyotypic females, precocious puberty, acne, hirsutism, male-pattern hair loss, and hypofertility in both sexes, oligo-amenorrhea and menstrual irregularities in women, and oligospermia in men.[11-18] Glucocorticoid deficiency is rare and has been reported in adults with chronic fatigue,[16,19,20] in a child with hypoglycemic generalized tonic-clonic seizures during an episode of febrile illness,[21] and in a newborn with profound hypoglycemia, reported easy "fatigability" with feeding, and growth hormone deficiency.[22] It is worth noting that the increased CRH concentrations may cause anxiety and depression.[18]

The clinical heterogeneity of the condition is mostly due to differences in target tissue sensitivity to glucocorticoids, mineralocorticoids, and adrenal androgens among patients.[11-18] Furthermore, other molecules participating in steroid signaling pathways, such as hormone inactivating or -activating enzymes, immunophilins, and heat shock proteins, as well as genetic and epigenetic factors, may contribute substantially to variations in tissue response to steroid hormones.[15,17,18]

MOLECULAR PATHOGENESIS

In Generalized Glucocorticoid Resistance, the decreased target-tissue sensitivity to glucocorticoids has been primarily ascribed to inactivating point mutations, insertions or deletions in the *NR3C1* gene, which encodes the human glucocorticoid receptor (hGR).[11-18] For many years it was believed that the *NR3C1* gene encoded one protein. During the last three decades, this classic dogma changed dramatically with the demonstration that the alternative use of exon 9α or 9β of the *NR3C1* gene upon transcription generates the two main protein isoforms, the hGRα and the hGRβ, which have different properties in terms of localization, ligand-binding ability, and transcriptional activity.[23-27] Moreover, Lu and Cidlowski showed that the hGRα mRNA may be translated into eight receptor α isoforms (hGRα-A, hGRα-B, hGRα-C1, hGRα-C2, hGRα-C3, hGRα-D1, hGRα-D2, and hGRα-D3) because of the presence of eight alternative translation initiation sites.[28,29] It is likely that the hGRβ mRNA may also be translated into eight receptor β isoforms through the same molecular mechanisms.

The classic hGRα is a modular protein that consists of four functional domains: i) the amino-terminal or immunogenic domain (NTD), which is the largest domain of the receptor and consists of amino acids that undergo several post-translational modifications; ii) the DNA-binding domain (DBD), which contains the conserved motif of two zinc fingers enabling the receptor to bind to DNA sequences within the promoter regions of glucocorticoid-responsive genes; iii) the hinge region, which confers the appropriate structural flexibility to the receptor and contains critical lysine residues that undergo acetylation by the transcription factor CLOCK, the circadian locomotor output cycle kaput which, together with the brain-muscle-arnt-like protein 1 (BMAL1), regulate the circadian oscillations of gene expression; and iv) the ligand-binding domain (LBD), where the receptor binds to natural or synthetic glucocorticoids.[5,6,18,30] The LBD consists of twelve α helices and four β sheets and contains amino acid sequences important for the ligand-induced nuclear translocation of the receptor, as well as amino acids that interact with coactivators or corepressors in a ligand-dependent fashion.[5,6,18]

At the target cell, the glucocorticoid signaling cascade is triggered upon glucocorticoid-binding to the LBD of the receptor and leads to conformational changes that result in dissociation of the receptor from heat shock proteins and immunophilins (Figure 2).[5,6,18] The ligand-bound hGRα translocates into the nucleus, forms homo- or hetero-dimers, and binds to the specific DNA sequences, the glucocorticoid response elements (GREs), within the regulatory regions of glucocorticoid target genes, thereby inducing or repressing their expression. Alternatively, the activated hGRα can influence gene expression independently of DNA binding by physically interacting with other important transcription factors, such as the nuclear factor-κB (NF-κB), the activator protein-1 (AP-1), and signal transducers and activators of transcription (STATs) (Figure 2).[5,6,18]

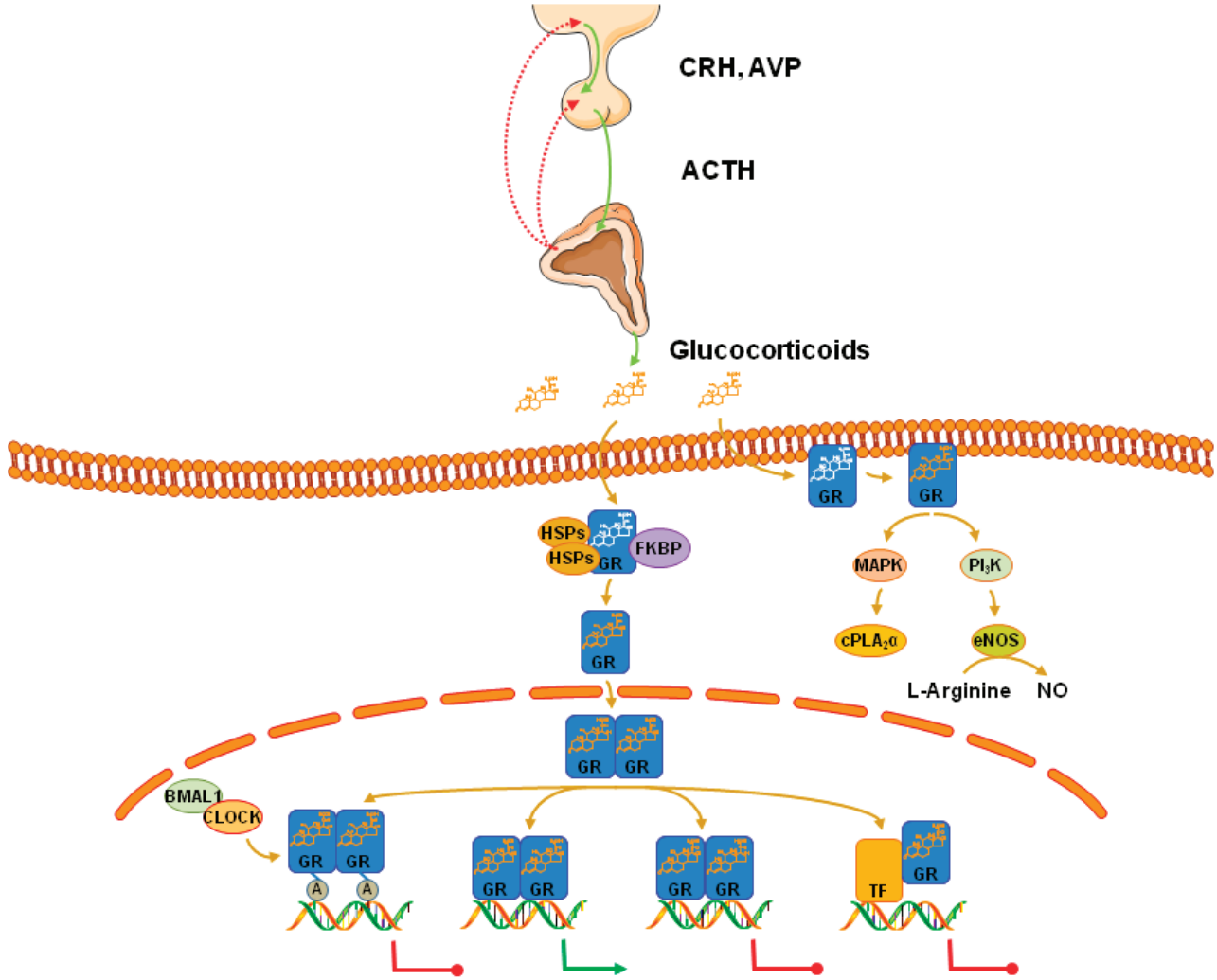

Figure 2. The HPA axis and the glucocorticoid signaling pathway. Upon stimulation of the HPA axis by numerous external or internal stressful stimuli, neurons of the paraventricular nuclei located in the hypothalamus release CRH and AVP, which both increase the production and secretion of ACTH by the anterior lobe of pituitary gland. ACTH then triggers the production of glucocorticoids, which reach every target-cell through the systemic circulation. In the target-cell, glucocorticoids bind to their cognate receptor which undergoes conformational changes, dissociates from HSPs and FKBPs, and translocates to the nucleus, where it binds as a homo- or heterodimer onto the GREs of target-genes, thereby inducing or repressing the expression of the latter. The hGR can alternatively regulate gene expression, independently of DNA binding, by physically interacting with other transcription factors (NF-κB, AP-1 or STAT5). Moreover, the hGR was recently shown to undergo acetylation by the transcription factor CLOCK in a lysine cluster of its hinge region. This CLOCK-mediated post-translational modification of the hGR may provide the basis for the circadian oscillations of glucocorticoid-target genes. In addition to their genomic actions, accumulating evidence suggests that glucocorticoids may induce some effects within seconds or minutes. These nongenomic glucocorticoid actions seem to be mediated by membrane-bound hGRs which activate the mitogen-activated protein kinase (MAPK) or the phosphatidylinositol 3-kinase (PI_3K) pathways. ACTH: adrenocorticotropic hormone; AVP: arginine-vasopressin; BMAL1: brain–muscle–arnt-like protein 1; CLOCK: circadian locomotor output cycle kaput; cPLA2α: cytosolic phospholipase A2 alpha; CRH: corticotropin-releasing hormone; eNOS: endothelial nitric oxide synthetase; FKBP: immunophilins; GR: glucocorticoid receptor; HSP: heat shock proteins; MAPK: mitogen-activated protein kinases; NO: nitric oxide; PI_3K: phosphatidylinositol 3-kinase; TF: transcription factor.

Primary Generalized Glucocorticoid Resistance is primarily caused by inactivating mostly heterozygous but also homozygous point mutations, insertions or deletions in the *NR3C1* gene that lead to a defective glucocorticoid receptor and impaired glucocorticoid signaling and cause generalized, partial tissue in-

sensitivity to glucocorticoids. Most of the reported *NR3C1* gene mutations are located in the LBD of the receptor, three of them, however, the hGRαV423A, the hGRαR469X and the hGRαR477H, having been identified in the DBD (Figure 3).[19,21,22,31-47] Over the last three decades, advances in molecular and structural biology have enabled the study of the molecular mechanisms through which the mutant hGRs impair glucocorticoid signal transduction and cause the variable clinical phenotype of Primary Generalized Glucocorticoid Resistance (Table 2).

THE MOLECULAR AND STRUCTURAL BIOLOGY OF THE NATURAL MUTANT RECEPTORS hGRαV423A, hGRαV575G AND hGRαH726R

We have recently identified three novel heterozygous inactivating point mutations in the *NR3C1* gene causing Primary Generalized Glucocorticoid Resistance and we have applied standard molecular and structural biology methods to elucidate the molecular mechanisms of action of the mutant receptors.[45-47] Specifically, we investigated: i) the ability of the mutant receptors to induce glucocorticoid-responsive genes through reporter assays; ii) the expression of the mutant receptors at the protein level via Western blotting; iii) the ability of the mutant receptors to exert a dominant negative effect upon the hGRα-mediated transcriptional activity using reporter assays; iv) the transrepressive activity of the mutant receptors upon the NF-κB-mediated transcriptional activity through reporter assays; v) the affinity of the mutant receptors for the ligand via dexamethasone-binding assays; vi) the subcellular localization of the mutant receptors in the absence of ligand and the time required to complete nuclear translocation following exposure to dexamethasone using green fluorescent protein (GFP)-fused plasmids; vii) the binding of the mutant receptors to GREs through *in vitro* binding assays; viii) the ability of the mutant receptors to interact with coactivators, such as the glucocorticoid receptor-interacting protein 1 (GRIP1) coactivator, using Glutathione-S-Transferase (GST)-pull down assays; and ix) the conformational changes of the mutant receptors causing Primary Generalized Glucocorticoid Resistance through computer-based 3-dimensional simulation using crystallographic data available in public.[45-47]

The first patient was a 9-year-old boy who presented with anxiety, fatigue, and hypertension.[45] He harbored a novel heterozygous mutation in the *NR3C1* gene that resulted in substitution of valine (V) by alanine (A) at amino acid position 423 in the LBD of the receptor.[45] *In vitro* functional studies showed that

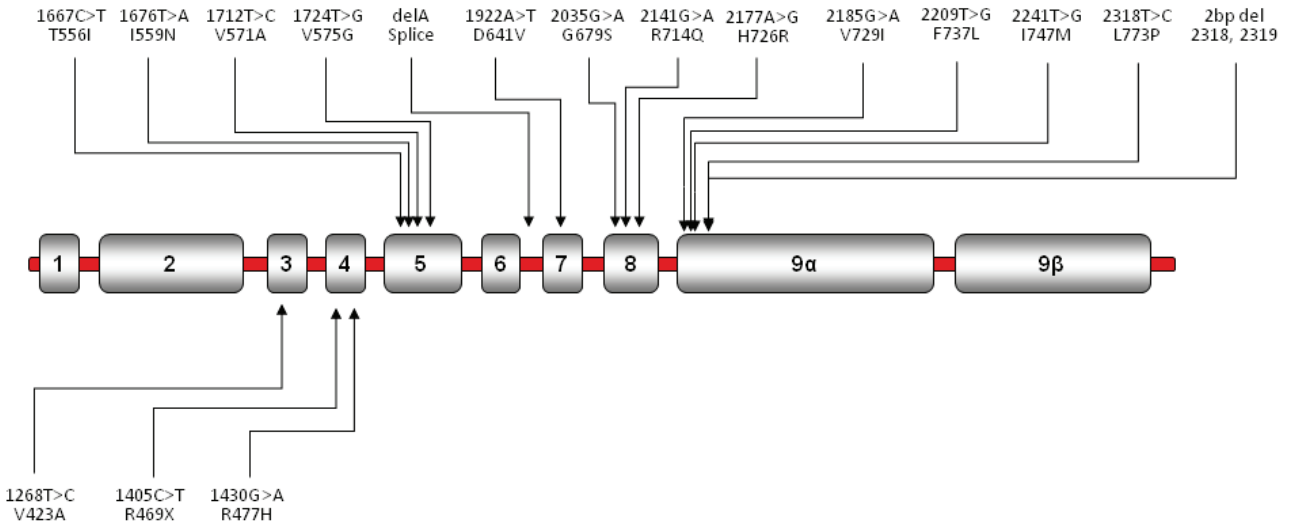

Figure 3. Schematic representation of the known mutations of the NR3C1 gene causing Primary Generalized Glucocorticoid Resistance. Mutations in the upper panel are located in the LBD of the receptor, while the V423A, R469X, and R477H mutations are located in the DBD of the receptor.

Table 2. Mutations of the human glucocorticoid receptor gene causing Primary Generalized Glucocorticoid Resistance

Author (Reference)	Mutation position		Molecular mechanisms	Genotype	Phenotype
	cDNA	Amino acid			
Chrousos et al[19] Hurley et al[32] Charmandari et al[39]	1922 (A→T)	641 (D→V)	Transactivation ↓ Affinity for ligand ↓ (x 3) Nuclear translocation: 22 min Abnormal interaction with GRIP1	Homozygous	Hypertension Hypokalemic alkalosis
Karl et al[33]	4 bp deletion in exon-intron 6		hGRα number: 50% of control Inactivation of the affected allele	Heterozygous	Hirsutism Male-pattern hair-loss Menstrual irregularities
Malchoff et al[34] Charmandari et al[39]	2185 (G→A)	729 (V→I)	Transactivation ↓ Affinity for ligand ↓ (x 2) Nuclear translocation: 120 min Abnormal interaction with GRIP1	Homozygous	Precocious puberty Hyperandrogenism
Karl et al[31] Kino et al[35] Charmandari et al[39]	1676 (T→A)	559 (I→N)	Transactivation ↓ Decrease in hGR binding sites Transdominance (+) Nuclear translocation: 180 Abnormal interaction with GRIP1	Heterozygous	Hypertension Oligospermia Infertility
Ruiz et al[36] Charmandari et al[41]	1430 (G→A)	477 (R→H)	Transactivation ↓ No DNA binding Nuclear translocation: 20 min	Heterozygous	Hirsutism Fatigue Hypertension
Ruiz et al.[36] Charmandari et al[41]	2035 (G→A)	679 (G→S)	Transactivation ↓ Affinity for ligand ↓ (x 2) Nuclear translocation: 30 min Abnormal interaction with GRIP1	Heterozygous	Hirsutism Fatigue Hypertension
Mendonca et al[37] Charmandari et al[39]	1712 (T→C)	571 (V→A)	Transactivation ↓ Affinity for ligand ↓ (x 6) Nuclear translocation: 25 min Abnormal interaction with GRIP1	Homozygous	Ambiguous genitalia Hypertension Hypokalemia Hyperandrogenism
Vottero et al[38] Charmandari et al[39]	2241 (T→G)	747 (I→M)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (x 2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Cystic acne Hirsutism Oligo-amenorrhea
Charmandari et al[40]	2318 (T→C)	773 (L→P)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (x 2.6) Nuclear translocation: 30 min Abnormal interaction with GRIP1	Heterozygous	Fatigue Anxiety Acne Hirsutism Hypertension
Charmandari et al[42]	2209 (T→C)	737 (F→L)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (x 1.5) Nuclear translocation: 180 min	Heterozygous	Hypertension Hypokalemia
McMahon et al[22]	2 bp deletion at nt 2318-9	773	Transactivation ↓ Affinity for ligand: absent No suppression of IL-6	Homozygous	Hypoglycemia Fatigability with feeding Hypertension

Table 2. (continued) Mutations of the human glucocorticoid receptor gene causing Primary Generalized Glucocorticoid Resistance

Author (Reference)	Mutation position		Molecular mechanisms	Genotype	Phenotype
	cDNA	Amino acid			
Nader et al[21]	2141 (G→A)	714 (R→Q)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (x 2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Hypoglycemia Hypokalemia Hypertension Mild clitoromegaly Advanced bone age Precocious pubarche
Bouligand et al[43]	1405 (C→T)	469 (R→X)	Transactivation ↓ Ligand-binding sites ↓ No DNA binding No nuclear translocation	Heterozygous	Adrenal hyperplasia Hypertension Hypokalemia
Zhu Hui-juan et al[44]	1667 (G→T)	556 (T→I)	Not studied yet	Heterozygous	Adrenal incidentaloma
Roberts et al[45]	1268 (T→C)	423 (V→A)	Transactivation ↓ Affinity for ligand: N No DNA binding Nuclear translocation: 35 min Interaction with GRIP1: N	Heterozygous	Fatigue Anxiety Hypertension
Nicolaides et al[46]	1724 (T→G)	575 (V→G)	Transactivation ↓ Transrepression Affinity for ligand ↓ (x 2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Melanoma Asymptomatic daughters
Nicolaides et al[47]	2177 (A→G)	726 (H→R)	Transactivation ↓ Transrepression ↓ Affinity for ligand ↓ (x 2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Hirsutism, Acne, Alopecia, Anxiety, Fatigue Irregular menstrual cycles

the hGRαV423A displayed reduced transcriptional activity, had a significant reduction in its ability to bind to DNA sequences within the promoter regions of glucocorticoid-target genes, and required a longer time to translocate into the nucleus following exposure to dexamethasone, compared with the wild-type receptor (Figure 4A and 4B).[45] Structural biology studies highlighted the critical role of the hydrophobic valine at this position within the first zinc finger of the DBD of the receptor. The hydrophobic nature of valine at amino acid position 423 protects the four zinc-binding cysteines (C421, C424, C438, and C441) from the destructive diffusion of water molecules. The substitution of valine by alanine results in water diffusion into the ion-binding region of the mutant receptor and causes reduced binding of the mutant receptor hGRαV423A to GREs.[45]

The second mutation in the *NR3C1* gene was identified in a 70-year-old man and his two daughters, who had increased urinary free cortisol excretion and showed resistance of the HPA axis to dexamethasone suppression without any symptoms or signs suggestive of Cushing syndrome.[46] Sequencing of the *NR3C1* gene revealed a substitution of valine (V) by glycine (G) at amino acid 575 in the LBD of the receptor.[46] Compared with the wild-type receptor, the hGRαV575G had 50% lower affinity for dexamethasone, displayed reduced transactivation of glucocorticoid-responsive genes, had a 2.5-fold delay in nuclear translocation, and interacted with the GRIP1 coactivator mostly through its AF-1

domain (Figure 4C).[46] This impaired interaction of the mutant receptor with the GRIP1 coactivator was further confirmed by structural biology assays which showed that the substitution of valine by glycine at amino acid position 575 resulted in the loss of two noncovalent bonds observed between the valine of the wild-type receptor and the LXXLL motif of the GRIP1 coactivator. Finally, the hGRαV575G demonstrated significantly increased ability to transrepress NF-κB-responsive genes (Figure 4C).[46]

The last point mutation in the *NR3C1* gene was identified in a 30-year-old woman with hirsutism, acne, alopecia, anxiety, fatigue, and irregular menstrual cycles without any clinical features of Cushing syndrome.[47] Endocrinologic evaluation revealed elevated 08:00 h plasma ACTH, serum cortisol concentrations, and increased urinary free cortisol (UFC) excretion. There was resistance of the HPA axis to overnight dexamethasone suppression, while a pituitary magnetic resonance imaging scan was normal.[47]

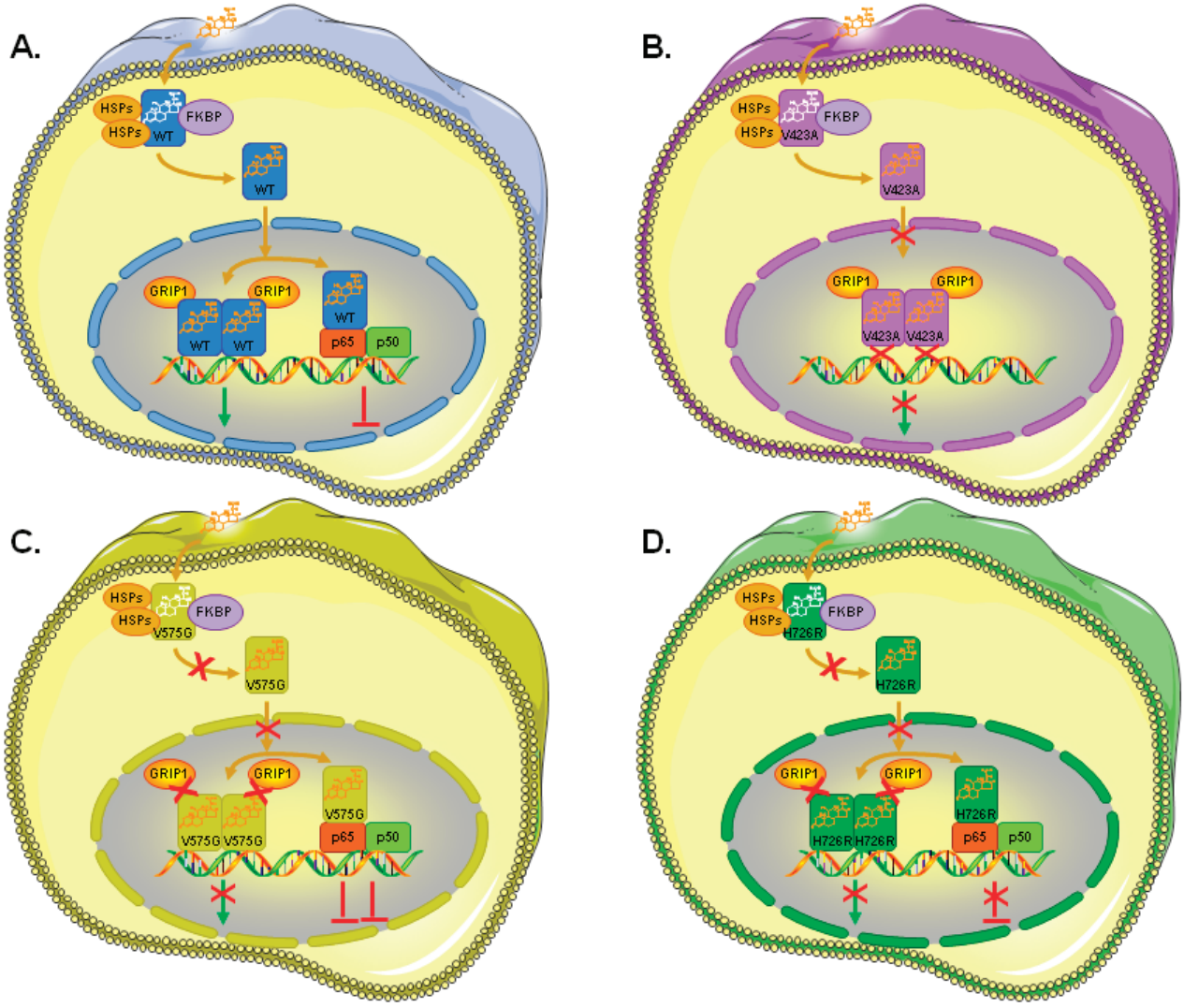

Figure 4. Molecular mechanisms of action of the mutant receptors hGRαV423A, hGRαV575G, and hGRαH726R, compared with the wild-type hGRα. **(A)** The hGRαWT-mediated signal transduction. **(B)** Molecular mechanisms of action of the hGRαV423A. **(C)** Molecular mechanisms of action of the hGRαV575G. **(D)** Molecular mechanisms of action of the hGRαH726R. FKBP: immunophilins; GRIP1: glucocorticoid receptor-interacting protein 1; H726R: human glucocorticoid receptor H726R; HSP: heat shock proteins; p50: transcription factor p50; p65: transcription factor p65; V423A: human glucocorticoid receptor V423A; V575G: human glucocorticoid receptor V575G; WT: wild-type human glucocorticoid receptor.

A novel heterozygous point mutation was identified in the *NR3C1* gene, which resulted in histidine (H) to arginine (R) substitution at amino acid position 726 in the LBD of the receptor.[47] We subsequently elucidated the molecular mechanisms of action of the mutant receptor hGRαH726R causing Primary Generalized Glucocorticoid Resistance. The hGRαH726R displayed reduced ability to transactivate target genes and to transrepress NF-κB-responsive genes, had 55% lower affinity for the ligand and a 4-fold delay in cytoplasmic-to-nuclear translocation following dexamethasone-induced activation, and interacted with the GRIP1 coactivator mostly through its AF-1 domain (Figure 4D).[47] Structural biology studies showed that the H726R mutation caused a structural shift in the rigidity of helix 10 within the LBD of the receptor, which resulted in reduced flexibility and decreased affinity of the mutant receptor for the ligand.[47]

DIAGNOSIS

When Primary Generalized Glucocorticoid Resistance is suspected, a detailed personal and family history should be obtained, placing particular emphasis on any clinical manifestations indicating alterations in the activity of the HPA axis.[11-13,15-18] Symptoms such as seizures, headaches or visual impairment should be carefully evaluated. The irregularity of menstrual cycles in women should be methodically documented. Furthermore, the growth, development and pubertal stage in children should be assessed. On clinical examination, particular attention should be paid to signs suggestive of mineralocorticoid and/or androgen excess.[11-13,15-18]

The endocrinologic evaluation includes determination of the 08:00h concentrations of serum cortisol, plasma ACTH, plasma renin activity (recumbent), serum aldosterone, androgens (testosterone, androstenedione, DHEA, DHEAS), and insulin.[11-13,15-18] The biochemical evaluation consists of measurement of the 08:00h concentrations of total cholesterol, HDL, LDL, triglycerides, and fasting glucose.[11-13,15-18] Patients with Primary Generalized Glucocorticoid Resistance have increased 24-hour serum cortisol concentrations and elevated 24-hour UFC excretion despite the absence of Cushingoid features; therefore, the 24-h UFC excretion should be determined on 2 or 3 consecutive days to enable accurate diagnosis of the condition.[11-13,15-18] It is also important to note that patients may display significant variations in the 24-hour UFC excretion and serum cortisol concentrations owing to variations in the impairment of glucocorticoid signal transduction. Indeed, serum cortisol concentrations may be up to 7- fold higher compared with the highest value of its normal range, while the 24-hour UFC excretion may be up to 50-fold higher when compared with the upper normal range. In addition, the 08:00h plasma ACTH concentrations may be normal or high, while the circadian pattern of secretion of both ACTH and cortisol, as well as their responsiveness to any external or internal stressful stimuli, are normal.[11-13,15-18]

The dexamethasone suppression test remains one of the most useful diagnostic tools to evaluate the responsiveness of the HPA axis and to determine the appropriate dose to be administered when treatment is commenced.[11-13,15-18] To this end, increasing doses of dexamethasone (0.3, 0.6, 1.0, 1.5, 2.0, 2.5, 3.0 mg) are administered *per os* at midnight every other day and serum cortisol and dexamethasone concentrations are determined at 08:00h the following morning. It is also important to determine the serum concentrations of dexamethasone concurrently in order to exclude the possibility of non-adherence to treatment, increased metabolic clearance or reduced absorption of the medication.[18] The HPA axis may display significant variation in its resistance to dexamethasone suppression depending on the impairment of the glucocorticoid signal transduction. The dose of dexamethasone required to suppress serum cortisol concentrations by 50% may be up to 7.5-fold higher than that required to achieve the same degree of HPA axis suppression in normal subjects.[18]

Dexamethasone-binding assays and thymidine incorporation assays remain the two main molecular biology methods that confirm the diagnosis of Primary Generalized Glucocorticoid Resistance.[11-13,15-18] In dexamethasone-binding assays, the administered tritiated dexamethasone binds to the mutated hGR of the patient's peripheral leukocytes with lower affinity compared with the wild-type hGR of the control subject in patients with mutations in the LBD of the receptor. In thymidine incorporation assays, the patient displays higher resistance to suppression of phytohemaglutinin-stimulated thymidine incorporation in response to dexamethasone compared with

the control subject. Finally, *NR3C1* gene insertions, deletions or mutations are identified by sequencing of the coding region (including the intron-exon junctions) of the gene in most but not all subjects with the condition.[11-13,15-18]

TREATMENT

The main aim of treatment in primary generalized glucocorticoid resistance is to suppress the increased secretion of ACTH, thereby suppressing the increased production of adrenal steroids with mineralocorticoid and androgenic activity. Treatment involves administration of high doses of mineralocorticoid-sparing synthetic glucocorticoids, such as dexamethasone (1-3 mg given once daily at night), which activate the mutant and/or wild-type hGRα and suppress the endogenous secretion of ACTH in affected subjects.[11-13,15-18] Clinicians should carefully titrate the dose of dexamethasone according to the clinical manifestations and biochemical profile of the patients. It is important to achieve adequate suppression of the HPA axis to prevent the development of an ACTH-secreting adenoma.[11-13,15-18]

BEYOND *NR3C1* GENE MUTATIONS: PRIMARY GENERALIZED GLUCOCORTICOID RESISTANCE IN THE ERA OF NEXT-GENERATION SEQUENCING

Although the clinical manifestations of primary generalized glucocorticoid resistance are primarily caused by point mutations, insertions or deletions in the *NR3C1* gene encoding defective hGRs, some patients with this condition do not harbor any *NR3C1* gene mutations, suggesting a possible role of other genes encoding proteins involved in the glucocorticoid signaling pathway or important hGR partners. One such protein is the FK506-Binding Immunophilin FKBP51, which forms a heterocomplex with the hGRα in the absence of glucocorticoids and is responsible for the cytoplasmic localization of the receptor. Interestingly, some New World primates had elevated expression of FKBP51 and decreased levels of FKBP52, which both contributed to the phenotype of glucocorticoid resistance.[48] It was subsequently shown that FKBP51 and FKBP52 have opposite effects in nuclear translocation of GR in mammalian cells, indicating that any imbalance between them could ultimately lead to glucocorticoid resistance or hypersensitivity.[49] In addition to the FKBP proteins, the chaperone proteins HSP90 and HSP70 are thought to play a role in determining tissue sensitivity to glucocorticoids. However, their role in glucocorticoid resistance is controversial, given that only a few studies have shown an association between abnormal expression of HSP90/HSP70 and glucocorticoid resistance.[50-53] In the era of next-generation sequencing, when a patient is suspected of having primary generalized glucocorticoid resistance, we suggest sequencing of the *NR3C1* gene as well as of other genes that encode proteins known to be involved in the glucocorticoid signaling cascade. The application of novel technologies, such as whole-exome sequencing and whole genome sequencing, may uncover other causes of Primary Generalized Glucocorticoid Resistance that may relate to the pathogenesis of this condition.

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