GLUCOCORTICOID RECEPTOR VARIANTS MODULATE THE SENSITIVITY TO CORTISOL

Cover: De stropdas is als het glucocorticoïdreceptor gen; het dragen van varianten geeft verschillende effecten.
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Glucocorticoid Receptor Variants Modulate the Sensitivity to Cortisol

Glucocorticoïdreceptor varianten beïnvloeden de gevoeligheid voor cortisol

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-wat zo verborgen is omzichtig naar boven te halen,
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wat zo veelomvattend is in het geheugen op te slaan,
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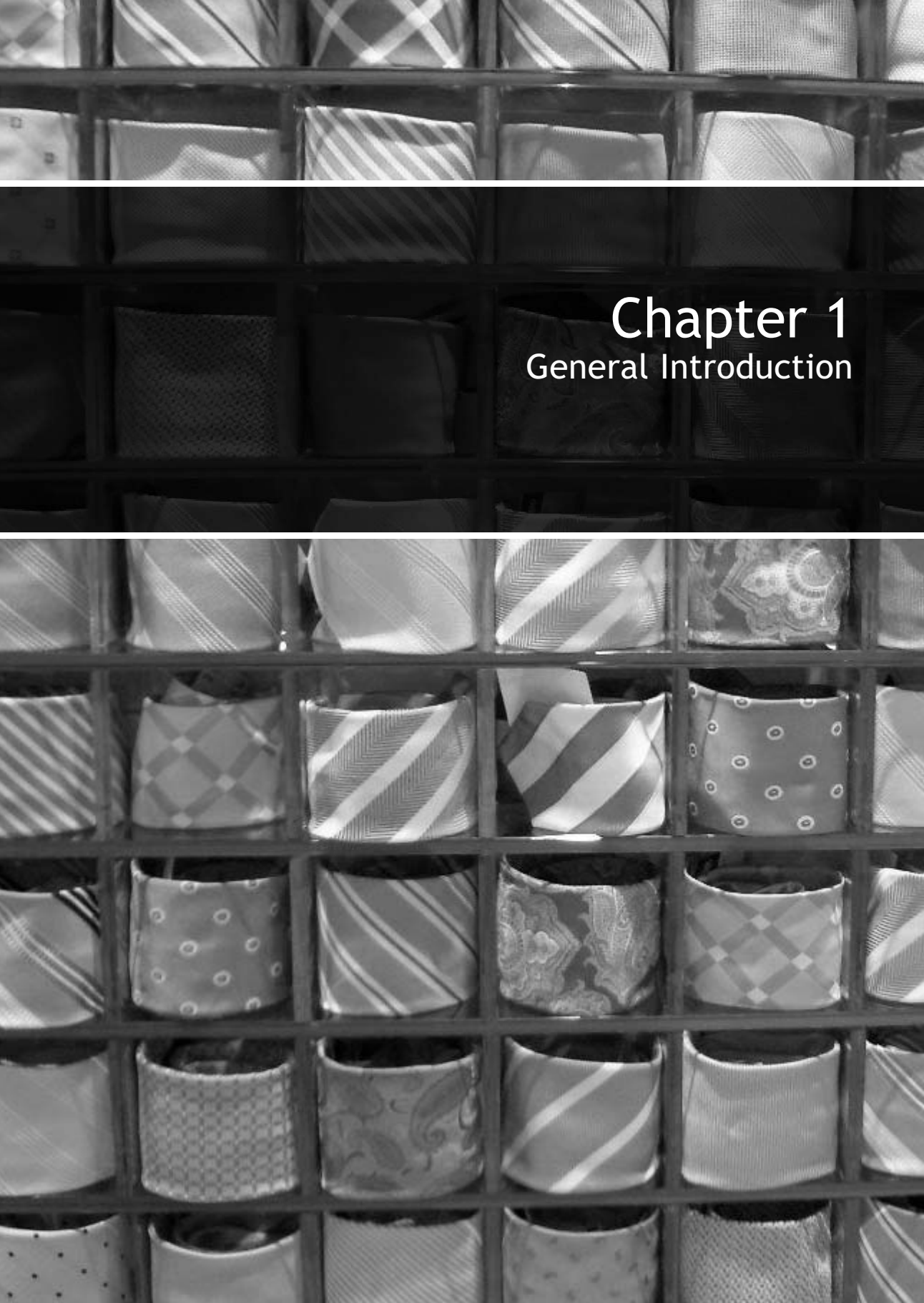
Desiderius Erasmus
Uit: "Lof van de Geneeskunde"

voor Annekee

Contents



Chapter 1
General Introduction

1.1 Glucocorticoids

The major glucocorticoid (GC) in man is cortisol and it's secretion increases in response to any stress in the body, whether physical (such as illness, trauma, surgery, or temperature extremes) or psychological (1, 2). However, this hormone is more than a simple marker of stress levels; it is necessary for the correct functioning of almost any part of the body. Excesses or deficiencies of this crucial hormone lead to various physical symptoms and disease states (3).

Cortisol and its synthetic analogs are also widely employed in the treatment of systemic autoimmune conditions. GCs were first successfully used in the treatment of rheumatoid arthritis in 1948 (4), and since this breakthrough, GCs have become some of the most important and frequently prescribed drugs in the control of acute and chronic inflammation, allergies and autoimmune diseases, in the treatment of leukemia and lymphomas and in the prevention of graft rejection after organ transplantation (5).

1.1.1 Glucocorticoid biosynthesis

GCs, and other steroids are generated in the adrenal glands by a process referred to as steroidogenesis (6). Adrenal glands are triangular organs situated above the kidneys and are composed of two parts: the cortex (outer part) and medulla (inner part) (Figure 1). The cortex, which makes up more than three-quarters of the adrenal mass, is made of three zones: the zona glomerulosa, the zona fasciculata, and the zona reticularis, and each zone has the capacity to secrete its own specific steroids. The fasciculata and reticularis zonae synthesize GCs and androgenic steroids, respectively, and the zona glomerulosa synthesizes aldosterone (7). Steroidogenesis starts with the non-steroid cholesterol and the subsequent biosynthetic pathways leading to cortisol, androgenic steroids, and aldosterone are outlined in Figure 2 (6). Cortisol is secreted from the adrenal cortex into the blood stream, were it is bound with high affinity to cortisol-binding globulin (CBG) (80-90%) and with low affinity to albumin (6-15%), whereas the remainder (4-5%) is unbound. The albumin-bound and free cortisol fractions are supposed to be biologically active, as they are readily available to cells (8).

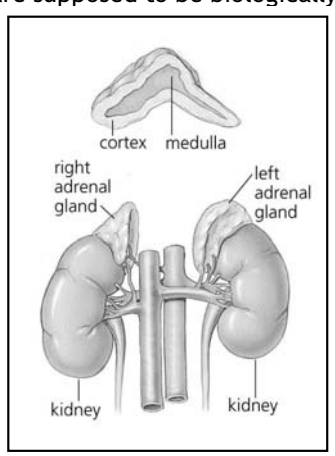


Figure 1
The adrenal glands are triangular organs situated above the kidneys and composed of the cortex and medulla.

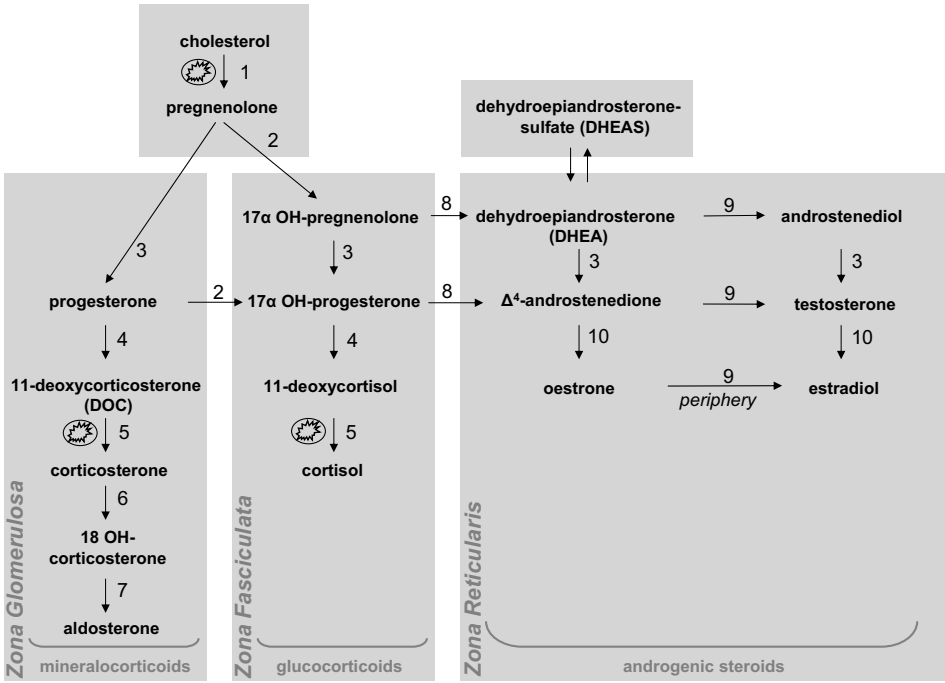


Figure 2 Biosynthesis of steroids in the adrenal cortex.
Mineralocorticoids are syntesized in the zona glomerulosa; Glucocorticoids are syntesized in the zona fasciculata; Androgenic steroids are synthesized in the zona reticularis. Conversions marked with occur in mitochondria, while the remaining reactions occur in the microsomal compartment. The numbers represent the enzymes involved in synthesis of the steroids: (1) StAR: steroidogenic acute regulatory protein and CYP11A1: cholesterol side chain cleavage enzyme. (2) CYP17: 17α-hydroxylase. (3) 3β-HSD-II: 3β-hydroxysteroid dehydrogenase enzyme II. (4) CYP21A2: 21-hydroxylase. (5) CYP11B1: 11β-hydroxylase. (6) CYP11B2: 18-hydroxylase. (7) 18-methyl-oxidase. (8) CYP17: 17,20-lyase. (9) 17β-HSD: 17β-hydroxysteroid dehydrogenase. (10) CYP19: aromatase.

1.1.2 Control of glucocorticoid release; the Hypothalamic-Pituitary-Adrenal-axis

Cortisol secretion and the regulation of the amount of cortisol in the bloodstream is controlled by an elaborate feedback system (Figure 3). Cortisol is secreted in response to adrenocorticotrophic hormone (ACTH), which is synthesized and released from the pituitary, a small organ at the base of the brain. ACTH is derived from the precursor molecule proopiomelanocortin (POMC) and binds to a G-protein-coupled ACTH-receptor in the plasma membrane of cells in the zona fasciculata, leading to elevated intracellular levels of cyclic AMP. cAMP ultimately leads to activation of enzyme systems involved in the biosynthesis of, among other steroids and mineralocorticoids, cortisol from cholesterol. ACTH is secreted under the control of hypothalamic peptide corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) in the central nervous system. The whole HPA-axis system is controlled by the circadian rhythm (cortisol levels are highest in the morning and lowest at night) and stressful events (physical and physiological). When cortisol is present in adequate (or excess) amounts, a negative feedback system operates on

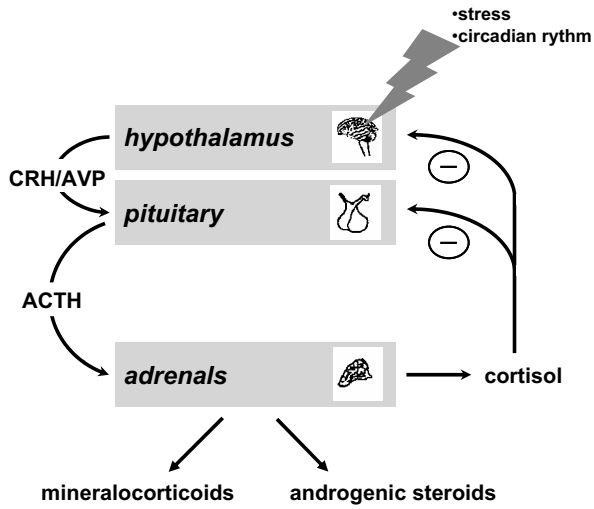


Figure 3 The Hypothalamic-Pituitary-Adrenal axis.
Feedback inhibition of stress/circadian rhythm induced corticotrophin-releasing hormone (CRH)-, arginine vasopressin (AVP)- and adrenocorticotropic hormone (ACTH)-mediated production and secretion of cortisol. CRH and AVP released by the hypothalamus stimulate the pituitary to produce ACTH, which in turn stimulates adrenal cortisol synthesis and release. To maintain homeostasis, cortisol feeds back on this pathway to achieve a perfect balance between cortisol need and production.

the pituitary and hypothalamus, alerting these areas to reduce the output of ACTH and CRH, respectively, in order to reduce cortisol secretion to normal homeostatic levels. In this way, a perfect balance between need and production could be achieved (1, 9-11).

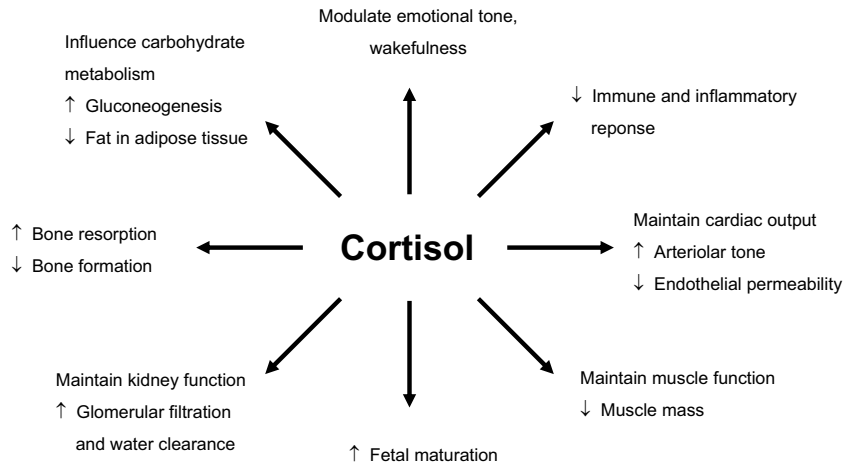


Figure 4 Overview of the actions of cortisol on various tissues, organs, and systems.

1.1.3 Effects of glucocorticoids

The effects of cortisol are summarized in Figure 4. The name glucocorticoid derives from early observations that these hormones were involved in glucose metabolism by enhancing the expression of key enzymes involved in the gluconeogenesis. Cortisol also stimulates the breakdown of fat in adipose tissue by enhancing the action of lipolytic agents such as adrenaline and growth hormone (12, 13). Separate from its metabolic function, cortisol influences the immune and inflammatory systems by decreasing the activity and production of immunomodulatory and inflammatory cells (14). Furthermore, cortisol influences fetal maturation (15, 16), maintains the contractility and work performance of skeletal and cardiac muscle (17, 18), maintains vascular tone (19), influences bone formation and kidney function (20, 21), and is known to alter mood, behavior and sleeping patterns (11, 22).

1.1.4 Mechanism of glucocorticoid action

Cortisol, as well as its synthetic analogs, exerts its effects by binding to the GC receptor (GR). In the absence of ligand, the receptor resides predominantly in the cytoplasm in a complex that includes heat-shock protein 90 (hsp90), which maintains the receptor in a form able to bind ligands. Upon ligand binding, the

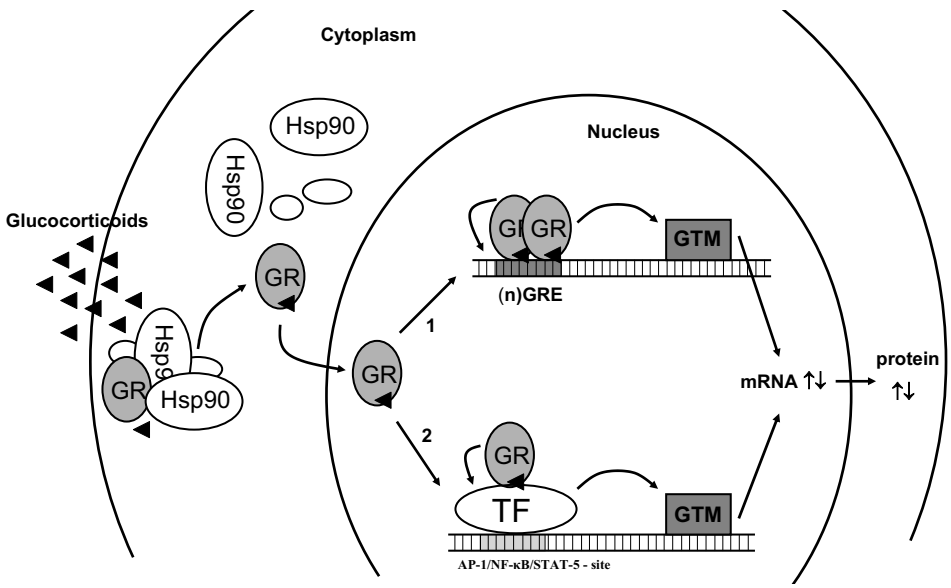


Figure 5 Simplified model of GR-mediated transcriptional modulation.
Glucocorticoids enter the cell by passive diffusion. Upon glucocorticoid binding, the GR dissociates from an Hsp90 containing multi-protein complex and translocates to the nucleus. Once there, GR modulates target gene transcription via (1) direct interaction with (negative) glucocorticoid response elements (GRE) as a dimer and (2) cross-talk with other DNA-bound transcription factors such as AP-1, NF-κB. The resulting modulation of target gene transcription leads to altered protein expression. GTM, general transcription machinery; TF, other transcription factors.

complex dissociates, whereafter the GR moves into the nucleus and regulates the transcriptional activity of hormonally responsive genes. Depending on the gene, this can be positive or negative and is accomplished through a variety of mechanisms (Figure 5). The GR acts as a homodimer by binding to conserved DNA motifs known as GC response elements (GREs). The recruitment of transcriptional coactivators, whose activities alter chromatin structure, further activates (GRE) or represses (negative GRE) gene expression. Alternatively, negative regulation can also be the result of protein-protein interactions with other transcription factors, such as activating protein-1 (AP-1) and nuclear factor-κB (NF-κB). In this pathway, the GR acts as a monomer, independently of direct binding to DNA, by inhibiting their ability to bind to DNA or activate the transcription apparatus (23-25). Also non-genomic actions of glucocorticoids are reported, which are mostly much faster than the genomic effects (26), but this aspect falls outside the scope of this thesis.

1.2 The Glucocorticoid Receptor

The GR is a member of the nuclear receptor family of ligand-regulated transcription factors. This family consists of receptors with identified ligands, including receptors for small hydrophobic molecules, such as the steroid hormones, thyroid hormones, vitamine D, retinoic acids, and metabolites of long-chain fatty acid, as well as a number of orphan receptors, for which still no endogenous ligands have been identified (27). The GR (NR3C1) gene is located on chromosome 5 (5q31) and is one of the approximately 30,000 genes of the human genome (28,29). This number of genes is surprisingly low compared to other species (The *Drosophila melanogaster* already has 15,000 genes and the mouse has only 300 genes less than the *Homo sapiens*). On the other hand, from one (human) gene, often a broad range of proteins can be formed, all having their own function. The use of different promoters, alternative mRNA splicing, alternative translation, and posttranslational modifications results in a broad diversity of GR proteins, which are all produced from one single gene (30, 31).

1.2.1 Glucocorticoid receptor structure

In common with the other nuclear receptors, the GR has a modular structure of three functionally separable domains: the amino-terminal transactivation domain (TAD), the central DNA-binding domain (DBD), and the carboxy-terminal ligand-binding domain (LBD) (Figure 6)(32-34).

The TAD contains an amino acid sequence (residues 77-262) important for constitutive transactivation, called tau1 (τ_1) or activating function-1 (AF-1). A smaller fragment comprises the activation core (residues 187-244) and is called τ_1c and represents the only autonomous transactivation activity within the larger AF-1 fragment (33, 35, 36).

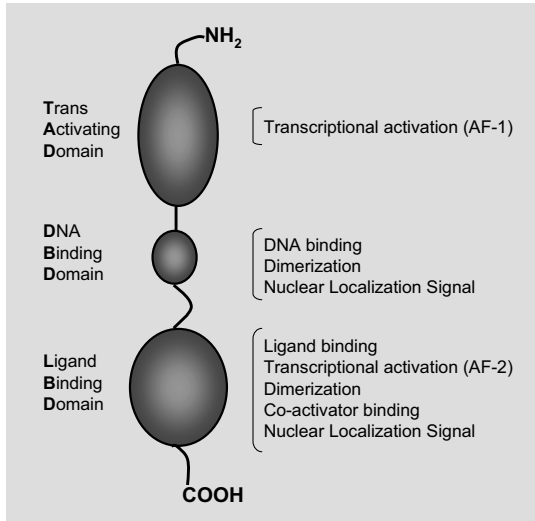


Figure 6 Modular structure of the GR. The GR consist of three functional domains with their specific functions as indicated (see also Figure 7).

The DBD is responsible for the interaction with DNA, and especially the response elements GRE and nGRE. The consensus sequence of the GRE is the palindromic 15 bp sequence 5'GGTACAnnnTGTTCT3' (where n is any nucleotide). The DBD contains two tetrahedral arrangements, each formed by four cysteines chelated to a single Zn^{2+} ion. These "zinc-clusters" contribute to the folding of this domain and consist of a region responsible for GRE-recognition (P-box) and a region responsible for dimerization (D-box). The carboxy-terminus of the DBD is rich in basic amino acids and is partially responsible for nuclear localization of the receptor, called the nuclear localization signal (NLS) (37-39).

The LBD contains a number of subdomains specifying not only the steroid-binding region, but also other functions including hsp-90 binding, a second transactivation subdomain (τ_2 or AF-2), a second NLS-2 subdomain, and subdomains that interact with other transcription factors such as AP-1 (c-jun/c-fos) and NF-κB. The LBD contains 11 α-helices and 4 small β-strands (41). Ligand binding induces a rearrangement of the most carboxy-terminal helix, closing the ligand-binding pocket and generating the binding site for co-activators (40-45).

1.2.2 Multiple promoters

The GR gene consists of 9 different exons (Figure 7) with exon 1 and the first part of exon 2 as the 5'-untranslated region (5'-UTR). Exon 2 contains the translation start site, while exon 9 contains the translation stop site and a large 3'-untranslated region (3'-UTR) (34). Exon 2 encodes the TAD, exon 3 and 4 the DBD, while exon 5-9 encodes the LBD. Several alternative exons 1 were identified and designated as exon 1A, 1B and 1C, which are each preceeded by their own promoter (46, 47). Moreover, exon 1A contains three separate alternative splice sites, resulting in

mRNA transcripts containing exons 1A1, 1A2, or 1A3, which means that at least 5 GR transcripts are expressed from three separate promoters (47). Footprint and functional analysis of the promoter regions reveal unique binding sites for several transcription factors, but only promoter 1A harbors a sequence resembling a GRE and could probably be autoregulated by the GR (48-50). Expression of 1A1, 1A2, 1B and 1C containing GR mRNA was found at various levels in a wide variety of cancer cell lines (47), while significant amounts of 1A3 containing transcripts were found in cell lines derived from hematological malignancies (47, 51). Although the regulation and downstream function of this promoter heterogeneity remains largely unclear, it is suggested that these differences might indicate that GR expression is cell type-specifically regulated (52).

1.2.3 Alternative splicing

Alternative splicing of the ligand binding domain of pre-mRNA GR transcript gives rise to various splice variants of the GR protein. The first two, termed GR-α and GR-β, were directly identified when the GR cDNA was cloned in 1985 (32). More recently, several additional splice variants have been described: GR-P, GR-γ, and GR-A (53-55).

1.2.3.1 GR-α and GR-β

GR-α is the 777-amino-acid protein and it is the functionally active isoform, mediating the effects of GCs on transactivation and transrepression. GR-α is the most abundant isoform and the two predominant GR-α transcripts observed on Northern blots are a 7.0 kb mRNA containing the full-length 3'-UTR, and a 5.5 kb mRNA containing only about 2.4 kb 3'-UTR. A GR-β mRNA transcript is generated by an alternative splicing pathway. In the default splicing pathway, the end of exon 8 is linked to the beginning of exon 9, resulting in the two GR-α transcripts (Figure 7). The GR-β mRNA transcript is generated by a splicing pathway, which links the end of exon 8 to downstream sequences in exon 9. Translation of the transcripts results in GR-α and GR-β proteins, which are identical through amino acid 727, but diverge in the carboxy-terminus, with GR-α having an additional 50 amino acids and GR-β having an additional unique 15 amino acids. This results in the absence of the last α-helix in the LBD of the GR protein, in the inability of GR-β to bind ligand, and hence in the lack of transactivating capacity of GR-β for GC-responsive genes (56, 57). In addition, GR-β does not repress the transcriptional activity of other transcription factors, such as NF-κB. The biological function of GR-β is not understood as yet; however, experimental evidence has shown that GR-β can exert a dominant negative effect on transactivation by GR-α (56, 58). The unique GR-β sequence contributes to this dominant negative function. Moreover, these 15 amino acids keep GR-β constitutively localized in the nucleus, and contrary to GR-α, GR-β does not undergo ligand-induced downregulation, further increasing the dominant negative capacity of GR-β on gene transcription (56, 57). Several possible mechanisms responsible for

this dominant negative effect have been suggested, including the following: (1) GR-β competes with GR-α for GRE binding (56), (2) GR-β forms trancriptionally inactive heterodimers with GR-α (56, 59), and (3) GR-β competes to bind to co-activators needed by GR-α for transcriptional activity (56, 60). GR-β mRNA transcript and protein have been detected in many human tissues, but at a considerably lower level than GR-α (0.2-1% of total GR) (56, 61-63). Furthermore, an abnormal level of GR-β, or GR-α to GR-β ratio, has been found in GC-resistant patients (64-70). Interestingly, mice totally lack GR-β (71). Nevertheless, the biological function of the GR-β isoform is still controversial (61, 72-76).

1.2.3.2 GR-P

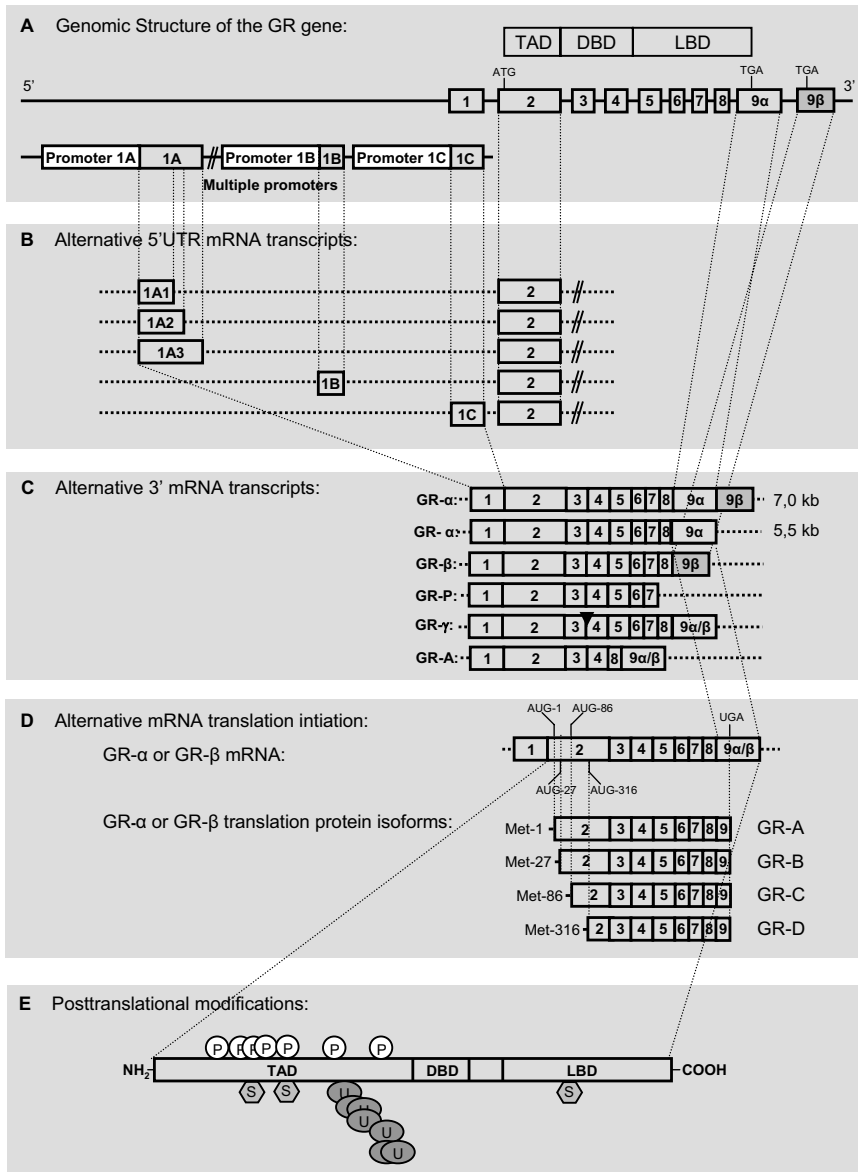
The GR-P mRNA splice variant encodes a protein of 676 amino acids and is formed by exon 2-7 plus several basepairs from intron 7. This truncated GR lacks a large portion of the ligand binding domain, and is therefore unable to bind GCs. This splice variant was found for the first time in tumor cells from a glucocorticoid resistant myeloma patient (53, 54). Studies in our laboratory (63, 77) suggest a widespread expression of GR-P in various tissues and cells at a level of approximately 10 – 20 % of that of GR-α, but the expression level is reported to be higher (up to 55%) in several hematological malignancies like ALL, non-Hodgkin's lymphomas and multiple myeloma (77-80). The biological function is still not fully understood. GR-P, in contrast to GR-β is reported to upregulate GR-α mediated gene transcription in some, but not all cell-lines (77), which suggests that the specific cellular environment plays a role in this. GR-P might form heterodimers with GR-α, which stimulate transcription of target genes more effectively than the regular GR-α homodimers.

1.2.3.3 Other GR-splice variants

GR-γ and GR-A are two less abundant splicing products of the GR and have been described to be expressed in certain cancer cell-lines, and various tissues.

GR-γ was found to be the result of the usage of an alternative mRNA splicing site, and an additional codon (453), GTA coding for arginine, was found to be inserted between exons 3 and 4 (55). This additional amino acid is situated between the two zinc-clusters of the binding domain of GR and subsequent transcriptional activity on an MMTV-reporter was reduced by 50%. The GR-γ seems rather ubiquitously expressed in healthy individuals in many tissues like brain, lung, heart, liver, lymphocytes and muscle and is expressed at relatively high levels (between 4-9% of the total GR transcripts) (55). The functional relevance of this splice variant is largely unknown, but may play a role in the observed poor GC-sensitivity in hematological malignancies like childhood ALL, and transcriptional control of GR-responsive genes (81).

The GR-A splice variant has an excision of exons 5, 6 and 7, resulting in the in frame juxtaposition of exon 8 to 4. No further information is available about expression levels and function of this variant (54).



Partially adapted from: Zhou et al. Steroids 70; 407-417 (2005) (30)

Figure 7 The many faces of the Glucocorticoid Receptor gene.

A. Genomic structure of the GR gene. The gene contains 9 exons, with three alternative exons 1 at the 5'-UTR, each controlled by an own specific promoter and two alternative exons 9 at the 3'-UTR.

B. Alternative 5'-UTR mRNA transcripts. 1A could be spliced into three different splice variants. The subsequent 5 exon 1 variants can join to the same acceptor site on exon 2, yielding transcripts containing various 5'-UTR sequences.

C. Alternative 3' mRNA transcripts. Alternative splicing of exon 9 gives rise to two mRNAs coding for GR-α and GR-β. Other splice variants are GR-P lacking exons 8 and 9, GR-γ containing a three-nucleotide insertion between exons 3 and 4, and GR-A missing exons 5-7.

D. Alternative mRNA translation. Translation initiation from the first AUG (Met-1) results in GR-A, from the second AUG (Met-27) results in GR-B, while translation from Met-86 and Met-316 results in GR-C and GR-D, respectively.

E. Postranslational modifications. P: phosphorylation, S: sumoylation, U: ubiquitination.

1.2.4 Alternative translation

The mRNA of the GR can be alternatively translated in at least four different GR translational isoforms: GR-A, GR-B, GR-C and GR-D. The GR-A isoform is the full-length GR of 777 amino acids, but alternative AUG usage can result in shorter proteins. Translation initiation from AUG-27 results in a receptor peptide of 751 amino acids, named GR-B, while initiation from AUG-86 and AUG-316 results in GR-C (83 kD) and GR-D (54 kD), respectively (82, 83). The mechanism(s) of production for the GR-B isoform is leaky scanning and for the GR-C isoform both leaky scanning and ribosomal shunting, while the GR-D isoform is thought to be produced by ribosomal shunting (82).

Ribosomal leaky scanning occurs in many as 5% of the transcribed mRNA molecules (84). It is generally postulated that the start codon is encountered by a scanning mechanism, which is initiated by binding of a 40S ribosomal subunit complex to the 5'-end 7-methyl guanosine caps of the mRNA. The 40S subunit subsequently migrates through the 5'-UTR. The scanning procedure stops when the first start codon (AUG) is recognized and a 60S ribosomal subunit joins the paused 40S subunit. This fixes that AUG as the initiation codon, whereafter translation is initiated (85). Two or more products from the same mRNA transcript are translated when an initiation start site is ignored in favour of a subsequent translation initiation start site. This ribosomal leaky scanning is provoked by weak Kozak consensus sequence surrounding the AUG start codon, resulting in weak 40S ribosomal subunit binding (86).

Ribosome shunting refers to discontinuous scanning of the mRNA by the translation initiation complex. It is thought that *cis*-acting elements within the structured region (the donor site) facilitate the translocation of the translation initiation complex to a downstream acceptor site, without translating the upstream coding sequence. However, the number of genes reported to utilize ribosomal shunting is scarce and the molecular mechanism of this translation regulatory process is poorly understood (31).

GR-A, -B, -C and -D were found to be widely expressed in different cell-lines and tissues, and were also detected when human GR-α is transiently expressed in COS-1 cells. Immunocytochemical analysis revealed that GR-A, -B and -C were located in the cytoplasm and efficiently translocated to the nucleus following dexamethasone treatment. In contrast, GR-D was located in the nucleus, regardless of the presence or absence of agonist. Furthermore, all GR isoforms activated GRE- or MMTV-driven luciferase expression in response to ligand, however, they differed in transcriptional efficacy (82). The transcriptional activity of GR-B has been investigated most extensively and appeared to be 1.4 – 2 fold higher on various promoters (i.e. a GRE-driven reporter gene, a reporter gene where two GREs are in tandem, and an MMTV promoter reporter gene) than the full-length GR-A isoform (83). Whether the isoforms are differentially expressed in a tissue and cell-type specific manner and how their expression levels are regulated or can be influenced is currently under investigation. However, Pederson *et al.* (49) observed that from a GR mRNA

containing the alternative exon 1A3 instead of the more common 1C, substantially more GR-B is translated and Breslin *et al.* (47) found that in T- and B-lymphoblasts the quantity of this 1A3 containing mRNA is increased 2.5-fold and decreased 5-fold upon GC-treatment, respectively. This suggests that the relative levels of the GR-A, GR-B, GR-C and GR-D isoforms in various tissues and cells might contribute to the tissue-specific effects of GCs.

1.2.5 Postranslational modifications

The transcriptional activity of a mature GR protein can be modulated by various posttranslational modifications, like phosphorylation, ubiquitination, sumoylation and nitrosylation. These modifications result in alterations of protein-protein interactions, nuclear receptor DNA binding, subcellular structures and degradation (30).

1.2.5.1 Phosphorylation

The GR is thought to be a substrate for several kinases and phosphatases and has been shown to be poly-phosphorylated on serine and threonine residues, embedded in a consensus phosphorylation site (87) in the N-terminal domain of the protein upon ligand binding (88, 89). The precise role of each specific phosphorylation event is unclear, but when multiple phosphorylation sites are destroyed, this has profound effects on receptor stability, protein half-life, and subcellular localization (90, 91). Furthermore, the transcriptional capacity of different GR phosphorylation states is highly promoter specific (90). Replacement of all eight phosphorylated residues in mouse GR did not alter the receptor's ability to induce an MMTV-driven reporter gene, while 50–75% less transcriptional activity was measured when using a GRE_2-driven reporter (90). Phosphorylation enhances the transactivation activity in a gene-specific manner. Different degrees of receptor phosphorylation, therefore, may extend the range of the gene regulatory capability of the GR (92).

1.2.5.2 Ubiquitination

Phosphorylated GR is susceptible to ubiquitination, targeting the protein for proteasomal degradation (93). A recent study on mouse GR showed that enzymes in the ubiquitin pathway recognise phosphorylated residues and catalyse the covalent binding of the 76-amino acid ubiquitin protein to lysine 426. This lysine residue lies within a PEST degradation element, rich in proline (P), glutamine (E), serine (S), and threonine (T) residues (residues 407-426). This PEST element is found to be conserved in mouse, rat and human GR. Ubiquitination allows the GR protein to be recognized by the multi-subunit protein complex known as the proteasome, which degrades the protein into small peptides and amino acids. Mutation of Lys 426 to alanine abrogates the ligand dependent down-regulation of GR and simultaneously enhances GR-induced transcriptional activation of gene expression, indicating that ubiquitination regulates GR turnover and terminates GR responses (93, 94).

1.2.5.3 Sumoylation

Sumoylation is a reversible and dynamic enzymatic process in which a small ubiquitin related modifyer 1 (SUMO-1), a 101-amino-acid polypeptide is covalently added to, mostly transcription factors. In contrast to ubiquitination, by which substrates are marked for protealysis, sumoylation of the GR induces transcriptional activity and at the same time decreases stability of the protein (95). These apparently contradicting effects emphasise that activation and degradation are closely coupled events, necessary for the continuous flexibility in GR-mediated responses (95).Sumoylation occurs at specific lysine residues, which are in most cases embedded in a consensus sequence ΨKXE, where Ψ is a large hydrophobic amino acid and X any amino acid (96). The major sumoylation sites of human GR are the N-terminal Lys^{277} and Lys^{293} residues, whereas the third site, the Lys^{703} residue functions as a poor SUMO-1 acceptor (96). The mechanisms that regulate sumoylation and the consequences on (dis)assembly of transcriptional active protein complexes dependent on sumoylation status of the GR are largely unknown.

1.2.5.4 Nitrosylation

Ligand binding capacity of the GR can be decreased by nitrosylation, which occurs on several –SH groups present in the LBD and DBD (97). NO is released in cells by the action of constitutive NO synthases (NOS), whose expression is induced by several endotoxins (like lipopolysacharide) and/or cytokines (98).

Reaction of NO with –SH groups yields S-nitrosothiols and two vicinal S-nitrosothiols can form a disulfide bridge upon NO release (99). The loss of the free –SH groups could cause conformational changes or restraints that would affect the physiological role of the GR, which might be important in protecting cells against specific actions of GCs.

1.3 Sensitivity to Glucocorticoids

1.3.1 Cortisol production and disease

Insufficient or excessive production of cortisol is harmfull (100, 101).

Insufficient production of cortisol, often accompanied by an aldosterone deficiency ("salt-loss") is called Addison's disease or hypoadrenocorticism. This disease can be caused by decreased enzyme function (congenital adrenal hyperplasia) in the steroidogenesis, or as a result of an infectious disease, or autoimmune destruction of the adrenal cortex. Symptoms include fatigue, low blood pressure, weight loss, weakness, loss of appetite, moodiness, nausea, vomiting, and diarrhea (102-104).

Signs and symptoms of GC-excess, called Cushing's syndrome include accentuated fatty tissue on the face ("moon" face) and upper back ("buffalo hump"), hirsutism, striae, thinning of the skin, acne, alopecia, purpura, and more

seriously, osteoporosis, cataracts, gastrointestinal bleeding, hypertension, diabetes, depression and moodiness (100, 105, 106). These excessive GC levels can be the result of an adrenal defect (adrenal neoplasm) or produced by ACTH-producing pituitary adenomas (Cushing's disease) or ectopic tumors producing ACTH or CRH (107). Also administration of exogenous GCs for therapeutic purposes may lead to cushingoid symptoms (108).

Table 1 Classification of human glucocorticoid resistance syndromes

Type of resistance	Underlying mechanism
I. Generalized inherited (familial) GC resistance (GIGR)	Mutations in GR gene
II. Pharmacologically induced GC resistance	
A. Administration of GR antagonist RU486	Blocks ligand binding to GR protein and activates HPA by preventing negative feedback mediated by GCs
B. Treatment of leukemic cell line with chemotherapeutic drugs	Deletion of GR gene
C. Treatment of leukemic cell line with chemical mutagens	Deletion of GR gene
III. Acquired GC resistance	
A. Neoplastic	
1. Ectopic ACTH syndrome	Decreased GR number, truncated GR, aberrant splicing and mutation of GR
2. Pituitary tumors (Nelson's syndrome)	Mutation of GR
3. Hematological malignancies	Mutations and aberrant splicing of GR
B. Transient	
1. Depression	Decreased number of GR
2. AIDS	Increased number of GR and reduced ligand affinity
3. Steroid-resistant asthma, rheumatoid arthritis, systemic lupus erythematosus	Number of GR abnormalities reported
IV. Physiological resistance to GCs and variation in sensitivity	
A. Receptor downregulation	GCs decrease rate of transcription of GR gene and decrease Sensitivity
B. Individual differences in GC sensitivity	Genetic variation in control of cortisol secretion, regulation of HPA-axis, and GR expression

Reprinted from Steroids ***61****, S. Werner and M. Bronnegard, Molecular basis of glucocorticoid-resistant syndromes, pp. 216-221 (1996)* (110)

1.3.1.1 Glucocorticoid resistance

Total GC resistance is not compatible with life, because cortisol is essential for survival. Transgenic mice, devoid of GR-α exhibit impaired lung development, reduced liver gluconeogenic enzyme expression, impaired proliferation, and elevated ACTH and corticosterone levels. The new-born mice died minutes after birth due to respiratory failure (109).

Partial resistance can be classified into generalized inherited, pharmacologically induced, acquired, physiological or into individually determined by genetic sensitivity variation (Table 1). This thesis will only focus on class I: generalized inherited (familial) GC resistance (1.3.2.1), and class IV.B: Individual differences in GC sensitivity (1.3.4) (110, 111)

Generalized Inherited (familial) GC resistance (GIGR)

In GIGR, a rare familial or sporadic condition, all tissues in the body are partially GC resistant (112-116). This overall resistance can be mimicked by the GR antagonist RU38486 (117) (contrary to other cases of acquired resistance in which only the affected tissue is resistant to GCs). Affected subjects have compensatory increased circulating cortisol, usually without the signs and symptoms of cortisol excess as seen in Cushing's syndrome, where the patient is GC sensitive. These elevated cortisol levels represent an adaptation or resetting of the HPA-axis at a higher

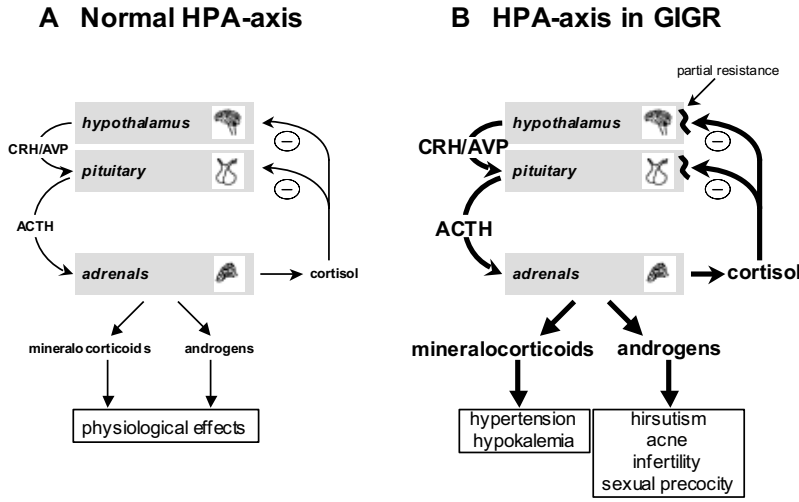


Figure 8 The HPA-axis under normal (A) and GIGR (B) conditions
As a result of partial generalized resistance to GCs, the HPA-axis is set at a higher level, resulting in adrenal overproduction of cortisol, mineralocorticoids, and androgens. The increased cortisol levels has a beneficial effect because it compensates for the GC resistance; the overproduction of androgens and mineralocorticoids leads to signs and symptoms of the clinical syndrome of GIGR.

level in order to overcome the decreased sensitivity in target tissues. However, the elevated ACTH levels not only stimulate the adrenals to secrete GCs, but also result in the overproduction of adrenal steroids with mineralocorticoid activity, such as deoxycorticosterone and corticosterone, and/or androgenic activity, such as androstenedione, dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS) (Figure 8) (112, 116, 118-123). Mineralocorticoid excess causes hypertension and hypokalemia, while androgen excess results in acne, hirsutism, and infertility in both sexes, abnormal spermatogenesis in men and male pattern baldness and oligoamenorrhoea in women (115, 124). In children premature adrenarche and ambiguous genitalia at birth were also reported (125). In adult males, the oligospermia and infertility are most likely caused by the increased androgenic activity suppressing luteinizing hormone (LH) and follicle-stimulating hormone (FSH) via the androgen receptor (AR) (116). The clinical spectrum of GIGR is quite broad, ranging from completely asymptomatic to severe cases of hyperandrogenism, fatigue, and/or mineralocorticoid excess (116).

The molecular cause for GIGR has mostly been attributed to mutations in the GR gene (116), affecting the receptor function of which the first case, a Dutch male, has been described by Hurley *et al.* (129). However, also cases have been reported with biochemical and clinical GC resistance without alterations in the GR gene, indicating that other functions in the signalling mechanism of GR could be affected (114). Steroid receptor coactivator defects may also account for resistance to other steroid hormones. New *et al.* (126, 127) for example, described two sisters with multiple, partial steroid resistance, whose clinical and biochemical findings were largely consistent with isolated GIGR. The absence of hyperandrogenic manifestations

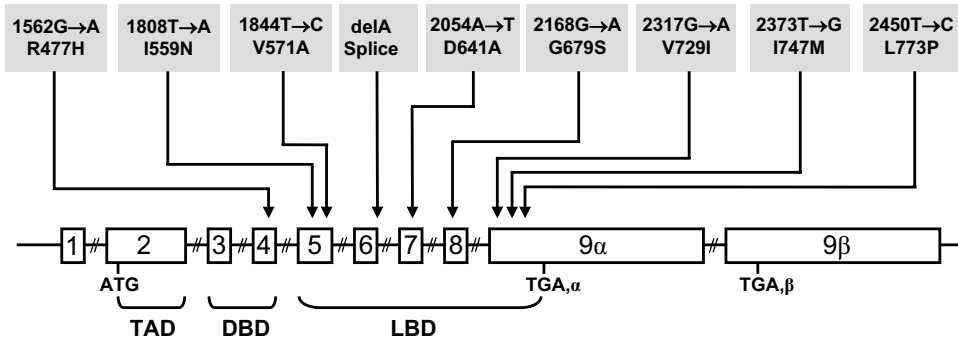


Figure 9 Schematic overview of mutations causing GIGR.
Locations of the known mutations of the human glucocorticoid receptor gene.

necessitated further evaluation and revealed also resistance to androgens, mineralocorticoids, and estrogens, but not vitamin D or thyroid hormones.

Mutations responsible for GIGR have been described in the DBD and LBD of the GR in 6 families and 3 sporadic cases (Figure 9). Although the limited number of mutations makes it difficult to generalize, it appears that all such mutations result in decreased receptor transactivating activity and/or decreased affinity for ligand (116). However, transrepressing activity by interaction with other transcription factors, is not always affected, again emphasizing that transrepression via protein-protein interaction is a different mechanism of GR action than transactivation (GRE) or transinhibition (nGRE) via direct interaction with DNA (42, 128). The molecular defects elucidated in the 9 reported cases so far and their biochemical and physical phenotype are summarized in Table 2 (123, 129-137).

1.3.1.2 Glucocorticoid hypersensitivity

Not much is known about glucocorticoid hypersensitivity and it must therefore be very rare. One patient with generalized glucocorticoid hypersensitivity has been reported (138). Iida *et al.* described a patient with this syndrome who displayed cushingoid features despite low plasma GC levels. The molecular mechanism underlying this defect has never been elucidated. In several reports, GC hypersensitivity was induced by *in vitro*-created point mutations, resulting in increased transcriptional activity of the GR and a diminished capacity to repress activator protein-1 (AP-1) induction (139, 140), but if such mutations are also responsible for GC hypersensitivity *in vivo* remains to be shown. Also natural point mutations that would enhance ligand binding, or defective or decreased dominant negative inhibitors, like the GR-β splice variant, have never been described to be the cause for GC hypersensitivity.

1.3.2 Variation in glucocorticoid sensitivity in the normal population

In the normal population a considerable variability in the sensitivity to GCs exists. Within an individual however, GC sensitivity is rather stable (141, 142). Normal individuals exhibit differential neuroendocrine and metabolic responses to stress and

Table 2 Mutations of the human glucocorticoid receptor gene causing glucocorticoid resistance.

Author	Mutation cDNA*	Position Amino Acid	Genotype	Biochemical Phenotype	Phenotype
Ruiz *et al* (134)	1562	R477H	Heterozygous Sporadic	Transactivation ↓	*Hirsutism *Fatigue
Karl *et al* (132) Kino *et al* (133)	1808	I559N	Heterozygous Sporadic	*Affinity for ligand ↓ *Transactivation↓ *Transdominance (+) *Transcriptional activity of LBD ↓ *DNA binding (+) *Abnormal interaction with GRIP1	*Hypertension *Oligospermia *Infertility
Mendonca *et al* (135)	1844	V571A	Homozygous Familial	*Affinity for ligand ↓ *Transactivation ↓ *Transcriptional activity of LBD ↓ *Transdominance (-) *Nuclear translaocation ↓ *DNA binding (+) *Abnormal interaction with GRIP1	*Ambiguous genitalia at birth *Hypertension *Hyperandrogenism *Hypokalemia
Karl *et al* (130)		4 bp exon-deletion in intron 6	Heterozygous Familial	*GR-α number: 50% of control *Inactivation of the affected allele	*Hirsutism *Male-pattern baldness *Menstrual irregularities
Chrousos *et al* (123) Hurley *et al* (129)	2054	D641V	Homozygous Familial	*Affinity for ligand *Transactivation ↓ *Transcriptional activity of LBD↓ *Transdominance (-) *Nuclear translocation ↓ *DNA binding (+) *Abnormal interaction with GRIP1	*Hypertension *Hypokalemic alkalosis
Ruiz *et al* (134)	2168	G679S	Heterozygous Sporadic	*Affinity for ligand ↓ *Transactivation↓	*Hypertension
Malchoff *et al* (131)	2317	V729I	Homozygous Familial	*Affinity for ligand ↓ *Transactivation ↓ *Transcriptional activity of the LBD ↓ *Transdominance (-) *Nuclear translocation ↓ *DNA binding (+) *Abnormal interaction with GRIP1	*Precocious puberty *Hyperandrogenism
Vottero *et al* (136)	2373	I747M	Heterozygous Familial	*Affinity for ligand ↓ *Transactivation ↓ *Transdominance (+) *Nuclear translocation ↓ *Transcriptional activity of LBD ↓ *DNA binding (+)	*Cystic acne *Hirsutism *Oligomenorrhea
Charmandari *et al* (137)	2450	L773P	Heterozygous Familial	*Transactivation ↓ *Transdominance (+) *Affinity for ligand ↓ *Nuclear translocation↓ *DNA binding (+) *Abnormal interaction with GRIP1	*Fatigue *Anxiety *Hypertension *Hyperandrogenism

* *numbering according to Hollenberg* et al. (32)
partly adapted from: Charmandari et al.*; Ann.N.Y.Acad.Sci.1024:168-181 (2004)* (116)

it has been suggested that these differences reside in the setpoint of the HPA-axis (142). By stressing the HPA-axis function by administering 0.25-mg dexamethasone and measuring the subsequent cortisol levels, or cortisol decrease, subtle differences in HPA-axis sensitivity could be revealed in normal individuals that were not apparent under standard test conditions. This variability is also clear in the wide range of responses to the same relative dose of exogenous GCs used in the treatment of various diseases. Some individuals develop severe adverse effects during hormonal therapy with low dose of GCs, such as osteoporosis, diabetes, myopathy, impaired wound healing, and fat redistribution, yet others do not develop side effects, or show no adequate response at all, even during long-term therapy with a much higher dose of GCs (113, 115, 142). Twin studies have clearly indicated the existence of a genetic component in the control of circadian rhythmicity of cortisol in terms of timing of the nocturnal nadir, an event that signals the beginning and rise of the cortisol concentration towards the morning maximum (143). Minor genetic variation within the population in the form of single nucleotide polymorphisms (SNPs) and other small changes at the level of the DNA sequence (e.g. deletions, insertions, repetitions) (144, 145) are thought to be responsible for such interindividual differences. With regard to GC-sensitivity these variations could be present in any of the genes encoding proteins and enzymes responsible for GC bioavailability (e.g. hydroxysteroiddehydrogenase converting the biologically inactive corticosterone to cortisol and *vice versa*, or enzymes having their function in steroidogenesis), systemic absorption of GCs (e.g. P-glycoprotein, exporting GCs from the inside of cells and from membranes to the extracellular space), pharmacokinetic handling (e.g. hepatic enzymes playing a role in the metabolic clearance of GCs), or factors playing a role in the intracellular GR signaling transduction pathway (i.e. co-regulators, splicing factors regulating transcriptional and translational GR variants). However, the most evident factor is the GR itself.

1.3.2.1 Single Nucleotide Polymorphisms in the GR gene

A rapidly increasing number of SNPs is being identified in the GR gene and most of them are stored in the National Center for Biotechnology Information SNP database at http://www.ncbi.nlm.nih.gov/SNP. Most of the reported SNPs are outside the coding region of the GR, or do not result in an amino acid change and are thought to have no effect on the receptor protein. Polymorphisms in the coding region of the GR gene that do change amino acids are mostly in exon 2, which encodes the transactivating domain and include: R23K, F29L, F65V, L112F, D233N, and N363S. SNPs studied in our laboratory are the ER22/23EK, two SNPs which are always linked, the N363S polymorphism in the coding region of the GR, a *TthIII*I RFLP in the promoter region, a *Bcl*I RFLP in intron 2, and a polymorphism in the 3'-UTR of exon 9β. Selection was based on allele frequency and on the description of functionality of these SNPs in other reports. Three of the five SNPs were found to be mutually exclusive, only the ER22/23EK was always present in combination with the *TthIII*I

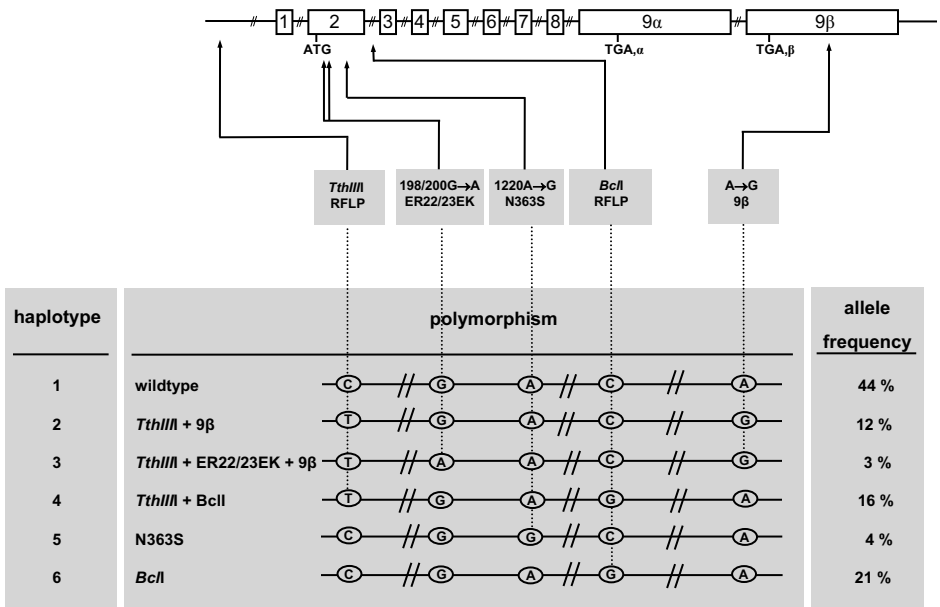


Figure 10 Single nucleotide polymorphisms in the GR gene studied in our laboratory with allele frequencies as indicated.

RFLP and the 9β SNP, but not the other way around (Figure 10).

ER22/23EK* (rs6189 and rs6190)*: This polymorphism consists of two single-nucleotide mutations in codons 22 and 23 in exon 2 of the GR gene that are always linked, with an allelic frequency of 3%. The first mutation is silent, changing codon 22 from GAG to GAA, both coding for glutamic acid (E). The second one, changing codon 23 from AGG to AAG results in a conservative amino acid change from arginine (R) to lysine (K). The ER22/23EK polymorphism is associated with relative resistance to GCs and the resulting phenotypic differences have been reviewed by Van Rossum *et al.* (146). In summary, ER22/23EK carriers react with a smaller decrease in morning cortisol levels after a 1-mg dexamethasone suppression test, and have lower total and low-density lipoprotein cholesterol levels, as well as lower fasting insulin concentrations and a better insulin sensitivity. Furthermore, C-reactive protein levels, which are positively related to cardiovascular damage (147), are lower in ER22/23EK carriers (146). These effects of the ER22/23EK polymorphism suggest a healthier cardiovascular and metabolic profile, which was confirmed in a follow-up study demonstrating an increased survival rate for carriers of the ER22/23EK polymorphism (148). The fact that the polymorphism is more prevalent in the older population (149) also indicates that ER22/23EK carriers have a higher chance to get older. Young-adult male ER22/23EK carriers are significantly taller and have more muscle strength, while in young-adult female carriers, waist circumference tended to be smaller (150). Furthermore, ER22/23EK carriers have a lower risk of dementia and have fewer white matter lesions in the brain, associated with small

vessel disease (146). The molecular mechanism through which this SNP exerts its effects is described in this thesis (Chapter 4).

***Tth*III (rs10052957)**: This SNP was identified in the promoter region of the GR gene, 2676 nucleotides upstream from exon 1B as a C/T nucleotide change (151). The *Tth*III RFLP, with an allelic frequency of 31% was not related to GC sensitivity and no associations with metabolic parameters or body compostion were found. However, this SNP appeared to be linked to the ER22/23EK, the 9β, or *Bcl*I polymorphism. Carriage of both *Tth*III and ER22/23EK SNPs was associated with a relative resistance to GCs, and a healthy metabolic profile (151).

***N363S* (rs6195):** This polymorphism (allele frequency: 4%) further downstream in exon 2 changes codon 363 from AAT to AGT, resulting in a serine (S) for asparagine (N) substitution (8). This SNP increases sensitivity to GCs, while an increased insulin response to DEX, and a tendency towards lower bone mineral density have also been observed. Some studies also found an association with increased body mass index (152, 153), but others did not (154, 155). The molecular mechanism through which the N363S SNP exerts its effects is unknown. It has been postulated that the SNP contributes a new serine residue for phosphorylation, whereby protein interactions with transcription cofactors might be altered.

***Bcl*I (no rs number assigned):** The *Bcl*I RFLP (allelic frequency 37%) is identified as a C to G nucleotide change in intron 2 of the GR gene and is also associated with increased GC sensitivity. In respect to its associations with body composition, conflicting data have been reported. However, in our laboratory, we found in middle-aged subjects, that the presence of this SNP was associated with increased abdominal obesity, while at older age, a lower BMI was found, accompanied by a tendency towards lower lean body mass (146). The molecular mechanism of the *Bcl*I SNP has not been clarified. No transfection experiments are possible to elucidate the mechanism, since the SNP is intronic. The *Bcl*I SNP could be linked to another SNP in the promoter region of the GR gene, which could result in enhanced expression, or a SNP in the 3'-UTR that could stabilize the mRNA.

9β (rs6198): The fifth polymorphism GR-9β is an A to G nucleotide substitution located in the 3'-UTR of exon 9β, the terminal exon of the mRNA of the GR-β isoform with an allelic frequency of 15%. This SNP is associated with an increased susceptibility to rheumatoid arthritis, reduced risk for *Staphylococcus aureus* nasal carriage, and an increased cardiovascular risk profile, caused by increased inflammation parameters (Van den Akker, unpublished observations). This indicates reduced capacity for immune suppression, probably caused by decreased GC sensitivity. The A to G nucleotide substitution (nucleotide 3669 in X03348) is located in an 'ATTTA' motif (to GTTTA), which is known to destabilize mRNA and decrease receptor protein expression *in vitro*. *In vitro* data show that this 9β polymorphism leads to a more stable GR-β mRNA, the dominant negative inhibitor of GR-α, leading to relative GC resistance (156).

1.4. Aim and outline of this theses

Glucocorticoids are the "hormones of life". They are important for the maintenance of metabolic homeostasis. It is not surprising that their pharmacological use is riddled with a number of effects, both desired and harmful, and the effectiveness of GC medication is highly determined by an individual's sensitivity to GCs. This variability is clearly reflected in the wide range of responses to the same relative dose of exogenous GCs used in the treatment of various diseases. Successful pharmacological use requires knowledge of GC synthesis, action, metabolism, and the complicated physiology.

This thesis will especially focus on GC action mediated by the GR and factors that could be responsible for the interindividual differences. One of the main urgent questions at this moment is to develop insight into the cause of differences in response between individuals to therapeutically applied GCs. Some patients respond to low doses, with or without side effects, while others do not respond at all.

Chapter 2 introduces a new bioassay for the determination of individual cellular sensitivity to GCs. We measured the effects of several clinically used GCs on the transcription of two GC-regulated genes on transactivation and transrepression using quantitative RT-PCR in peripheral blood mononuclear lymphocytes (PBMLs) isolated from only 5 mL whole blood.

Chapter 3 describes how this bioassay was used to measure the effects of the ER22/23EK and N363S polymorphisms on cellular GC sensitivity and shows that the effects of the polymorphisms in the GR gene previously observed in population studies were also detected at the level of gene expression.

Chapter 4 deals with the molecular mechanism of how the ER22/23EK polymorphism decreases the receptor's sensitivity to GCs.

Chapter 5 focusses on the GR-9β polymorphism and attempts to explain why the effect and associations of the 9β polymorphism are completely different from that found for the ER22/23EK polymorphism, while both polymorphism are reported to decrease cellular GC sensitivity.

Chapter 6 shows that regulation of the GR-α, GR-β and GR-P expression could partly occur through the use of multiple promoters of the GR gene and that this is another way how cells and tissues can be sensitized to the different activities of the GR splice variants.

Chapter 7 is a description of our investigation of 9 patients with seriously affected GC sensitivity and sets out strategies to characterise these GC sensitivity disorders. Patients diagnosed with mutations found in the LBD of the GR could provide us with valuable information about GC action and how the beneficial properties of GCs could be optimised while minimizing some of their more serious side effects.

Chapter 8 provides the general discussion of the data described in this thesis.

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Chapter 2

The Development of a New Bioassay to Measure Cellular Glucocorticoid Sensitivity *ex vivo*

Based on:

1. "Differential Regulation of Synthetic Glucocorticoids on Gene Expression Levels of GILZ and IL-2"
Pauline Smit, Henk Russcher, Frank H. de Jong, Albert O. Brinkmann, Steven W.J. Lamberts and Jan W. Koper
The Journal of Clinical Endocrinology and Metabolism 90(5):2994-3000
2. "Simplified *in vitro* Bioassay for Glucocorticoid Sensitivity made Applicable for Large Scale Use"
Master of Science thesis of Marieke A. van Leeuwen

Abstract

Context: Within the healthy population considerable interindividual variation in glucocorticoid (GC) sensitivity exists, which implies that each subject, when treated with GCs needs an individually optimized dose to maintain a balance between beneficial and adverse effects associated with GC-treatment.

Objective/design: We developed a new bioassay for the determination of individual cellular sensitivity to GCs.

Setting and Subjects: Academic hospital, three independent study groups of 10, 15 and 15 healthy individuals without a history of GC medication.

Interventions: Cellular GC sensitivity was determined *ex vivo* by measuring the effects of several clinically used GCs on the GC-specific transactivation of the GC-induced leucine zipper (GILZ) gene and on transrepression of the Interleukin-2 (IL-2) gene using quantitative real-time PCR in peripheral blood mononuclear lymphocytes (PBMLs). *Ex* vivo responses were compared with the *in vivo* response by testing the hypothalamic-pituitary-adrenal (HPA)-axis sensitivity in a dexamethasone suppression test (DST).

Results: The transcriptional effects on GILZ and IL-2 are GC-specific since they can be abrogated by addition of GR-antagonists and are not evoked by other steroids. The maximal effect (E_{max}) and half maximal effect concentration (EC_{50}) parameters could be obtained from dose response curves of GILZ and IL-2 gene expression. Furthermore, when PBMLs were incubated with hydrocortisone, dexamethasone, budesonide, and prednisolone, a clear difference in relative potencies for transactivation and transrepression of various GCs was observed, suggesting differential effects. The response to hydrocortisone was highly correlated to that of the other GCs regarding both GILZ and IL-2 expression, but not with *in vivo* sensitivity of the HPA-axis.

Conclusions: The large interindividual variations in GC sensitivity could be quantified by using an expression assay representing GC-mediated transactivation and transrepression. The maximal expression of GILZ and IL-2 induced by pharmacological doses of GCs (E_{max}) is a more reliable variable to categorize individuals into GC-sensitive and GC-resistant groups than the variable that shows the concentration of half-maximal expression (EC_{50}). Furthermore, the responses of GILZ and IL-2 expression to GCs did not correlate with each other, nor with the results of the DST. This suggests that regulation of the HPA-axis is more complex. Although this assay remains quite laborious, it could be a helpful tool to predict the most optimal GC dosage to obtain balance between the beneficial and adverse effects.

2.1 Introduction

Glucocorticoids (GCs) play a crucial role in the regulation of transcription of many genes, and are important regulators of diverse physiological systems, including the immune and cardiovascular systems (1-3). The effects of GCs are exerted through the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily (4). Upon ligand-induced dimerization of the GR, the complex translocates to the nucleus and binds to conserved DNA-motifs known as glucocorticoid response elements (GREs) and negative GREs (nGREs), to stimulate (GREs) or suppress (nGREs) gene expression. GC-mediated regulation also occurs independently of interactions of the GR with DNA, mediated by protein-protein interactions of the GR with other transcription factors such as activating protein-1 (AP-1) and nuclear factor-κB (NF-κB) (4, 5). The anti-inflammatory effects of GCs are routinely used in the pharmacological GC-treatment of patients with chronic inflammatory or autoimmune diseases. However, severe side effects (including diabetes, skin atrophy, arteriosclerosis and osteoporosis) are associated with GC-treatment, limiting its therapeutic usefulness (4, 6). Conventional GCs do not dissociate transactivation from transrepression and it is generally thought that transactivation is the predominant mechanism by which GCs exert many of their metabolic and cardiovascular actions and that these actions are mainly responsible for their side effects (7). Due to the considerable variability in GC sensitivity existing in the normal population, it is currently impossible to define such a GC dose for a given patient that a good balance could be achieved between the unwanted adverse and the desired beneficial effects.

To investigate the interindividual differences in cellular sensitivity, we developed a bioassay that evaluates changes in GC-responsive gene expression by means of quantitative real-time RT-PCR (Q-PCR). We selected the glucocorticoid-induced leucine zipper (GILZ) gene, which is upregulated through GRE-mediated transactivation and the interleukin-2 (IL-2) gene, which is downregulated via protein-protein interaction of the liganded GR with NF-κB and AP-1 (8, 9). With this bioassay, we aimed to assess which clinically used GC (i.e. dexamethasone, budesonide, hydrocortisone, and prednisolone) fully maintain the beneficial effects through transrepression, in the absence of GRE-dependent transactivation. Because we were also interested whether these *ex vivo* outcomes could predict *in vivo* potencies of GCs, a group of 15 healthy volunteer underwent a 0.25-mg dexamethasone suppression test (DST) while determining GILZ and IL-2 expression levels in their PBMLs incubated with hydrocorticone, dexamethasone (DEX), budesonide, and prednisolone.

2.2 Materials and Methods

2.2.1 Materials and subjects

The steroids hydrocortisone (HC), dexamethasone (DEX), prednisolone, triamcinolone acetonide (AC), budesonide, methylprednisolone, beclomethasone dipropionate (DP), deoxycorticosterone, d-aldosterone, megestrol acetate, progesterone, 6α-methyl-17α-hydroxy-progesterone acetate (MPA), and 17β-estradiol (E2) were all purchased from Sigma-Aldrich (Zwijndrecht, the Nederlands). The GR-antagonists RU 38486 (mifepristone) and ZK 98299 (onapristone) were from Sigma-Aldrich, whereas Org 31806 was obtained from Organon (Oss, the Netherlands).

For the determination of transactivation and transrepression activities of GCs, peripheral blood from volunteers (all Caucasian) was used. All subjects were healthy, and none were using exogenous GCs. From all subjects, informed consent was obtained and the Medical Ethics Committee of Erasmus MC, the Netherlands approved this study.

2.2.2 Dexamethasone suppression test

The 0.25-mg dexamethasone suppression test (DST) was performed as previously described (10). Briefly, venous blood for serum cortisol measurements was obtained between 08.00 and 09.00 h after an overnight fast. Subjects were asked to ingest a tablet of 0.25-mg DEX at 23.00 h. The next morning, fasting blood was drawn by venapuncture at the same time as the previous day.

2.2.3 Blood cell preparations

Peripheral blood was collected by venipuncture in heparinized tubes, and PBMLs were obtained after density centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) as previously described (11). For the hormone measurements, blood was drawn by venipuncture and allowed to coagulate for at least 30 minutes. Subsequently, serum was separated by centrifugation and quickly frozen at –20°C.

2.2.4 Cortisol measurements

Serum cortisol concentrations were determined using the Immulite 2000 (Diagnostic Products Corporation, Los Angeles, CA, USA). Between-run variability was below 10.4%.

2.2.5 Cells and culture conditions

The acute lymphoblastic T cell leukemia cell line CCRF-CEM (no. CCL-119; American Type Culture Collection (ATCC), Manassas, Virginia, USA) was cultured in RPMI 1640 medium containing L-glutamine (Invitrogen, Breda, The Netherlands) supplemented with 4.5 g/L glucose, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (Invitrogen). Cortisol levels in the culture medium were below detection limits (data not shown). Cells (4 x 10^6 per incubation) were incubated at

a density of 10 x 10^6 cells/ml for 4 hours at 37°C with 100 nM steroids (including GR-antagonists), after which they were collected.

PBMLs were suspended in RPMI 1640 medium containing L-glutamine (Invitrogen) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (Invitrogen). Cells were incubated for 30 min at 37°C in a shaking waterbath in order to remove endogenous cortisol. Afterwards, medium was replaced and the indicated number of cells per well were pre-cultured overnight in 48- or 96- wells plates at a density of 4.0 x 10^6 cells/ml. The next day, PBMLs were incubated for 4 hours with increasing amounts of GCs together with 10 µg/ml phytohemagglutinin (PHA) (Sigma-Aldrich). Afterwards, cells were collected.

2.2.6 RNA isolation

Total RNA was isolated from CCRF-CEM cells and PBMLs using an RNA isolation kit (High Pure RNA Isolation Kit; Roche, Mannheim, Germany), and directly frozen at –80°C.

2.2.7 RT-PCR

An RT-PCR was performed using 200 ng of total RNA per reaction (400 ng for CCRF-CEM cells). For this, we used a 50 µl reaction volume, containing the desired amount of RNA, 5.5 mM $MgCl_2$, 5 µl Reverse Transcriptase buffer, 2 mM dNTP mixture (0.5 mM each), 5 µM random hexamers, 0.2 µM oligo $d(T)_{16}$, 20 U RNAse inhibitor and 62.5 U Reverse Transcriptase (Taqman Reverse Transcriptase Reagents; Applied Biosystems, Nieuwerkerk a/d IJssel, The Nederlands).

2.2.8 Quantitative real-time PCR

For quantitative real-time PCR analysis, the Taqman technology (7700 Sequence Detector; Applied Biosystems) was applied according to the manufacturer's instructions. We determined the gene expression levels of GILZ and IL-2 while correcting for the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT). Primers and probes (Biosource International, Camarillo, California, USA) were chosen using the Primer Express software (Applied Biosystems) and are listed in

Table 1 Primer and probe sequences for GILZ, IL-2 and HPRT used in quantitative real-time PCR

GILZ: forward primer	5'-GCA CAA TTT CTC CAT CTC CTT CTT-3'
GILZ: reverse primer	5'-TCA GAT GAT TCT TCA CCA GAT CCA-3'
GILZ: probe	5'-6FAM-TCG ATC TTG TTG TCT ATG GCC ACC ACG-TAMRA-3'
IL-2: forward primer	5'-TTT GAA TGG AAT TAA TAA TTA CAA GAA TCC-3'
IL-2: reverse primer	5'-TTC TAG ACA CTG AAG CTG TTT CAG TTC-3'
IL-2: probe	5'-6FAM-CAG GAT GCT CAC ATT TAA GTT TTA CAT GCC C-BHQ-3'
HPRT: forward primer	5'-CAC TGG CAA AAC AAT GCA GAC T-3'
HPRT: reverse primer	5'-GTC TGG CTT ATA TCC AAC ACT TCG T-3'
HPRT: probe	5'-FAM-CAA GCT TGC GAC CTT GAC CAT CTT TGG A-TAMRA-3'

Table 1. A reaction volume of 25 µl was used, containing 2.5 µl cDNA (obtained from RT-PCR), 12.5 µl Universal Master Mix (Roche, Branchburg, New Jersey, USA), 0.3 pmol/µl forward and reverse primer (0.5 pmol/µl for HPRT) and 0.1 pmol/µl probe (0.2 pmol/µl for HPRT). Standard PCR conditions, as supplied by the manufacturer, were used for analysis on the 7700 Sequence Detector. For calculation of the relative amounts of GILZ, IL-2 and HPRT mRNA, the comparative treshold method was used (12).

2.2.9 Statistical analysis

Data were analyzed using SPSS for Windows, release 10.1 (SPSS, Chicago, Illinois, USA). Spearman rank correlation was used for analyzing relationships between data, and data points were fitted with regression-lines using the least-squares method. Statistical significance was set at $p<0.05$. Maximal effect (E_{max}) and concentration half maximal effect (EC_{50}) values were calculated using Instat software version 2.01 (GraphPad Software, Inc, San Diego, USA).

2.3 Results

2.3.1 GILZ and IL-2 expression in PBMLs

We derived PBMLs from a healthy volunteer and used these cells to investigate if GILZ and IL-2 were expressed in these cells, how they can be respectively up- and downregulated by DEX, and how their expression can be optimized in time. Figure 1 shows GILZ and IL-2 expression during 6 hours and how their expression was influenced by 100 nM DEX. The effect of DEX was optimal when cells were incubated for 4 hours, and increased GILZ expression by 500%, while IL-2 expression was repressed by more than 50%.

The response curves of GILZ and IL-2 expression to increasing DEX concentrations are shown in Figure 2A and 2B respectively. From these curves the parameters EC_{50} (half maximal effect concentration) and E_{max} (maximal effect) were obtained. The GILZ and IL-2 mRNA expression levels were, apart from using the same amount of

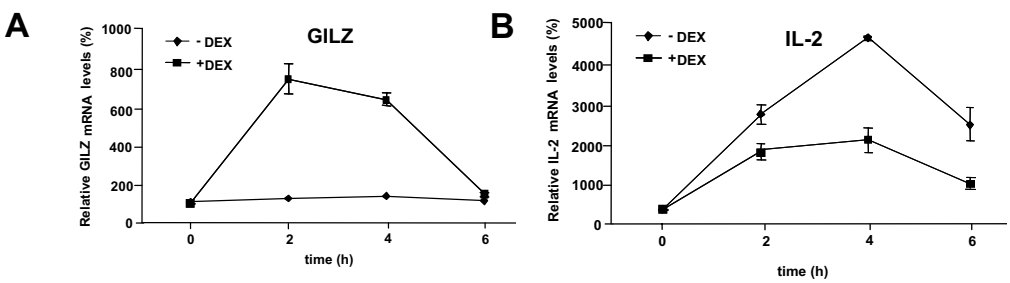

Figure 1 Relative GILZ (A) and IL-2 (B) mRNA levels after a 0-6 h incubation with 100 nM DEX in PBMLs of a healthy volunteer. Bars represent means ± S.E.M.

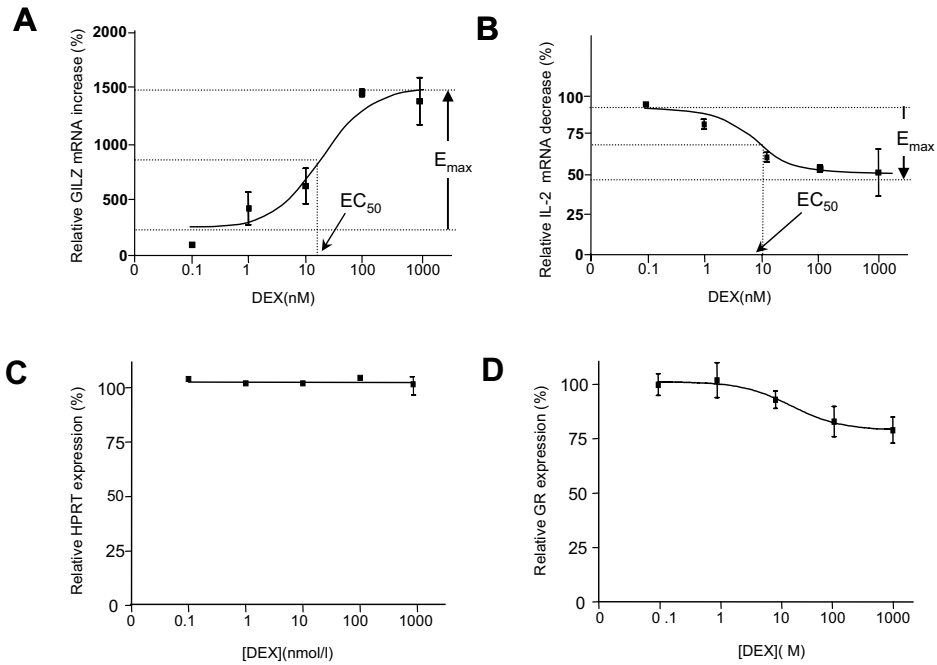

Figure 2 GILZ (A), IL-2 (B), HPRT (C) and GR-α (D) mRNA expression levels in response to the indicated concentrations of DEX during a 4 h-incubation in PBMLs of a healthy volunteer. Bars represent means ± S.E.M. of at least two independent experiments performed in duplicate.

total RNA to obtain cDNA samples, normalized by correcting them for the amount of HPRT mRNA (see also methods section). Figure 2C shows that this correction is justified because HPRT levels were not affected by DEX. Figure 2D shows that DEX treatment decreased GR mRNA levels with 21±6% ($p<0.02$).

2.3.2 GC specific GILZ induction

In order to investigate whether GCs specifically regulate gene expression of these marker genes, GILZ mRNA induction was explored by incubating CCRF-CEM cells at 37°C with 100 nM of different steroids for 4 hours. This concentration was chosen because sub-optimal effects were achieved with 100 nM and intra- and interindividual differences were more pronounced. The GCs DEX and hydrocortisone were both able to raise GILZ mRNA levels by 5028% and 1869% respectively, relative to non-stimulated cells (Figure 3). When cells were incubated with DEX together with RU 38486, the increase in GILZ mRNA was highly suppressed. The GR-antagonists (RU 38486, Org 31806, ZK 98299), the progesterone receptor (PR)-agonist progesterone, the mineralocorticoid receptor (MR)-agonists (deoxycorticosterone, aldosterone), and the estrogen receptor (ER)-agonist 17β-estradiol (E2) were all unable to increase GILZ mRNA levels. However, incubation of the cells with the PR-agonists megestrol acetate and 6α-methyl-17α-hydroxyprogesterone acetate (MPA) led to a slight induction of GILZ gene expression levels compared to non-stimulated cells (Figure 3).

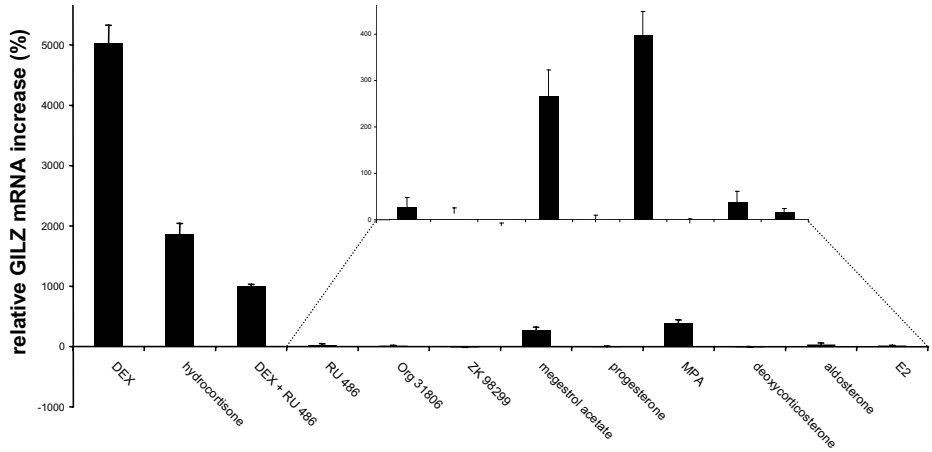

Figure 3 Relative increase in GILZ mRNA levels in CCRF-CEM cells incubated for 4 h with 100 nM of the indicated steroids. Bars represent means ± S.E.M. of two independent experiments performed in duplicate.

2.3.3 Inter- and intraindividual variation in GILZ and IL-2 expression

Next, we assessed the inter- and intraindividual variation of GILZ and IL-2 expression in response to DEX in PBMLs by using 2 x 10^6 or 0.25 x 10^6 cells per incubation. When using 2 x 10^6 cells per well, dose response curves were created by using 40 x 10^6 cells per assay, corresponding to 40 mL whole blood, while for incubations with 8 times less cells, only 5 mL whole blood was needed. This would be more patient-friendly and even measuring GC sensitivity in children would then be possible. The EC_{50} and E_{max} parameters calculated from the dose response are shown in Figure 4. The measurements for which 2 x 10^6 cells were used were independent from those when only 0.25 x 10^6 cells were used, however, 5 healthy individuals who participated in the first study also participated in the latter one.

Table 2 The interindividual and intraindividual variation of the GILZ and IL-2 response

Variation (% of total)	GILZ response				IL-2 response			
	2x10^6 cells		0.25x10^6 cells		2x10^6 cells		0.25x10^6 cells	
	E_{max}	Log EC_{50}	E_{max}	Log EC_{50}	E_{max}	Log EC_{50}	E_{max}	Log EC_{50}
Inter-Individual mean ± Std. Deviation	1577 ± 364	0.91 ± 0.43	1517 ± 394	0.94 ± 0.33	74.9 ± 15.6	0.60 ± 0.46	68.3 ± 9.6	0.26 ± 0.79
Intra-Individual mean ± Std. Deviation	1232 ± 54	0.83 ± 0.23	1154 ± 161	0.89 ± 0.11	43.7 ± 3.4	0.39 ± 0.20	42.7 ± 1.9	0.57 ± 0.52
Inter-Individual Variation (% of total)	23%	47%	26%	35%	21%	74,9%	14%	302%
Intra-Individual Variation (% of total)	4.4%	27%	13.9%	12%	7.7%	51.4%	4.4%	92%
	↓	↓	↓	↓	↓	↓	↓	↓
F-ratio	45.4	3.5	6.2	9	21.7	5.3	26,4	2.3
F test P	<0.01	n.s	<0.05	<0.05	<0.05	n.s	<0.05	n.s.

n.s. = not significant

The F test (variance ratio test) is based on the null hypothesis that two populations which are normally distributed have equal variances. The variance ratio is the ratio of the sample variances or the square of the sample standard deviations.

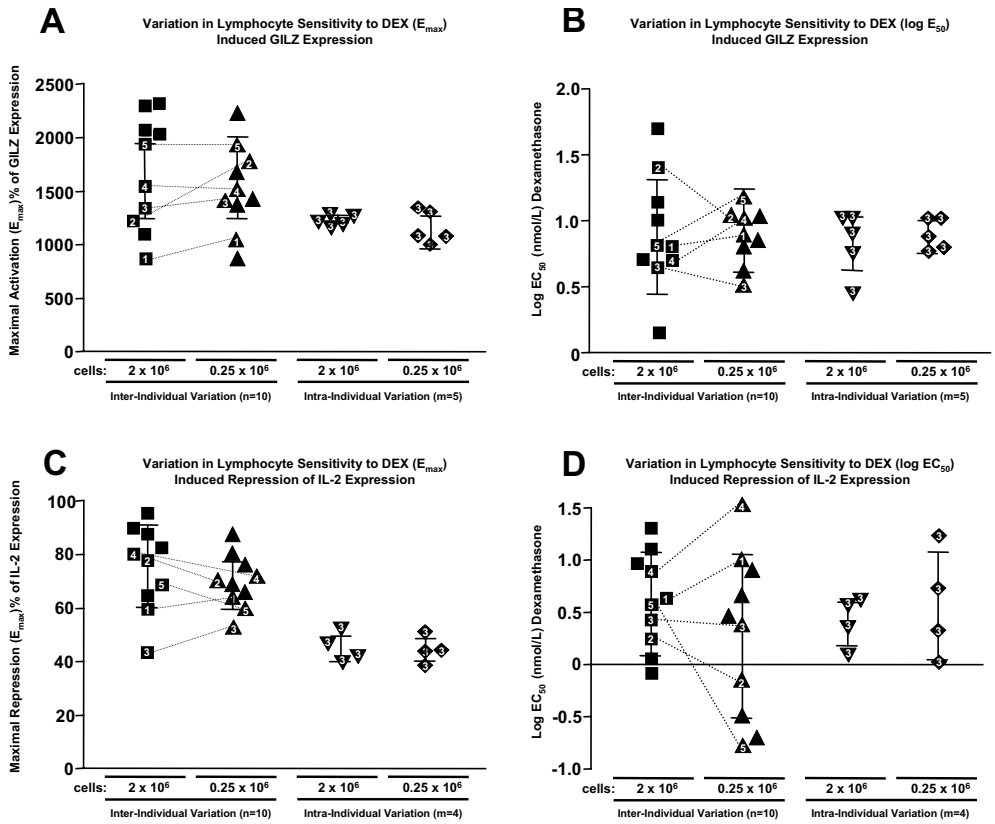

Figure 4 Inter- and intraindividual differences in E_{max} and EC_{50} parameters of GILZ (A,B) and IL-2 (C,D) responses to DEX during a 4 h-incubation in PBMLs two different groups of 10 healthy individuals. For one group, 2 x 10^6 cells per incubation were used, while for the other group 0.25 x 10^6 cells per incubation were used. Five controls participated in both studies and their parameters are connected with each other with dashed lines.

Figure 4A shows the inter- and intraindividual variation in GILZ expression for E_{max}. Considerable interindividual variation was found, but comparing this with repeated measurements within an individual at different time points, relatively little intraindividual variation was found. Using comparisons of variance (%CV), within-individual variation in E_{max} accounted only 4.4% of total variation, whereas interindividual variation contributed 23% (for 0.25 x 10^6 cells 13.9% and 26%, repectively). Applying the variance ratio test (F-test) confirmed that inter- and intraindividual variation differed significantly ($p<0.01$) (for 0.25 x 10^6 cells: $p<0.05$), indicating wide variation in E_{max} between individuals, but relative stability within each individual over time (see also Table 2). The variation of E_{max} of IL-2 repression (Figure 4B) showed an interindividual %CV of 21% and an intraindividual %CV of 7.7% (for 0.25 x 10^6 cells 14% and 4.4%, respectively). The variance ratio test also indicated that inter- and intraindividual variation of IL-2 repression were significantly different (for 2 x 10^6 cells and for 0.25 x 10^6 cells: $p<0.05$).

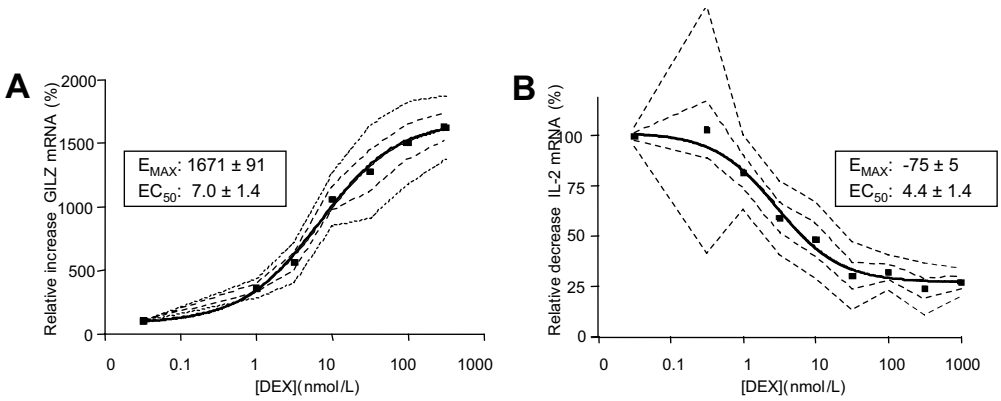

Figure 5 The average GILZ (A) and IL-2 (B) mRNA expression levels in response to the indicated concentrations of DEX during a 4 h-incubation in PBMLs of 10 healthy individuals. The corresponding average E_{max} and EC_{50} parameters are indicated. The 95% C.I. interval and S.E.M. values are indicated with the dotted outer and inner lines, respectively.

The %CV for the EC_{50} values of the GILZ and IL-2 responses were much wider than for the E_{max} values (Figure 4B and 4D). Analysis of variance (Table 2) indicated that for EC_{50}, interindividual variation was as great as intraindividual variation, indicating that they could not be separated from each other. The EC_{50} values calculated for the 5 indicated individuals who participated in the 0.25 as well as in the 2 million cells-per-incubation-assay were much less consistent than for the E_{max} values. Furthermore, the %CV of the inter- and intraindividual variation in EC_{50} values calculated from the IL-2 response using only 0.25 x 10^6 cells was higher than when 2 x 10^6 cells were used, however, no significant differences were shown in the variance ratio test.

The average response of GILZ and IL-2 mRNA expression in the 10 healthy volunteers (2 x 10^6 cells per incubation) and the corresponding E_{max} and EC_{50} parameters are shown in respectively Figure 5A and 5B. The 95% confidence interval (C.I.) and standard error of the mean (S.E.M.) are indicated with the dotted outer and inner lines, respectively. These average values could be used as references indicating the healthy population when comparisons should be made with patients with disease states in whom cellular GC sensitivity could be severely affected.

2.3.4 GILZ and IL-2 mRNA regulation by various GCs

When incubating PBMLs from a healthy volunteer for 4 hours at 37°C with 1 nM, 10 nM, and 100 nM of seven clinically used GCs (hydrocortisone, DEX, prednisolone, triamcinolone AC, budesonide, methylprednisolone, beclomethasone DP), all GCs were able to induce GILZ gene expression levels and to repress IL-2 gene expression levels. E_{max} and EC_{50} values were calculated and show that there existed considerable differences between the used GCs (Table 3), both with respect to the EC_{50} and to E_{max}. Interestingly, high transactivating activity (low EC_{50} / high E_{max} in the GILZ

Table 3 Maximal relative effect (E_{max}) and half maximal effect concentration (EC_{50}) values calculated from dose response curves to the indicated GCs of GILZ and IL-2 mRNA expression in PBMLs from a healthy control donor. Data represent means ± S.E.M of two independent experiments performed in duplicate.

Glucocorticoid	GILZ EC_{50} (nM)	GILZ E_{max} (%)	IL-2 EC_{50} (nM)	IL-2 E_{max} (%)
hydrocortisone	**56.7 ± 1.3**	**698 ± 79**	**1.1 ± 2.4**	**-33.9 ± 4.4**
dexamethasone	**4.1 ± 1.9**	**1341 ± 160**	**14.3 ± 1.5**	**-76.3 ± 8.3**
prednisolone	**88.5 ± 4.0**	**1680 ± 1075**	**6.1 ± 2.0**	**-38.0 ± 5.2**
triamcinolone AC	**2.8 ± 2.3**	**1074 ± 145**	**1.5 ± 3.2**	**-61.6 ± 10.2**
budesonide	**0.3 ± 1.9**	**1216 ± 85**	**0.3 ± 2.3**	**-73.8 ± 6.7**
methylprednisolone	**13.8 ± 1.7**	**998 ± 139**	**0.2 ± 6.5**	**-64.0 ± 7.6**
beclomethasone DP	**2.8 ± 2.2**	**806 ± 114**	**2.2 ± 1.7**	**-63.6 ± 5.2**

Table 4 Relative increase of GILZ and relative decrease of IL-2 mRNA levels compared in PBMLs from 15 healthy subjects stimulated for 4 h with 100 nM hydrocortisone, DEX, budesonide, or prednisolone. Data represent means ± S.E.M. of two independent experiments performed in duplicate.

	Relative increase in GILZ mRNA levels (%)				Relative decrease in IL-2 mRNA levels (%)			
Subjects	hydrocortisone	dexamethasone	budesonide	prednisolone	hydrocortisone	dexamethasone	budesonide	prednisolone
1	**1057 ± 412**	**2248 ± 159**	**1914 ± 167**	**1907 ± 892**	**18 ± 25**	**-32 ± 5**	**-47 ± 11**	**-9 ± 24**
2	**265 ± 81**	**1619 ± 330**	**870 ± 72**	**474 ± 174**	**-61 ± 12**	**-61 ± 5**	**-84 ± 4**	**-41 ± 20**
3	**662 ± 19**	**1250 ± 117**	**2223 ± 257**	**1228 ± 129**	**2 ± 3**	**-46 ± 13**	**-52 ± 1**	**-22 ± 4**
4	**273 ± 55**	**776 ± 71**	**612 ± 72**	**312 ± 82**	**-24 ± 7**	**-73 ± 2**	**-74 ± 3**	**-62 ± 3**
5	**268 ± 22**	**1399 ± 525**	**1038 ± 547**	**n.d.**	**-63 ± 20**	**-79 ± 3**	**-90 ± 3**	**n.d.**
6	**816 ± 120**	**1022 ± 135**	**1554 ± 174**	**n.d.**	**-62 ± 11**	**-66 ± 2**	**-72 ± 2**	**n.d.**
7	**1099 ± 190**	**3503 ± 392**	**1992 ± 562**	**n.d.**	**-70 ± 12**	**-76 ± 5**	**-80 ± 1**	**n.d.**
8	**209 ± 20**	**1362 ± 28**	**2312 ± 238**	**n.d.**	**-43 ± 6**	**-68 ± 7**	**-83 ± 2**	**n.d.**
9	**1058 ± 146**	**2765 ± 448**	**2726 ± 88**	**n.d.**	**-63 ± 5**	**-73 ± 4**	**-86 ± 5**	**n.d.**
10	**532 ± 78**	**2050 ± 75**	**1539 ± 77**	**1052 ± 157**	**-73 ± 2**	**-95 ± 1**	**-83 ± 1**	**-78 ± 3**
11	**307 ± 52**	**2355 ± 39**	**708 ± 77**	**515 ± 183**	**-47 ± 14**	**-30 ± 0**	**-79 ± 7**	**-72 ± 2**
12	**520 ± 94**	**2121 ± 370**	**1386 ± 83**	**n.d.**	**17 ± 25**	**-57 ± 9**	**-22 ± 7**	**n.d.**
13	**803 ± 149**	**2226 ± 659**	**2215 ± 319**	**n.d.**	**-43 ± 5**	**-64 ± 8**	**-72 ± 8**	**n.d.**
14	**648 ± 2**	**2571 ± 144**	**944 ± 96**	**548 ± 23**	**-51 ± 1**	**-33 ± 6**	**-67 ± 2**	**-58 ± 7**
15	**537 ± 28**	**1874 ± 319**	**2466 ± 120**	**1357 ± 208**	**-70 ± 2**	**-60 ± 5**	**-81 ± 1**	**-68 ± 8**

n.d. = not determined

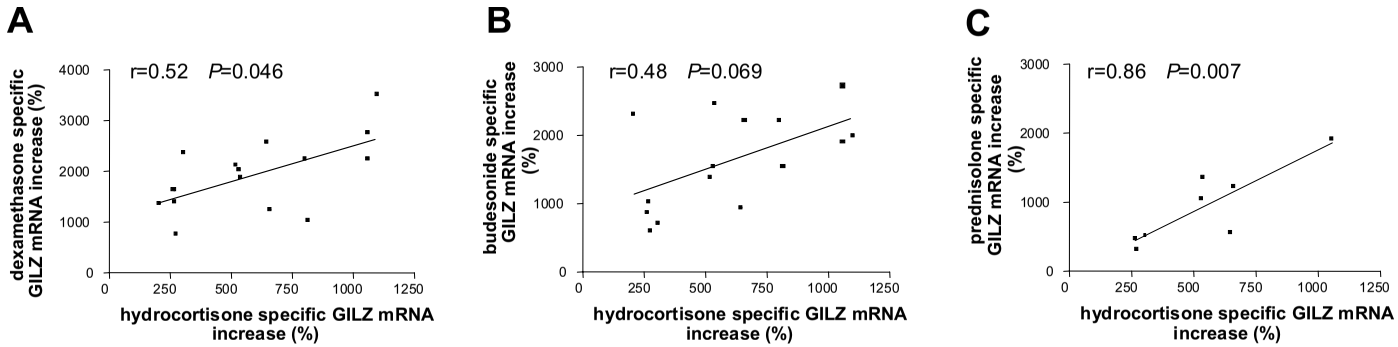

Figure 6 Correlation analysis (Spearman's correlation) between induction of GILZ mRNA levels by 100 nM of hydrocortisone and DEX (A), budesonide (B) and prednisolone (C) in PBMLs of fifteen healthy volunteers.

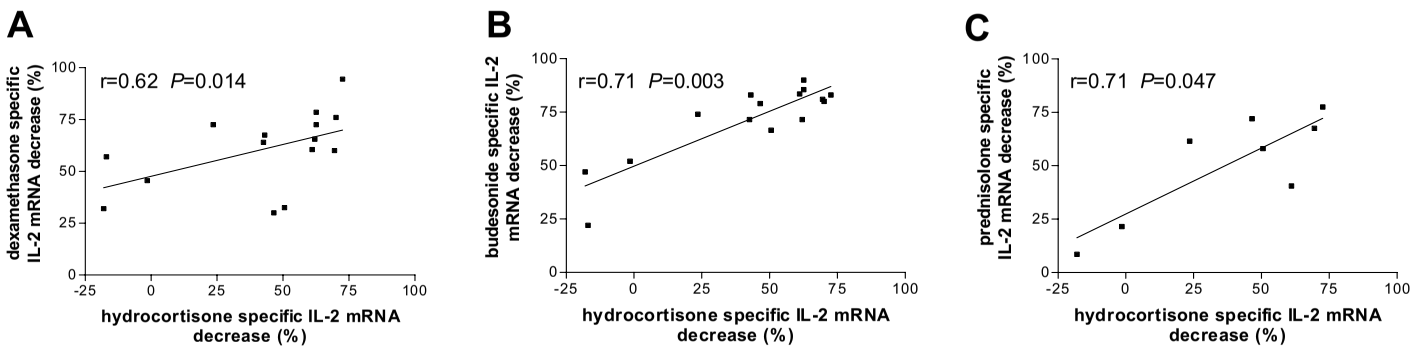

Figure 7 Correlation analysis (Spearman's correlation) between repression of IL-2 mRNA levels by 100 nM hydrocortisone and DEX (A), budesonide (B) and prednisolone (C) in PBMLs of fifteen healthy volunteers.

Table 5 Mean rank and mean effect of 100 nM of hydrocortisone, dexamethasone, budesonide and prednisolone in the GILZ expression assay (A) and the IL-2 expression assay (B). data were calculated from Table 4 and represent means ± S.E.M. of two independent experiments performed in duplicate.

A GILZ

Glucocorticoid	Mean rank	Mean activation (%)
hydrocortisone	3.7 ± 0.1	603 ± 80
dexamethasone	1.3 ± 0.1	1943 ± 187
budesonide	1.7 ± 0.1	1633 ± 178
prednisolone	3.1 ± 0.1	924 ± 196

B IL-2

Glucocorticoid	Mean rank	Mean repression (%)
hydrocortisone	3.4 ± 0.2	-42 ± 8
dexamethasone	2.3 ± 0.3	-61 ± 5
budesonide	1.1 ± 0.1	-71 ± 5
prednisolone	2.9 ± 0.2	-51 ± 9

expression assay) did not necessarily correspond to high transrepression activity (low EC_{50} / high E_{max} in the IL-2 expression assay).

When incubating PBMLs from 15 healthy volunteers (a different study group than used for Figure 4 and 5) for 4 hours at 37^{0}C with 100 nM hydrocortisone, DEX, budesonide and prednisolone (n=8), also large interindividual differences in transactivation and transrepression capacities were found for the different GCs (Table 4). However, studying the whole group, we found correlations in transactivation levels (GILZ) between hydrocortisone-DEX (r=0.52, p=0.046), hydrocortisone-budesonide (r=0.48, p=0.069) and hydrocortisone-prednisolone (r=0.86, p=0.007) (Figure 6), and in transrepression levels (IL-2) between hydrocortisone-DEX (r=0.62, p=0.014), hydrocortisone-budesonide (r=0.71, p=0.003), hydrocortisone-prednisolone (r=0.71, p=0.047) (Figure 7). However, no correlations were found between the two expression assays.

Furthermore, we observed a difference in mean ranking of the relative potencies and in the absolute effects (Table 5) of the GCs tested between the GILZ and IL-2 expression assays. In the GILZ expression assay, the order in potencies for the different GCs was DEX>budesonide>prednisolone>hydrocortisone, whereas this was budesonide>DEX>prednisolone>hydrocortisone in the IL-2 expression assay.

2.3.5 Sensitivity to DEX *in vivo*

All 15 subjects underwent a 0.25-mg overnight DST, and serum cortisol levels before and after the administration of DEX were measured (Table 6). No correlations were found between the results of the DST and GILZ and IL-2 expression assays.

Table 6 Serum cortisol levels of 15 healthy volunteers before and after a 0.25-mg overnight DST

	Cortisol concentrations (nmol/l)		
Subjects	before DEX	after DEX	Before - after
1	465	170	295
2	472	228	244
3	370	298	72
4	692	71	621
5	394	231	163
6	373	274	99
7	727	416	311
8	284	255	29
9	540	400	140
10	425	570	-145
11	570	358	212
12	469	448	21
13	351	225	126
14	457	197	260
15	362	142	220

2.4 Discussion

In this study, we developed a new assay for the determination of individual cellular sensitivity to GCs in transactivation and transrepression by using Q-PCR analysis. Tonko *et al.* (13) identified several GC-regulated genes in the CCRF-CEM cell-line using DNA chip technology. We chose the glucocorticoid-induced leucine zipper (GILZ) protein, first identified by D'Adamio *et al.* (14), for our experiments on transactivation, as this protein showed the highest levels of mRNA upregulation induced by dexamethasone (DEX) in these cells. We selected IL-2 for studying transrepression activity, as it is the prototype of a key immune gene repressed by GCs via NF-κB (15-17). We wanted to determine whether the mRNA of these marker genes could be used in bioassays to determine GC potency and GC sensitivity of patient-derived lymphocytes *ex vivo*.

To determine whether GC-effects on GILZ gene expression are really mediated by the GR, we incubated CCRF-CEM cells with GCs (DEX, hydrocortisone), GR-antagonists (RU 38486, Org 31806, ZK 98299), PR-agonists (megestrol acetate, progesterone, MPA), MR-agonists (deoxycorticosterone, aldosterone) and the ER-agonist E2. The results demonstrate that GILZ gene expression is indeed regulated via the GR,

as only the GCs and the steroids with known GC-activity (megestrol acetate and MPA) were able to induce GILZ mRNA levels. Furthermore, co-incubation of DEX-stimulated cells with the GR-antagonist RU 38486 led to a considerable suppression of the transactivation of GILZ by DEX.

When exploring the inter- and intraindividual regulation of GILZ and IL-2 gene expression by GCs, large differences in both EC_{50} and E_{max} values were shown. Such a variation was also found in other studies using different tests of *ex vivo* sensitivity (18, 19). In a given individual however, the variation of E_{max} of GILZ and IL-2 response calculated at various time points, was much less. In contrast, for EC_{50}, interindividual variation could not be distinguished from within-individual variation (Figure 4, Table 2). Thus, E_{max} is a more suitable marker to categorize individuals into steroid sensitive and steroid resistant groups than EC_{50}. As it is thought that most known side effects of GCs are driven by actions that are regulated by transactivation rather than transrepression (7, 20, 21), the ideal GC would be a relatively weak inducer of GILZ gene expression while at the same time being a strong suppressor of IL-2 gene expression. The average GILZ and IL-2 responses in the healthy control group (Figure 5A and 5B) can be used as reference when patients with disease states in whom cellular GC sensitivity could be affected are explored.

We were also interested whether there is a relationship between the *ex vivo* outcomes from our expression assay and the *in vivo* effects of GCs. We subjected a group of fifteen healthy volunteers (not using GCs or oral contraceptives) to a 0.25-mg DST, and in parallel, measured *ex vivo* the induction of the expression of GILZ and the suppression of the expression of IL-2 by hydrocortisone, DEX, budesonide, and prednisolone in their PBMLs. No significant correlations were found between the cortisol response to DEX in the 0.25-mg DST and the outcomes from the GILZ and IL-2 expression assays. However, in the whole study group correlations were found for GILZ and IL-2 mRNA levels for hydrocortisone with DEX, budesonide and prednisolone. So, for an individual person, the potency of DEX, budesonide, and prednisolone can be predicted from knowing only the potency of hydrocortisone. However, it is uncertain whether this also accounts for other GCs. Furthermore, differences in mean ranking of the relative potencies of the GCs were observed between the two expression assays. In the GILZ expression assay, the order was DEX >budesonide>prednisolone>hydrocortisone, whereas it was budesonide>DEX>predni solone>hydrocortisone in the IL-2 expression assay. Whelan *et al.* compared in their study the potency of budesonide, DEX and hydrocortisone (and beclomethasone dipropionate) in inhibition of IL-5 and IFN-γ and found a ranking of, from greatest to least, budesonide, DEX, and hydrocortisone, which is similar to our results in the IL-2 expression assay.

To study individually determined cellular GC sensitivity in large population based studies and in small children, the GILZ and IL-2 assays needed to be downscaled in order to reduce the required number of cells, reduce costs, and making the assay less laborious without giving in accuracy. Unfortunately, due to statistical

reasons, especially EC_{50} values could not be calculated with less than 6 different concentrations. This indicates that the way in which the assay is currently performed could not substantially be simplified with respect to labour and costs, but reliable E_{max} values were obtained (Figure 4) by using only 5 million instead of 40 million cells, which means that only 5 mL of whole blood is necessary instead of 40 mL.

The present report describes the use of a new mRNA expression assay to determine an individual's cellular GC sensitivity using Q-PCR. This study shows the large interindividual variation in GC sensitivity existing in the healthy population. The intraindividual variation for E_{max} is low, compared to other assays, which determine cellular GC sensitivity. The intra-variation in EC_{50} values is too large to be used as independent marker for GC-sensitivity and could be attributed to the variable physical condition in time of an individual. Furthermore, we show that in a larger group (n=15), correlations exist between the potency of hydrocortisone and DEX, budesonide and prednisolone in the GILZ and IL-2 expression assays. As we expected, differences were found between the GCs with respect to their relative potencies for transactivation and transrepression. But surprisingly, the order of potencies was not only different for transactivation and transrepression, but also between individuals. Also neither the results of the GILZ nor the IL-2 expression assay correlated with the results of the DST. This lack of correlation between the DST and the GILZ and IL-2 expression assays may reflect the mechanistic differences between stimulation and repression of gene expression *in vitro* and the process of HPA-axis regulation *in vivo*. However, these assays may be useful in determining the optimal type and dosage of GC in individual patients.

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Chapter 3

Two Polymorphisms in the Glucocorticoid Receptor Gene Directly Affect Glucocorticoid-Regulated Gene Expression

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Abstract

Context: Interindividual variation in glucocorticoid (GC)-sensitivity can be partly explained by polymorphisms in the GC receptor (GR) gene. The ER22/23EK and N363S polymorphisms have been described to be associated with lower and higher GC sensitivity, respectively.

Objective and Design: We examined the basis of this altered GC sensitivity by expressing GR(N363S) and GR(ER22/23EK) in COS-1 cells and investigating their transactivating and transrepressing capacities using a GC response element-luciferase reporter and a p65-activated nuclear factor κB-luciferase reporter, respectively. Furthermore, we evaluated the transactivating and transrepressing capacities of the GR in peripheral blood mononuclear lymphocytes of homozygous and heterozygous carriers of these polymorphisms by determining the maximum effect of dexamethasone on transactivation of the GC-induced leucine zipper and transinhibition of the IL-2 gene by means of real-time RT-PCR.

Results: The effects of the polymorphisms in the GR gene previously observed in population studies were also detected at the level of gene expression. The ER22/23EK polymorphism resulted in a significant reduction of transactivating capacity, in both transfection experiments (–14 ± 5%, $p<0.05$) and peripheral blood mononuclear lymphocytes of carriers of this polymorphism (homozygous: –48 ± 6%, $p<0.01$, $n=1$; heterozygous: –21 ± 4%, $p=0.08$, $n=3$). The N363S polymorphism, associated with increased GC sensitivity, resulted in a significantly increased transactivating capacity, both *in vitro* (8 ± 3%; $p<0.02$) and *ex vivo* (homozygous: 204 ± 19%, $p<0.0001$, $n=1$; heterozygous: 124 ± 8%, $p=0.05$, $n=3$). Neither the ER22/23EK nor the N363S polymorphism seemed to influence the transrepressing capacity of the GR.

Conclusion: The presence of these and other GC sensitivity-modulating polymorphisms may have consequences for the use of GCs in a clinical setting.

3.1 Introduction

Glucocorticoids (GCs) (cortisol in humans, corticosterone in rodents) are key hormones in metabolic and immunological homeostasis. They regulate many physiological processes and especially their immunosuppressive and anti-inflammatory actions explain the widespread use of synthetic GCs in a variety of (auto)immune diseases (1, 2).

Within the healthy population, considerable interindividual variation in GC sensitivity exists, as is demonstrated by a variable suppressive response to 0.25 mg dexamethasone (DEX) (3). This implies that each subject, when treated with GCs, needs an individually optimized dose to maintain a balance between beneficial and adverse effects (e.g. diabetes mellitus, peptic ulcer, osteoporosis, skin atrophy, psychosis, glaucoma, and many others) associated with GC treatment (4). Within an individual person, however, GC sensitivity is rather stable (3), and the response to cortisol is correlated with that of several other corticosteroids (5). This implies that a set point for GC sensitivity with respect to the feedback system exists, which might be genetically determined.

One of the genetic factors involved is the occurrence of polymorphisms, generally defined as common variations at the DNA level with a frequency of more than 1% in the normal population. GC action is mediated by the GC receptor (GR). Two polymorphisms in the open reading frame of the GR gene have been described to be associated with altered sensitivity to GCs and may contribute to the interindividual differences (6, 7).

The most intriguing polymorphism is ER22/23EK present in exon 2, consisting of two linked single nucleotide mutations in codons 22 and 23: GAG AGG (GluArg) -> GAA AAG (GluLys) (rs6189 and rs6190) (8). This polymorphism reduces sensitivity to GCs and results in a phenotype that can be summarized as a more favorable metabolic profile, resulting in an increased survival rate for carriers of the ER22/23EK polymorphism (7, 9, 10). The polymorphism probably alters the secondary structure of the mRNA of the GR, resulting in a higher expression of the GR-A (94 kDa) at the expense of the GR-B (91 kDa) isoform, of which the latter has more transactivating capacity. The shift in GR-A to GR-B expression ratio leads to an overall decrease in transcriptional activity (11).

Further downstream in exon 2, a polymorphism was identified that changes codon 363 from AAT to AGT (rs6195), resulting in a serine for asparagine substitution (8). This polymorphism increases sensitivity to GCs, whereas an increased insulin response to DEX and a tendency toward lower bone mineral density have also been observed. Some studies also found an association with increased body mass index (12, 13), but others did not (14, 15). The molecular mechanism through which the N363S polymorphism exerts its effects is unknown. It has been postulated that the polymorphism contributes a new serine residue for phosphorylation, whereby protein interactions with transcription cofactors might be altered (16).

In this study, we set out to establish the effects of these polymorphisms in functional bioassays. Previous reports showed that transient transfection assays by calcium phosphate precipitation did not show significant differences in activation or repression of gene expression driven from various promoters (17, 18). However, improvement in transfection methods (e.g. cationic liposome-mediated transfection) and possibilities to correct for transfection efficiency (e.g. renilla luciferase) encouraged us to reinvestigate the transactivating and transrepressing capacities of GR(ER22/23EK) and GR(N363S) from a GC response element (GRE)-driven or a p65-activated nuclear factor-κB (NF-κB) luciferase reporter, respectively. In addition, we investigated the effects of these polymorphisms on the regulation of two GC-sensitive genes: the GC-induced leucine zipper (GILZ), which is up-regulated (19, 20), and IL-2, which is down-regulated by GCs (21, 22, 23).

3.2 Materials and Methods

3.2.1 Materials, plasmids, and subjects

Dexamethasone was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Oligonucleotide primers for mutagenesis and quantitative PCR (Q-PCR) were synthesized by Biosource Europe S.A. (Nivelles, Belgium).

The pcDNA3 and pRL-cytomegalovirus (CMV) vectors were purchased from Invitrogen (Breda, The Netherlands) and Promega (Leiden, The Netherlands), respectively. The pRShGRα expression plasmid, the GRE-luciferase (LUC) and NF-κB-LUC reporter plasmid, p65 plasmid, and pTZ plasmid were described previously (17, 24).

Peripheral blood from three heterozygous and one homozygous ER22/23EK carriers and three heterozygous and one homozygous N363S carriers was used to study the transactivating and transrepressing capacities of the GR variants. Peripheral blood of 10 volunteers, all noncarriers of both polymorphisms, was used as control material. All subjects were healthy, and none were using exogenous GCs. From all subjects, informed consent was obtained and the Medical Ethics Committee of Erasmus MC, The Netherlands, approved this study.

3.2.2 Plasmid construction

pcDNA3hGRα was generated by digesting pRShGRα with *Kpn*I and *Xho*I and cloning the resulting fragment into the *KpnI* and *Xho*I sites of pcDNA3. The ER22/23EK and N363S polymorphisms were introduced independently into pcDNA3hGRα by using a QuickChange site-directed mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands) according the manufacturer's guidelines.

3.2.3 Cell culture

Monkey kidney (COS-1) cells were maintained in a 5% CO_2 humidified incubator at 37°C in DMEM tissue culture medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/liter penicillin, 100 mg/liter streptomycin, and 1.25 mg/liter fungizone and passaged every 3–4 d.

3.2.4 Transfections

For transcription regulation studies and Western immunoblot analysis, COS-1 cells (6.0 x 10^5/ml) were plated at 3.0 x 10^5 cells/well (2.8 cm^2) and grown for 24 h. Cells were transfected using FuGENE6 reagent (Roche Diagnostics, Almere, The Netherlands). Per well, 0.7 µl of reagent was diluted in 100 µl serum-free medium and mixed with 215 ng plasmid DNA. For the GRE-LUC measurements, this pool of plasmid DNA contained the indicated human (h)GR-α expression plasmids (7.5 ng), GRE-LUC reporter (50 ng), CMV-renilla expression (2 ng/well), and pTZ carrier plasmid and for the NF-κB-LUC measurements, the indicated hGR-α expression plasmid (4.0 ng), NF-κB-LUC reporter (50 ng), p65 expression (10 ng), CMV-renilla expression (2 ng), and pTZ carrier plasmid. After an incubation period of 30 min at room temperature, the mixture was added to the cells. Cells were subsequently returned to the incubator until the reporter luciferase assay or Western immunoblot analysis.

3.2.5 Reporter luciferase assay

Five hours after transfection, the indicated concentrations of DEX were added. Twenty hours later, cells were lysed in 100 µl lysis buffer [25 mM trisphosphate (pH 7.8), 15% glycerol, 1% Triton X-100, 1 mM dithiothreitol, and 8 mM $MgCl_2$]. Luciferase activity was measured in 25 µl in a TOPCOUNT luminometer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), using the Dual-Glo luciferase assay system (Promega). By using the Stop&Glo reagents, luminescence was also measured from the pCMV-renilla expression plasmid to correct for transfection efficiency.

3.2.6 Western immunoblot analysis

Cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 0.1% sodium dodecyl sulfate, 150 mM NaCl, 5 mM dithiothreitol, and protease inhibitors (Complete, Roche Diagnostics). After a 10-min high-speed (100,000 rpm) spin to remove cellular debris, protein concentrations were determined by a Bradford assay. Equal amounts (30 µg) of protein were separated on SDS-PAGE (8%) (25) and transferred to nitrocellulose membranes. The membranes were washed in TBS-T (Tris-buffered saline with 0.1% Tween 20) and blocked in TBS-T with 5% nonfatty milk powder for 1 h at room temperature. Blots were incubated overnight at 4°C in block buffer, supplemented with the anti-hGR 57 (1:2500) (10P's, Breda, The Netherlands) or anti-hGR (P-20) (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA). Thereafter, blots were washed in TBS-T and subsequently incubated with horseradish peroxidase goat antirabbit IgG secondary antibody (1:10,000) (DakoCytomation, Glostrup, Denmark) for 2 h at

room temperature. After washing the blots in TBS-T, the proteins were visualized by enhanced chemoluminescence (Amersham Pharmacia Biotech, Roosendaal, The Netherlands).

3.2.7 Blood cell preparations

Peripheral blood was collected by venipuncture in heparinized tubes, and peripheral blood mononuclear lymphocytes (PBMLs) were obtained after density centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) as previously described (26). PBMLs were resuspended in RPMI 1640 medium with L-glutamine (300 mg/liter) (Invitrogen) supplemented with 100 U/liter penicillin, 100 mg/liter streptomycin, and 10% fetal bovine serum. Cells were incubated for 1 h at 37˚C in a shaking water bath to remove endogenous cortisol. Afterward medium was replaced, and 2 x 10^6 cells/well were precultured overnight (5% CO_2, 37˚C) in a 48-well plate at a density of 4 x 10^6 cells/ml. The next day PBMLs were incubated for 4 h with increasing DEX concentrations together with 10 µg/ml phytohemagglutinin (PHA).

Table 1 Primer and probe sequences for GILZ, IL-2, HPRT, and GR-α used in quantitative real-time PCR.

	sequence (5'-3')
GILZ forward primer	GCA CAA TTT CTC CAT CTC CTT CTT
GILZ reverse primer	TCA GAT GAT TCT TCA CCA GAT CCA
GILZ probe	6FAM-TCG ATC TTG TTG TCT ATG GCC ACC ACG-TAMRA
IL-2 forward primer	TTT GAA TGG AAT TAA TAA TTA CAA GAA TCC
IL-2 reverse primer	TTC TAG ACA CTG AAG CTG TTT CAG TTC
IL-2 probe	6-FAM-CAG GAT GCT CAC ATT TAA GTT TTA CAT GCC C-BHQ
HPRT forward primer	CAC TGG CAA AAC AAT GCA GAC T
HPRT reverse primer	CTC TGG CTT ATA TCC AAC ACT TCG T
HPRT probe	6FAM-CAA GCT TGC GAC CTT GAC CAT CTT TGG A-TAMRA
GR-α forward primer	TGT TTT GCT CCT GAT CTG A
GR-α reverse primer	TCG GGG AAT TCA ATA CTC A
GR-α probe	FAM-TGA CTC TAC CCT GCA TGT ACG AC-TAMRA

3.2.8 RNA isolation, RT reaction, and Q-PCR

PBMLs were washed with 0.15 M NaCl, and total RNA was isolated using a high pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol.

cDNA was synthesized in a reverse transcription reaction with Taqman reverse transcriptase reagents (Applied Biosystems) as described previously (5).

GILZ, IL-2, and GR-α mRNA expression levels were determined in a Q-PCR, by using primers and probes (Biosource International, Camarillo, CA), that were designed by using the Primer Express software (Applied Biosystems, Foster City, CA). Correction for assay variability was performed using the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) of which expression levels are stable and not influenced by GCs in this cell system (data not shown). The primer

sequences used are presented in Table 1. The reaction to determine GILZ, IL-2, and HPRT mRNA expression levels, with a total volume of 25 µl, contained 2.5 µl cDNA template (corresponding to 10 ng total RNA in the RT-PCR), 12.5 µl universal master mix (Roche, Branchburg, NJ), 0.3 pmol/µl forward and reverse primers (0.5 pmol/µl for HPRT), and 0.1 pmol/µl probe (0.2 pmol/µl for HPRT), whereas the reaction to determine GR-α levels contained 2.5 µl cDNA template, 7.5 pmol/µl of each primer, 5 pmol/µl probe in a Q-PCR-core kit (Eurogentec, Liege, Belgium). Standard PCR conditions, as supplied by the manufacturer, were used for analysis on an ABI 7700 sequence detector system (Applied Biosystems). The expression levels of GILZ, IL-2, GR-α, and HPRT were calculated using the comparative threshold method, according to the manufacturer's guidelines.

3.2.9 Statistical analysis

Data were analyzed statistically using Instat software version 2.01 (GraphPad Software, Inc., San Diego, CA). The differences in transcriptional activity of GR variants measured *in vitro* were determined using one-way ANOVA. Bonferroni *post hoc* tests were used to test for differences between each GR variant and to correct for multiple comparisons. The differences in total response of DEX-induced up- and down-regulation of GILZ and IL-2 mRNA levels in carriers of the indicated GR polymorphisms, compared with noncarriers, were analyzed by the Student's *t* test using the area under the curve. Data were expressed as mean ± S.E.M. $p<0.05$ was considered as significant.

3.3 Results

3.3.1 Transfection of GR variants

The GR(ER22/23EK) and GR(N363S) variants were expressed in COS-1 cells, a system known to be devoid of endogenous GR (17). To examine overall receptor expression, immunoblot analysis was performed with GR antibody 57, which is raised against amino acid 346–367 (27). Figure 1A shows that all GR constructs were expressed. The 94-kDa band is the full-length GR (amino acids 1–777), also called GR-A (Met-1), whereas the 91-kDa band represents the translation variant GR-B (Met-27). The 82-kDa band was thought to be a degradation product of GR-B (28); however, recently additional translation variants have been reported, and the 82-kDa band has been named GR-C (Met-86) (29). We have previously shown that the ER22/23EK polymorphism leads to a modest shift in translation in favor of GR-A over GR-B, but this difference is difficult to detect by semiquantitative Western blotting (11). Densitometric scanning indicates that the GR-B band of GR(ER22/23EK) is slightly weaker than the band of GR[wild type (WT)]; this difference, however, did not reach statistical significance. Because the epitope of GR antibody 57 contains codon 363, proper expression of

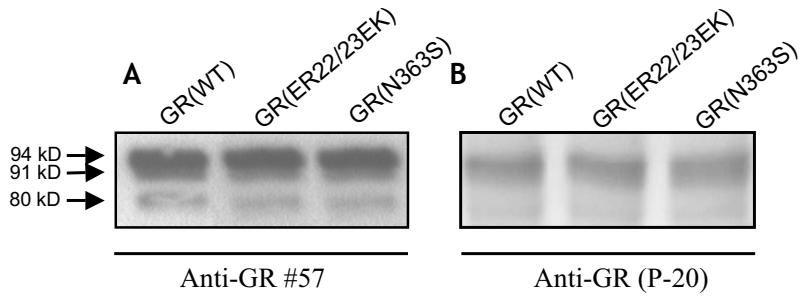

Figure 1 Expression of GR(WT), GR(ER22/23EK), and GR(N363S) variants from recombinant constructs. The pcDNA3hGR(WT), pcDNA3hGR(ER22/23EK), and pcDNA3hGR(N363S) vectors were transfected to COS-1 cells. After 24 h, cells were lysed, and 30 µg of proteins were electrophoresed on an 8% polyacrylamide gel and subsequently transblotted to a nitrocellulose membrane. After incubation with GR antibody 57 (A) or GR antibody P20 (B) followed by a secondary antibody, protein bands were visualized by enhanced chemoluminescence. The 94-kDa band represents the full-length GR protein, whereas the 91- and 80-kDa bands represent translational variants.

GR(N363S) was further confirmed by using an antibody (GR antibody P20) directed against a C-terminal epitope (Figure 1B).

3.3.2 Transactivation by the GR variants

The expressed GR(ER22/23EK) and GR(N363S) proteins were activated by increasing amounts of DEX, whereafter their capacity to transactivate GRE-driven transcription was investigated. The maximal response of the GR(WT) to activate transcription was set to 100%, and Figure 2A shows that the N363S polymorphism increased maximal transactivation by 8.0 ± 3% ($p<0.02$), whereas a reduction of the maximal response by 14 ± 5% ($p<0.05$) occurred when the ER22/23EK polymorphism was present. No significant differences in EC_{50} were found.

3.3.3 NF-κB transcriptional repression by the GR variants

Transfection of the 5 x NF-κB response element-LUC reporter gene in COS-1 cells established a basal luciferase activity that could not be repressed by GR(WT) in the presence of DEX (data not shown). After cotransfecting a plasmid expressing the p65-subunit, luciferase expression was increased 5-fold, which was maximally repressed to 57 ± 8% by GR(WT) (Figure 2B). Figure 2B also shows that the ER22/23EK and N363S receptor protein variants seemed to repress the p65-dimer activity to a similar extent, as did the wild type. However, the variability in these experiments was higher than in the transactivation experiments, and any effects might be obscured by noise.

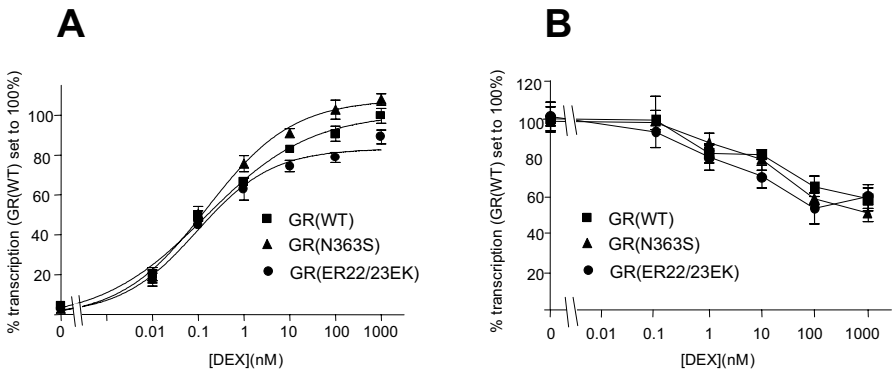

Figure 2 Transcriptional activation and repression capacities of GR(WT) (■), GR(ER22/23EK) (●), and GR(N363S) (▲). COS-1 cells were cotransfected with a GRE-LUC reporter construct (A) or a p65-activated NF-κB reporter (B) and vectors expressing GR(WT), GR(ER22/23EK), or GR(N363S). Five hours after transfection, cells were treated with the indicated amounts of DEX for 20 h, and luciferase activity was measured. Data represent means ± S.E.M. of four experiments, each with quadruplicate measurements.

3.3.4 Transactivating and transrepressing capacities in PBMLs of homozygous and heterozygous GR variant carriers

GILZ and IL-2 are GC-responsive genes in PBMLs. GILZ expression is strongly up-regulated by GCs, whereas IL-2 is down-regulated (5). PHA was necessary to induce transcription of the IL-2 gene but did not affect expression of GILZ or GR mRNA levels (data not shown). The effects of GILZ and IL-2 are GC specific because they can be abrogated by addition of RU38486 and are not evoked by other steroids (e.g. progesterone, estradiol, etc.) (5). The response to DEX in expression of these genes is therefore a measure for the transactivating and transrepressing capacities of the GR variants.

PBMLs from carriers of the polymorphisms or noncarrier controls were stimulated with PHA and the indicated doses of DEX. The changes in mRNA levels for GILZ and IL-2 (relative to the values in the absence of DEX) are shown in Figure 3. No systematic differences between genotypes were observed for the values in the absence of DEX (data not shown).

The total response of GILZ expression to DEX in cells of the homozygous GR-N363S carrier was two times higher (204 ± 19%; $p<0.0001$) than the average response in the control group, whereas the average total response measured in PBMLs of the heterozygous carriers was 124 ± 8% ($p=0.05$). In PBMLs of the homozygous GR-ER22/23EK carrier, a response of 52 ± 6% ($p< 0.01$) was measured, whereas in the heterozygous GR-ER22/23EK carriers, this response was 79 ± 4% ($p=0.08$). IL-2 down-regulation in PBMLs of the homozygous and heterozygous ER22/23EK, and heterozygous N363S carriers did not significantly differ from the control group, but in the homozygous N363S carrier, the total DEX-induced capacity to transrepress was decreased by 19 ± 4% ($p<0.05$).

Overall expression levels of the GR were not significantly different between controls and heterozygous or homozygous carriers of the two polymorphisms as measured by [^{3}H]DEX binding capacity of the cells [n (receptors/cell) = 5547 ± 991 in controls; n = 5844 ± 640 and 5213 in, respectively, hetero- and homozygous N363S carriers; n = 6258 ± 300 and 5785 in, respectively, hetero- and homozygous ER22/23EK carriers] and quantitation of the GR mRNA by real-time RT-PCR (data not shown). On the indicated DEX treatment, GR mRNA levels decreased with 21 ± 6% ($p<0.02$), and this decrease was equal in PBMLs of controls, ER22/23EK, and N363S carriers (data not shown).

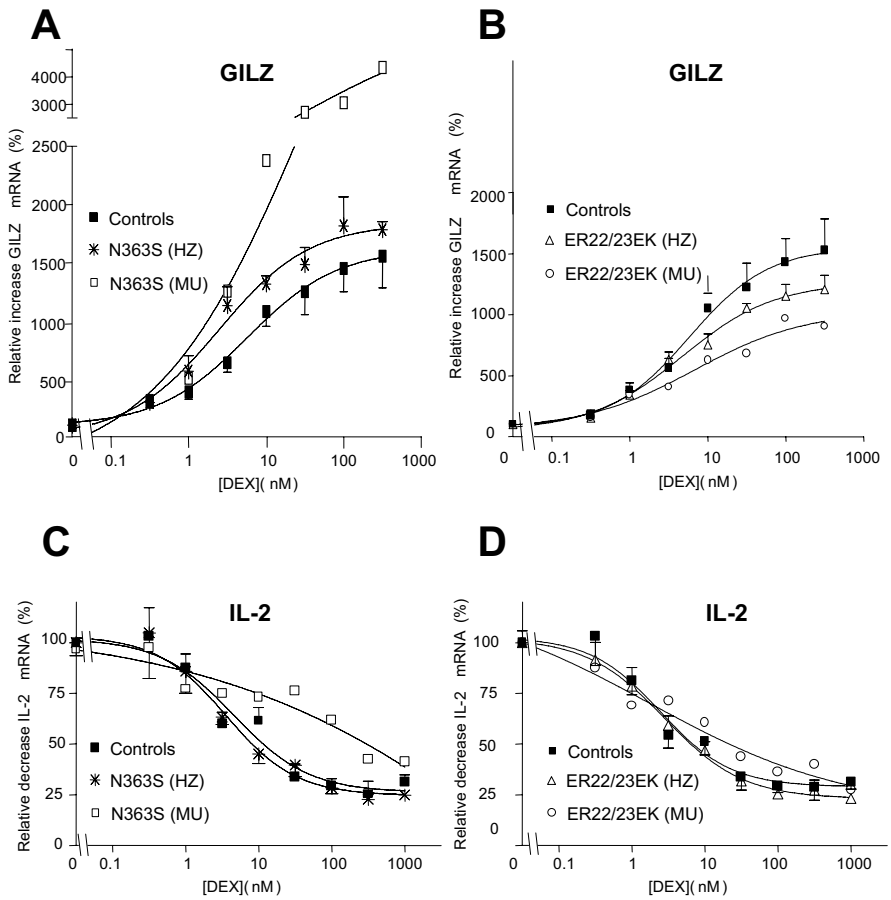

Figure 3 Relative responses of GILZ and IL-2 mRNA expression to DEX in PBMLs of noncarriers and carriers of either the ER22/23EK or N363S polymorphism in the GR gene. PBMLs of homozygous (MU, n=1) and heterozygous (HZ, n=3) carriers of either the ER22/23EK or N363S polymorphisms in the GR gene were incubated for 4 h with PHA and the indicated concentrations of DEX, followed by mRNA isolation and quantitation by real-time RT-PCR. Healthy noncarriers served as controls (n=10). Data are presented as the increase of GILZ (A and B) and decrease of IL-2 (C and D) mRNA relative to the values in the absence of DEX and represent means ± S.E.M. of the average response in PBMLs of the indicated number of subjects. No systemic differences between genotypes were observed for the values in the absence of DEX (data not shown). DEX incubations were performed in duplicate, and duplicate RT-PCR was performed for every sample. For all genotypes, PHA treatment in the absence of DEX led to a 12- to 17-fold stimulation in IL-2 mRNA levels but did not affect GILZ mRNA levels (data not shown).

3.4 Discussion

The ER22/23EK polymorphism is associated with a relative resistance to GCs (7, 9, 10, 30, 31, 32). It is situated in the τ1-transactivating domain but is not within its core region, which is variably defined as amino acid 77–262 (33) or 98–305 (34). This study shows that the polymorphism influences the transactivating capacity of the GR: in transfected cells we found a reduced capacity for GRE-driven LUC activation (Figure 2A), whereas in PBMLs from hetero- and homozygous carriers of this polymorphism treated with increasing concentrations of DEX, a significant reduction in the activation of GILZ transcription was observed (Figure 3A). This was not mediated through differences in the regulation of the GR expression because the decrease in GR mRNA levels during the 4 h DEX treatment was not different from that in the controls. We could not detect differences in the transrepression of NF-κB activity (Figure 2), and although variability in these experiments was rather high, this result was supported in the experiments in PBMLs, in which transrepression of IL-2 was equal to that in the control group (Figure 3D).

We have recently shown that the ER22/23EK polymorphism leads to a modest shift in translation in favor of the translation variant GR-A over GR-B (11), of which the latter had a stronger transactivating effect in transient transfection experiments (28). This possibly explains the decreased GC sensitivity in GR(ER22/23EK) carriers. GR-A and GR-B are equally potent in inhibiting NF-κB activity (28), explaining the unchanged transrepression.

With respect to the N363S polymorphism, we found that it increased the transactivating capacity of the GR. In transfection experiments, the N363S polymorphism increased transcription from the GRE-LUC reporter (Figure 2A), whereas in PBMLs, expression of GILZ mRNA was increased in cells from both heterozygous and homozygous carriers of this polymorphism (Figure 3A). GR(N363S) down-regulation during DEX treatment was not different from that in controls.

The effects of the N363S polymorphism on transrepression are more difficult to interpret. This polymorphism did not significantly interfere with the NF-κB-driven transcription of the LUC reporter gene (Figure 2B) in transfection experiments, although the variability in these experiments was rather high, possibly obscuring any effects. However, the response of PBMLs from heterozygous carriers with respect to IL-2 transcription also did not differ from that of normal controls (Figure 3C), but cells from the homozygous carrier showed a reduced response to DEX, suggesting a decreased sensitivity. The GR can interfere in at least two ways with IL-2 expression: by direct inhibition of the activator function of NF-κB and up-regulation of inhibitory-κBα (21, 22, 23). We assume that, like in the transfection assay, direct inhibition of NF-κB is not affected by the N363S polymorphism. Therefore, the observed effect on IL-2 may be due to GR-induced up-regulation of inhibitory-κBα. However, it is also possible that GILZ (35, 36) or other aspects of the signaling network are affected. Finally, it is possible that this effect is due to the homozygous presence of the

polymorphism or rather to the absence of the wild-type allele. The results also differ from those previously observed in nine heterozygous carriers whose PBMLs were tested in a mitogen-stimulated proliferation assay (6). There we found a tendency to increased sensitivity (lower IC_{50} values) for the carriers. However, whereas IL-2 production certainly plays a role in that assay, it is carried out over a much longer time scale (4 d, rather than 4 h in the current assays), and the outcome is formed by the integration of many processes, including apoptosis.

The exact molecular mechanism underlying the increased sensitivity in N363S carriers is still unknown. The GR has been shown to be poly-phosphorylated on serine and threonine residues in the N-terminal domain of the protein (35, 36), and it has been suggested that the N363S variant introduces a new phosphorylation site, possibly altering interactions with other transcription factors (16). However, this serine residue is not in one of the presently known consensus phosphorylation sites (36).

The allele frequencies of the ER22/23EK (2.5%) and N363S (4.5%) polymorphisms are relatively low (30). In the (Caucasian) population that we studied, approximately one of 1000 subjects is homozygous for the ER22/23EK or N363S polymorphism (6, 8, 30). Therefore, the possibility to investigate the transactivating and transrepressing properties *ex vivo* is rather exceptional. This can partly be overcome by also performing transfection experiments. Clearly these assays show less sensitivity and more variation than the experiments with PBMLs from carriers. On the other hand, in the transfection experiments in COS cells, the polymorphisms are the only variable, whereas in PBMLs of our subjects, variables other than the GR polymorphisms might also influence GC-mediated gene regulation. Overall, both types of experiments showed similar results.

In conclusion, the ER22/23EK and N363S polymorphisms in the GR gene can partly explain interindividual variation in sensitivity to GCs. These polymorphisms alter sensitivity to GCs, which is mainly caused by alterations in the transactivating capacity of the receptor. In our population-based studies, carriers of the ER22/23EK or N363S polymorphisms showed impressive effects on cardiovascular and metabolic profiles (30). Recent studies suggested that GC excess *in utero* is linked to cardiovascular and metabolic diseases in adulthood due to changes in fetal programming of the hypothalamic-pituitary-adrenal axis (37, 38). This programming might also be influenced by the GR polymorphisms predisposing for the described cardiovascular and metabolic profiles. The relatively small direct effects of the polymorphisms reported in this study, to which carriers are indeed exposed for life, could add up to these programmed effects, together causing the dramatic phenotypes observed.

Acknowledgements

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Chapter 4

Increased Expression of the Glucocorticoid Receptor-A Translational Isoform as a Result of the ER22/23EK Polymorphism

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Abstract

One of the most intriguing polymorphisms in the GR [glucocorticoid (GC) receptor] gene is in codons 22 and 23 [GAG AGG(GluArg) -> GAA AAG (GluLys)]. This polymorphism is associated with a reduced GC sensitivity, a better metabolic and cardiovascular health profile, and an increased survival rate. Recently, Yudt and Cidlowski reported that two different methionine codons in the GR mRNA may be used as initiation codon: AUG-1 and AUG-27, resulting in two isoforms, the GR-A and the GR-B proteins, respectively. They also showed that the GR-B protein had a stronger transactivating effect in transient transfection experiments.

In this study, we elucidated the molecular basis for the reduced GC sensitivity by investigating the influence of the ER22/23EK polymorphism on synthesis of GR-A and GR-B by expressing them independently from constructs with and without the polymorphic site. Binding studies with [^{3}H]-dexamethasone and transactivation studies showed that, when the ER22/23EK polymorphism is present, approximately 15% more GR-A protein was expressed, whereas total GR levels (GR-A + GR-B) were not affected. These results show that the transcriptional activity in GR(ER22/23EK) carriers is decreased because more of the less transcriptionally active GR-A isoform is formed. This is probably caused by secondary mRNA structure.

4.1 Introduction

Glucocorticoids (GCs) are widely used in clinical practice to treat immune diseases such as asthma, chronic intestinal inflammations, and prevention of rejection of organ transplants. It is well known that the effects of treatment vary between patients. Some patients develop side effects even on relatively low doses of therapeutically administered GCs, whereas others need a high dose to establish clinical effects without manifestation of side effects at all (1, 2). Factors like variations in systemic resorption and pharmacokinetic handling of GCs can be responsible for these differences (3). Also, an individual's cellular sensitivity to GCs is a factor with GC receptor (GR) number, regulation of splice or translational GR variants, mutations and/or polymorphisms in the GR gene, and the availability of cofactors as important variables (4, 5, 6).

A striking example of a factor that influences cellular sensitivity is the ER22/23EK polymorphism in the GR gene (7). This polymorphism consists of two single-nucleotide mutations in codons 22 and 23 in exon 2 of the GR gene that are always linked. The first mutation is silent, changing codon 22 from GAG to GAA, both coding for glutamic acid (E). The second one, changing codon 23 from AGG to AAG, results in a conservative amino acid change from arginine (R) to lysine (K). The ER22/23EK polymorphism is associated with relative resistance to GCs, and the resulting phenotypic differences have been reviewed by Van Rossum *et al.* (6). In summary, ER22/23EK carriers react with a smaller decrease in morning cortisol levels after a 1-mg dexamethasone suppression test and have lower total and low-density lipoprotein cholesterol levels, as well as lower fasting insulin concentrations and a better insulin sensitivity. Furthermore, C-reactive protein levels, which are positively related to cardiovascular damage (8), are lower in ER22/23EK carriers (6). These effects of the ER22/23EK polymorphism suggest a healthier cardiovascular and metabolic profile, which was confirmed in a follow-up study demonstrating an increased survival rate for carriers of the ER22/23EK polymorphism (9). The fact that the polymorphism is more prevalent in the older population (10) also indicates that ER22/23EK carriers have a higher chance to get older. Young adult male ER22/23EK carriers are significantly taller and have more muscle strength, whereas in young adult female carriers, waist circumference tended to be smaller (11). Furthermore, ER22/23EK carriers have a lower risk of dementia and have fewer white matter lesions in the brain, associated with small vessel disease (6, 12).

The molecular mechanism underlying the relatively decreased GC sensitivity associated with GR(ER22/23EK) is unknown. In addition to GR-splicing isoforms (e.g. GR-α, GR-β, and GR-P) of which GR-α is the functional active one (13, 14), also two translational isoforms have been described (15). The mRNA is subjected to alternative translation initiation, resulting in a longer isoform (GR-A), initiated from the first AUG codon (Met-1), and a shorter isoform, GR-B, initiated from an internal, in frame AUG codon (Met-27). Due to a weak Kozak translation initiation

consensus sequence, the ribosomal scanning mechanism does not always recognize the suboptimal first translation initiation codon, and translation is subsequently initiated from methionine 27. Transient transfection studies showed that GR-B was 1.4- to 2-fold more effective as a transactivator than GR-A on GC-responsive promoters containing a single GC response element (GRE), two GREs in tandem, or the mouse mammary tumor virus promoter (15).

The ER22/23EK polymorphism is in close proximity to the Met-1 and Met-27 translation initiation start sites. In this study, we show that this polymorphism may affect the ratio in which GR-A and GR-B are synthesized, suggesting that this may cause a relative decrease in GC sensitivity.

4.2 Materials and Methods

4.2.1 Materials and plasmids

Dexamethasone was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). [^{3}H]-dexamethasone (88 Ci/mmol) and L-[^{35}S]-methionine (977 Ci/mmol) were purchased from Amersham Biosciences (Roozendaal, The Netherlands). Oligonucleotide primers for mutagenesis and Q-PCR were synthesized by Biosource Europe S.A. (Nivelles, Belgium).

The pcDNA3.1 and pCMV-renilla vectors were purchased from Invitrogen Life Technologies (Breda, The Netherlands) and Promega Benelux B.V. (Leiden, The Netherlands), respectively. The pRShGRα expression plasmid, the GRE-LUC reporter plasmid and pTZ plasmid were described previously (16, 32).

4.2.2 Construction of GR plasmids

pcDNA3.1hGRα was generated by digesting pRShGRα with *Kpn*I and *Xho*I. The resulting 3000-bp fragment was subsequently cloned into the *Kpn*I and *Xho*I sites of pcDNA3.1. The pcDNA3.1hGRα(M27T) and pcDNA3.1hGRα(M1T) were generated to uniquely express GR-A and GR-B, respectively. The thymidine (T) residues at respectively cDNA positions 134 [numbering according to Hollenberg *et al.* (33)] and 212 of the pcDNA3.1hGRα vector were replaced by a cytidine (C). This mutagenesis was performed by using a QuikChange Site-Directed Mutagenesis Kit (Stratagene Europe, Amsterdam, The Netherlands) according to the manufacturer's protocol. To mutate position 134 the forward primer: 5'-GCC AGA GTT GAT ATT CAC TGA CGG ACT CCA AAG AAT C-3' was used in combination with the reverse primer: 5'-GAT TCT TTG GAG TCC GTC AGT GAA TAT CAA CTC TGG C-3'. Position 212 was mutated with 5'-GAG AGG GGA GAT GTG ACG GAC TTC TAT AAA ACCCTA AG-3' as forward primer and 5'-CTT AGG GTT TTA TAG AAG TCC GTC ACA TCT CCC CTC TC-3' as reverse primer. To introduce the ER22/23EK polymorphism in the pcDNA3.1hGRα, pcDNA3.1hGRα(M1T) and pcDNA3.1hGRα(M27T) vectors, the guanosine (G) residues at positions 198 and

200 were replaced by an adenosine (A), by using 5'-CCC AGC AGT GTG CTT GCT CAG GAA AAG GGA GAT GTG-3' as forward primer and 5'-CAC ATC TCC CTT TTC CTG AGC AAG CAC ACT GCT GGG-3' as the reverse. A strong Kozak consensus site of the AUG-1 start site of pcDNA3.1hGRα and pcDNA3.1hGRα(ER22/23EK) was created by replacing a cytidine (C) residue at cDNA position 130 by a guanosine (G) by using 5'-GCC AGA GTT GAT ATT CAG TCA TGG ACT CCA AAG AAT C-3' as forward primer and 5'-GAT TCT TTG GAG TCC ATC ACT GAA TAT CAA CTC TGG C-3' as the reverse. The constructed GR plasmids: pcDNA3.1hGRα(wt), pcDNA3.1hGRα(M27T), pcDNA3.1hGRα(M1T), pcDNA3.1hGRα(ER22/23EK), pcDNA3.1hGRα(ER22/23EK,M27T), and pcDNA3.1hGRα(M1T,ER22/23EK) are designated as phGR-wt, phGR-A-wt, phGR-B-wt, phGR-ER22/23EK, phGR-A-ER22/23EK, and phGR-B-ER22/23EK plasmids, respectively.

4.2.3 *In vitro* transcription and translation

GR-A and/or GR-B proteins were formed from the phGR-wt, phGR-A-wt, phGR-B-wt, phGR-ER22/23EK, phGR-A-ER22/23EK, phGR-B-ER22/23EK, and Kozak mutant plasmids *in vitro* by using a TnT Quick Coupled Transcription/Translation System (Promega) using [^{35}S]-methionine. A mixture of 20 µl TnT Quick Master Mix, 20 µCi [^{35}S]-methionine, 1 µg plasmid DNA template, and nuclease-free water to a final volume of 25 µl was incubated at 30°C for 90 min. The result of translation was analyzed by SDS-PAGE (34) and visualized by exposure to high-performance autoradiography film (Amersham Pharmacia Biotech, Chalfont, UK) for 5 h at –70°C.

4.2.4 Cell culture

Monkey kidney (COS-1) cells were maintained in a 5% CO_2 humidified incubator at 37°C in DMEM tissue culture medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/liter penicillin, 100 mg/liter streptomycin, and 1.25 mg/liter fungizone and passaged every 3–4 d.

4.2.5 Transfections

For transcription regulation studies, [^{3}H]-dexamethasone-binding studies, and quantitation of mRNA transcription, COS-1 cells (6.0 x 10^6/ml) were plated at 3.0 x 10^5 cells per well (2.8 cm^2) and grown for 24 h. Cells were transfected using FuGENE6 reagent (Roche Diagnostics Nederland B.V., Almere, The Netherlands). Per well, 0.7 µl of reagent was diluted in 100 µl serum-free medium and mixed with 215 ng plasmid DNA. This pool of plasmid DNA contained the indicated GR expression plasmids (7.5 ng), GRE-LUC reporter plasmid (50 ng), CMV-renilla expression plasmid (5 ng), and pTZ carrier plasmid (32). After an incubation period of 30 min at room temperature, the mixture was added to the cells. Cells were subsequently returned to the incubator until the reporter luciferase assay, [^{3}H]-dexamethasone binding studies, or quantitative mRNA analysis.

4.2.6 [^{3}H]-Dexamethasone binding capacity

Twenty-four hours after transfection, the cells were incubated in triplicate with 100 nM [^{3}H]-dexamethasone for quantification of the total dexamethasone-binding capacity (no unlabeled dexamethasone) and the nonspecific dexamethasone binding (in the presence of 40 μM of unlabeled dexamethasone). After an incubation period of 2 h at 37°C, cells were washed two times with cold 0.15 M NaCl and lysed in 150 μl 1 M NaOH. After neutralization with 150 μl HCl, 1 ml Microscint-40 scintillation solution (Packard Biosciences B.V., Groningen, The Netherlands) was added and radioactivity was counted in a liquid scintillation counter (TOPCOUNT). Total binding minus nonspecific binding was taken to represent specific, receptor-mediated binding (35). Luminescence from the pCMV-renilla expression plasmid was measured to correct for transfection efficiency. When this procedure was carried out with untransfected COS-1 cells (no endogenous GR), there was no difference between total and nonspecific binding (not shown). This procedure is essentially the same as described for Scatchard analysis of the GR, albeit that only the maximal binding capacity is estimated (35).

4.2.7 Reporter luciferase assay

Four to six hours after transfection, the indicated concentrations of dexamethasone were added. Twenty hours later, cells were lysed in 50 μl lysis buffer [25 mM trisphosphate (pH 7.8), 15% glycerol, 1% Triton X-100, 1 mM dithiothreitol, 8 mM $MgCl_2$]. Luciferase activity was measured in 20 μl in a TOPCOUNT (Packard, Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) luminometer, using the Dual-Glo Luciferase Assay System (Promega). By using the Stop&Glo reagents, luminescence was also measured from the pCMV-renilla expression plasmid, to correct for transfection efficiency.

4.2.8 Quantitative analysis of transcribed GR mRNA

Twenty-four hours after transfection, cells were washed with 0.15 M NaCl. Total RNA was isolated using a High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol.

cDNA was synthesized in a reverse transcriptase reaction with Taqman Reverse Transcriptase Reagents (Applied Biosystems). The reaction contained 500 ng RNA, 5.5 mM $MgCl_2$, 5 μl 10x reverse transcriptase buffer, 2 mM deoxynucleotide triphosphates, 5 μM random hexamers, 0.2 μM oligo deoxythymidine)$_{16}$, 20 U ribonuclease inhibitor and 62.5 U MultiScribe reverse transcriptase in a total volume of 50 μl.

Q-PCR was performed using qPCR Core Kit (Eurogentec, Maastricht, The Netherlands) in a total reaction volume of 25 μl. The reaction contained 2.5 μl 10x reaction buffer, 5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 300 nM forward primer, 300 nM reverse primer, 200 nM probe, 0.625 U HotGoldStar PCR enzyme, and 2 μl cDNA template, corresponding to 20 ng total RNA in the reverse transcriptase reaction. The reactions were carried out in an ABI 7700 Sequence Detector (Applied

Biosystems). After an initial heating at 95°C for 8 min, samples were subjected to 40 cycles of denaturation at 95°C for 15 sec and annealing for 1 min at 60°C. The primer sequences used included: GR forward 5'-TGT TTT GCT CCT GAT CTG A-3' and GR reverse 5'-TCG GGG AAT TCA ATA CTC A-3'. The probe sequence for GR mRNA was: 5'-FAM-TGA CTC TAC CCT GCA TGT ACG AC-TAMRA-3'. The expression levels of the GR were calculated according to the comparative threshold method, according to the manufacturer's guidelines.

4.2.9 Statistical analysis

Data were analyzed statistically using Instat software version 2.01 (GraphPad Software, Inc., San Diego, CA). The differences in transcriptional activity and mRNA expression levels were determined using ANOVA. When significant overall effects were obtained by ANOVA, multiple comparisons were made using the Bonferroni test. Differences in [^{3}H]-dexamethasone binding between the GR variants were analyzed nonparametrically using the Mann-Whitney test.

4.3 Results

4.3.1 Expression of the human GR constructs

To investigate the influence of the ER22/23EK polymorphism on translation of GR mRNA to GR-A and GR-B proteins, we constructed the phGR-wild-type (wt), phGR-A-wt, phGR-B-wt, phGR-ER22/23EK, phGR-A-ER22/23EK, and phGR-B-ER22/23EK expression vectors (Table 1).

Table 1 Variant GR expression vectors coding for the GR-A and/or GR-B isoforms.

Vector	*mRNA*	*Protein*
	GR-A ↓ GR-B ↓	
phGR-wt	-----(AUG)$_1$-------//-----(GAG AGG)---(AUG)$_{27}$---	GR-A + GR-B
phGR-A-wt	-----(AUG)$_1$-------//-----(GAG AGG)---(A**C**G)$_{27}$---	GR-A
phGR-B-wt	-----(A**C**G)$_1$-------//-----(GAG AGG)---(AUG)$_{27}$---	GR-B
phGR-ER22/23EK	-----(AUG)$_1$-------//-----(GA**A** A**A**G)---(AUG)$_{27}$---	GR-A + GR-B
phGR-A-ER22/23EK	----(AUG)$_1$-------//-----(GA**A** A**A**G)---(A**C**G)$_{27}$---	GR-A
phGR-B-ER22/23EK	---(A**C**G)$_1$-------//-----(GA**A** A**A**G)---(AUG)$_{27}$---	GR-B

We first investigated whether proper receptor protein is expressed from these constructs by performing *in vitro* transcription and translation using rabbit reticulocyte lysate and incorporation of [^{35}S]-methionine. From the phGR-wt and phGR-ER22/23EK constructs both 94 kDa (GR-A) and 91 kDa (GR-B) proteins were expressed as shown by the double band in Figure 1, lanes 2 and 5. From the phGR-A-wt and phGR-A-ER22/23EK vectors, only the 94-kDa isoform was expressed (Figure 1, lanes 3 and 6), whereas from the phGR-B-wt and phGR-B-ER22/23EK vectors, only the 91-kDa isoform was produced (Figure 1, lanes 1 and 4). Optical densitometry scanning showed that GR-A expression from phGR-ER22/23EK and from phGR-A-ER22/23EK was approximately 10% higher than from the corresponding wt vectors; however, this difference was not significant (data not shown).

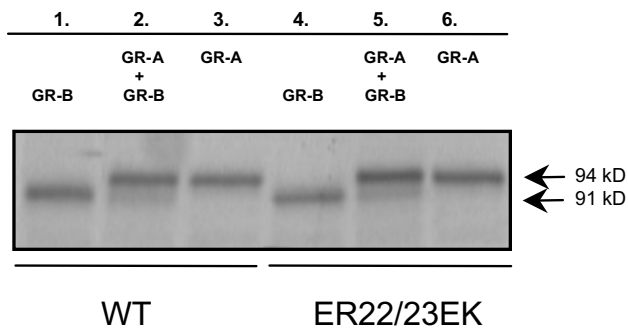

Figure 1 *In vitro* expression of GR-A and GR-B isoforms from recombinant construct. The wt GR, the wt start site mutants, the polymorphic GR and the polymorphic start site mutants were prepared by *in vitro* translation using [^{35}S]-methionine and reticulocyte lysates. Expressed proteins were electrophoresed on a 7% polyacrylamide gel and visualized by exposure to high-performance autoradiography film. The synthesis from methionine 1 (in mutant M27T) using either the wt (lane 3) or polymorphic (lane 6) construct is referred to as GR-A isoform, whereas the protein synthesized from methionine 27 (in mutant M1T) using either the wt (lane 1) or polymorphic (lane 4) construct is referred to as GR-B isoform. From the wt (lane 2) and polymorphic (lane 5) constructs, both isoforms were expressed.

4.3.2 [^{3}H]-Dexamethasone binding to GR variants

To confirm that more GR-A is expressed from the vector containing the ER22/23EK polymorphism than from the wt construct, we transfected COS-1 cells, a cell system known to be devoid of endogenous GR protein (16), with the wt and polymorphic constructs individually expressing the GR-A and GR-B isoforms and incubated them with 100 nM [^{3}H]-dexamethasone. Because the dissociation constant of the GR is about 8 nM, at this concentration, the receptors will be almost fully occupied. Therefore, the amount of specifically bound [^{3}H]-dexamethasone (= total binding – nonspecific binding) is a measure for GR levels expressed in the cells. As shown in Figure 2, the amount of total GR protein (GR-A + GR-B) expressed from the phGR-wt

and phGR-ER22/23EK vectors did not differ significantly. However, from the phGR-A-ER22/23EK vector, 15 ± 6% ($p<0.05$) more GR-A protein was expressed than from the phGR-A-wt vector. Levels of GR-B protein expressed from the phGR-B-wt and phGR-B-ER22/23EK did not differ (Figure 2).

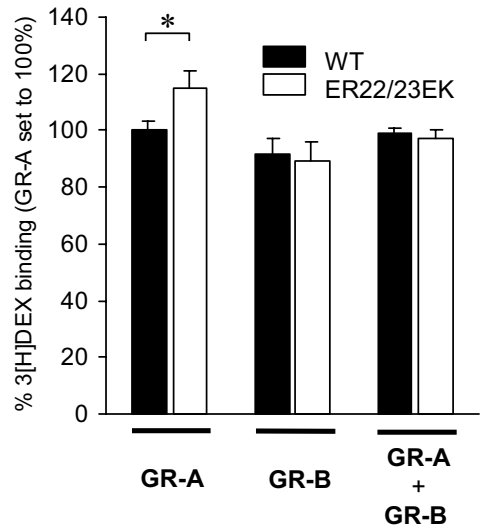

Figure 2 [^{3}H]-Dexamethasone binding to GR-A and GR-B isoforms expressed from wt and polymorphic contructs. COS-1 cells were transfected with each of the constructs from Table 1 expressing the indicated GR isoforms. After 24 h, 100 nM [^{3}H]-dexamethasone without (total binding) or with (nonspecific binding) a 400-fold excess of unlabeled dexamethasone was added and incubated for 2 h at 37°C. Bars represent the amount of specifically bound [^{3}H]-dexamethasone relative to binding to the GR(A+B) expression from the phGR-wt plasmid (100%). (means ± S.E.M.) of four representative experiments. *, $p<0.05$ by nonparametric Mann-Whitney test.

4.3.3 Transcriptional activity

If the polymorphism influences the ratio in which the less transcriptionally active GR-A and the more transcriptionally active GR-B isoforms are formed, then also the effectiveness of the activation of gene transcription by GR might be changed. The phGR-wt and phGR-ER22/23EK constructs, which can both express the GR-A and GR-B isoforms, were transfected together with a GRE-LUC reporter gene. Increasing amounts of dexamethasone were added to create dose response curves. Figure 3 shows that the presence of the ER22/23EK polymorphism resulted in a reduction of the maximal transcriptional activity by 14 ± 5% ($p<0.05$). No significant difference in EC_{50} was detected.

To investigate whether the ER22/23EK polymorphism selectively stimulates or represses expression of the GR-A or/and GR-B isoform, we determined the maximal transcriptional activity expressed from the AUG-1 and the AUG-27 translation initiation start codon. We transfected these four constructs together with the luciferase reporter gene into COS-1 cells, treated them with 100 nM dexamethasone

and measured the luciferase activity. Figure 4 shows that the transcriptional activity of GR-B expressed from the wt construct was 146 ± 5%, ($p<0.01$) of that of GR-A, which is in line with results previously reported by Yudt and Cidlowski (15). Furthermore, the phGR-A-ER22/23EK construct showed 17 ± 5% ($p<0.05$) more transcriptional activity than the phGR-A-wt construct. For the transactivating capacity of GR-B, it did not matter if it was expressed from the wt or the polymorphic plasmid.

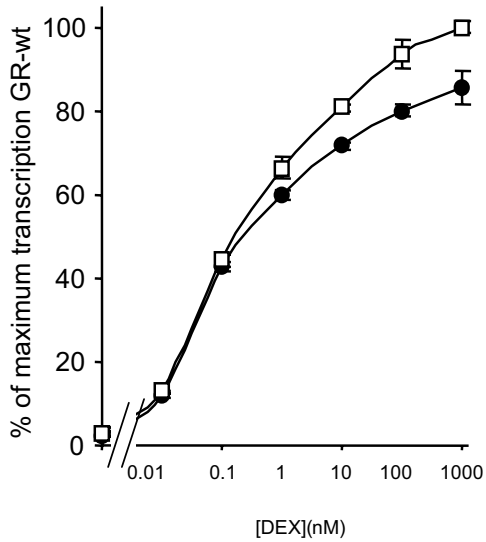

Figure 3 Transcriptional activation of a GRE containing reporter gene by GR wt (□) and GR-ER22/23EK (●). COS-1 cells were cotransfected with the GRE-LUC reporter construct and either the wt or polymorphic GR expression vector. After 5 h, cells were treated with the indicated amounts of dexamethasone for 20 h and luciferase activity was measured. Data represents means ± S.E.M. of three representative experiments. *, $p<0.05$ by ANOVA.

The ER22/23EK polymorphism does not affect the expression of total GR protein (Figure 2) but seems to change the ratio in which GR-A and GR-B are expressed. To investigate the interaction of both translation variants, we cotransfected phGR-A and phGR-B and varied the ratio of GR-A to GR-B, while keeping total GR expression plasmid levels constant. The transfected cells were stimulated with 100 nM dexamethasone and total transcriptional activity on a GRE-LUC promoter was measured (Figure 5). The results show a linear interrelationship, suggesting that the resulting activity is solely dependent on the relative contribution of each of the translation variants, without other mutual effects. The increased transcriptional activity expressed from the phGR-A-ER22/23EK construct seems to be related to higher GR-A expression levels. However, also the ER22/23EK polymorphism itself might affect transcriptional activity. Although the amino acid change in codon 23 is not within the τ1-region of the transactivation domain, which is variably defined as amino acid 77–262 (17) or 98–305 (18), it could cause subtle alterations in secondary

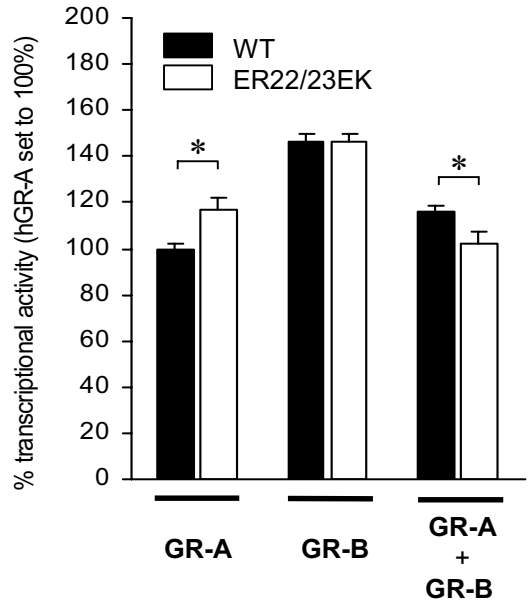

Figure 4 Transcriptional activation of a GRE containing reporter gene by GR-A and GR-B isoforms expressed from wt and polymorphic constructs. COS-1 cells were co-transfected with each of the constructs from Table 1 expressing the indicated GR isoform(s). After 5 h, cells were treated with 100 nM dexamethasone for 20 h and luciferase activity was measured. Results are expressed relative to wt GR-A, expressed from phGR-A-wt (100%). Data represent means ± S.E.M. of three representative experiments. *, $p<0.05$ by ANOVA.

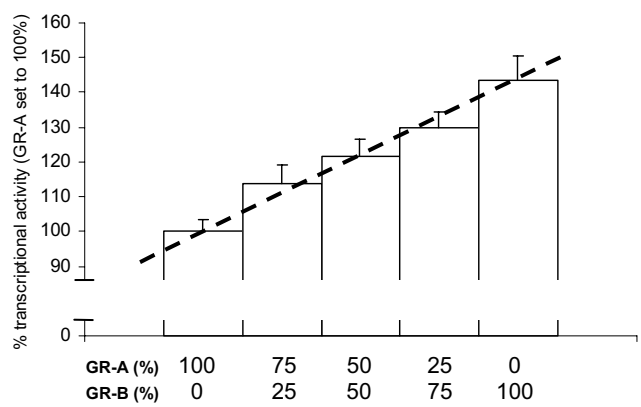

Figure 5 Interrelationship of GR-A and GR-B on transcriptional activity. COS-1 cells were cotransfected with the phGR-A-wt and phGR-B-wt constructs expressing the GR-A and GR-B isoforms at the indicated ratio. After 5 h, cells were treated with 100 nM dexamethasone for 20 h and luciferase activity was measured. Results are expressed relative to wt GR-A (100%). Data represent means ± S.E.M. of four experiments.

structure of the protein influencing interaction with cofactors and/or DNA. This change in intrinsic activity can only be investigated if the influence of the ER22/23EK polymorphism on leaky scanning has been abolished, which was done by mutating the Kozak sequence of the AUG-1 start codon from a weak consensus sequence to a strong one. The -3 position (when ATG codon represents bases +1, +2, and +3, respectively) was mutated from a C- to a G-nucleotide (Figure 6A). Expressing the phGR-wt and phGR(ER22/23EK)-ATG1-Kozak mutants in an *in vitro* transcription and translation assay, GR-B production was not completely blocked (Figure 6B) but significantly reduced with about 70% as determined by optical densitometry (data not shown). Figure 6C shows that the transcriptional activity expressed from phGR-ER22/23EK of which the AUG-1 start site is embedded in a strong consensus sequence is reduced to that expressed from the wt construct. This suggests that the increased transcriptional activity from the phGR-A-ER22/23EK construct is related to higher GR-A expression levels rather than the intrinsic activity of the ER22/23EK polymorphism itself. In Figure 6C, the polymorphism appears to be even more effective than the strong Kozak sequence in initiating translation of GR-A. However, to make this comparison, the effects of the polymorphism and the Kozak consensus sequence would have to be compared in the context of the M27T plasmid.

4.3.4 GR mRNA levels transcribed from the GR constructs

Increased GR-A expression from the phGR-A-ER22/23EK vector might be caused by differences in mRNA stability. To investigate differences in mRNA stability, mRNA levels were measured by quantitative real-time PCR in COS-1 cells expressing the GR variants. Furthermore, in peripheral blood mononuclear lymphocytes (PBMLs) of two heterozygous ER22/23EK carriers, the amounts of mRNA transcribed from both the wt and polymorphic allele were determined. Total RNA was isolated, cDNA was synthesized, and quantitative PCR (Q-PCR) was performed. No differences in mRNA levels transcribed from the different constructs were detected (Figure 7A) and the total GR mRNA measured in heterozygous ER22/23EK-carriers consisted of 50% expressed from the wt and 50% expressed from the polymorphic allele (Figure 7B). This implies that the ER22/23EK polymorphism does not influence mRNA stability.

4.4 Discussion

Various polymorphisms of the human GR gene have been reported to be associated with variations in GC sensitivity (for reviews see Refs. 6 and 19), but for none of these, not even missense polymorphisms, a mechanism of action has been elucidated.

One of the most intriguing polymorphisms in the GR gene is the ER22/23EK polymorphism (7, 9, 10, 11), which is associated with a reduced GC sensitivity, and

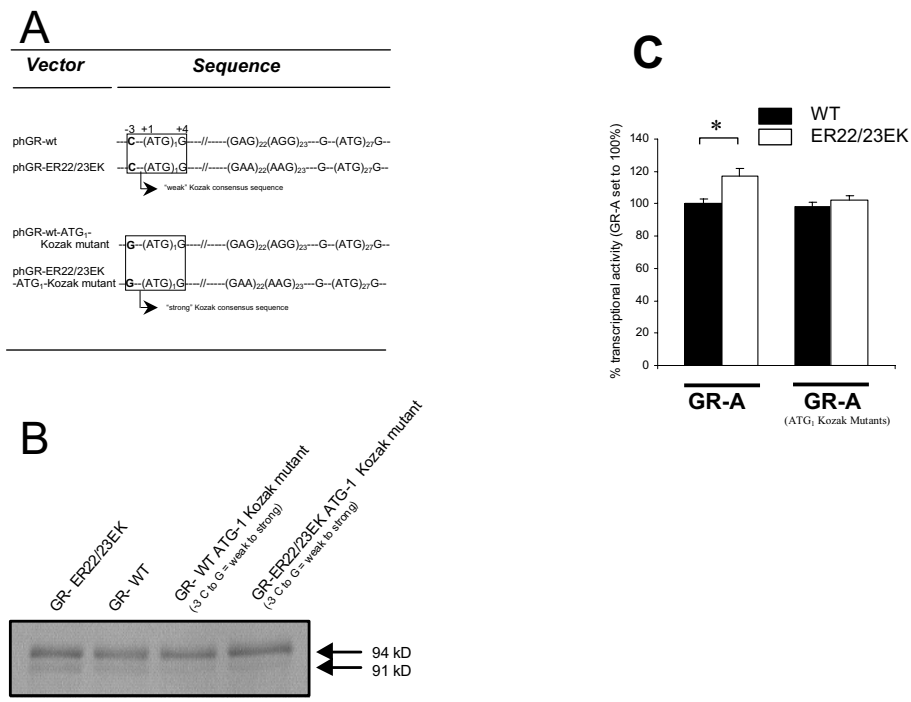

Figure 6 Expression and transcriptional activity of phGR-wt- and phGR-ER22/23EK-ATG_1-Kozak mutants. A, AUG-1, the translation initiation start site of the GR represents bases +1, +2 and +3. The bases at position –3 and +4, relative to ATG-1 have been found to represent either a weak or strong Kozak consensus sequence, as indicated. B, *In vitro* translation of the GR-A and GR-B isoform from the indicated constructs using [^{35}S]-methionine and reticulocyte lysates. Expressed proteins were electrophoresed on a 7% polyacrylamide gel and visualized by exposure to high-performance autoradiography film. C, COS-1 cells were cotransfected with each of the constructs phGR-A-wt, phGR-A-ER22/23EK (Table 1) and phGR-wt-ATG_1-Kozak and phGR-ER22/23EK-ATG_1-Kozak (Figure 6A). After 5 h, cells were treated with 100 nM dexamethasone for 20 h and luciferase activity was measured. Results are expressed relative to wt GR-A, expressed from phGR-A-wt (100%). Data represent means ± S.E.M. of four representative experiments. *, $p<0.05$ by ANOVA.

results in a phenotype that could be summarized as a more favorable metabolic profile (10), and, eventually, in survival benefits for carriers of this polymorphism (9). However, measurement of GR parameters in PBMLs from carriers of this polymorphism did not show any differences, nor did transient transfection assays by calcium phosphate precipitation (16). This transfection method is probably not sufficiently reproducible to distinguish between wt GR and this polymorphism, compared with current cationic liposome-mediated transfection methods (i.e. FuGENE6 used in this study).

Recently, Yudt and Cidlowski reported the existence of two translational variants of the GR: the GR-A, resulting from translation of the GR mRNA starting at the first AUG codon (Met-1) and the GR-B, for which translation starts at the second in-frame AUG

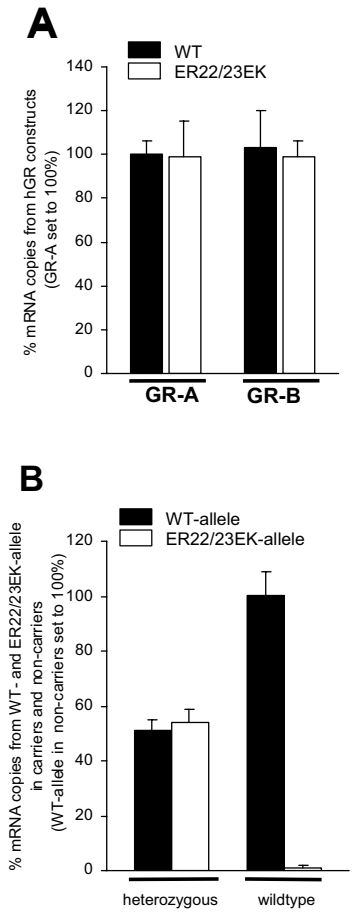

Figure 7 mRNA Levels transcribed from the GR variants. A, COS-1 cells were transfected with either the wt or polymorphic vector coding for either the GR-A (phGR-A-wt and phGR-A-ER22/23EK) or GR-B (phGR-B-wt and phGR-B-ER22/23EK) isoform. Twenty-four hours later, transcribed mRNA was isolated, cDNA was synthesized and expression levels of GR were determined by Q-PCR. Data were normalized to phGR-A-wt. B, From PBMLs of two heterozygous ER22/23EK-carriers and two wt carriers, total mRNA was isolated, cDNA was synthesized and levels of GR mRNA expressed from the wt allele and polymorphic allele was determined by Q-PCR. Data were normalized relative to the wt allele in homozygous wt carriers. Data represent means ± S.E.M. of two experiments.

codon (Met-27). These authors also showed that the GR-B protein is approximately 1.5-fold more active in transactivation from GREs or the mouse mammary tumor virus promoter than the GR-A protein (15). Selection of the translation start site by the ribosome is generally accepted to be dictated by the context of the AUG codon: a Kozak sequence (typically RNNAUGG; see Figure 6A) should be present and there should be no in-frame stop codons nearby, whereas the secondary structure of the mRNA also plays a role in this process (20, 21). Suboptimal Kozak-sequences, such as the one surrounding the first AUG codon in the GR mRNA (CtgAUGG), can lead to

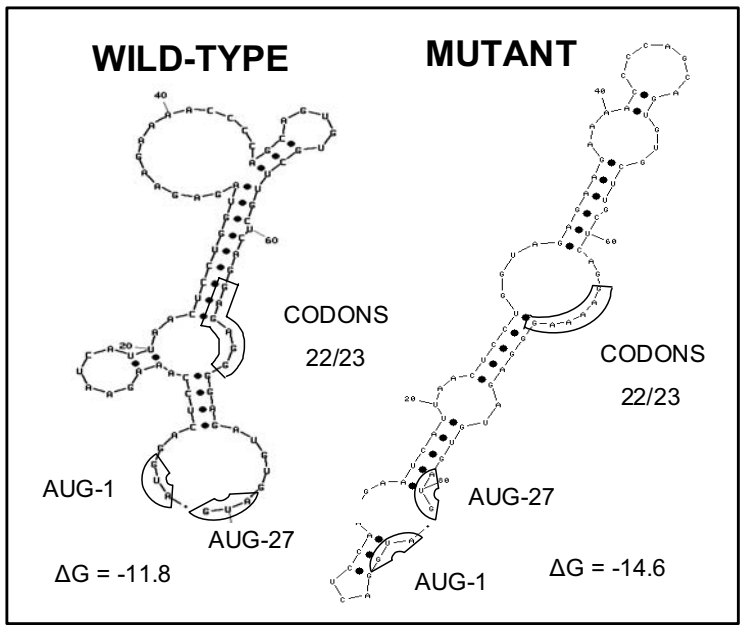

Figure 8 The change in secondary structure of the GR mRNA caused by the ER22/23EK Polymorphism. The most favorable structures computed for nucleotides 133–213 (numbering according to Hollenberg *et al.*) for wt [(GAG)22(AGG)23] and mutant [(GAA)22(AAG)23], respectively.

slippage of the ribosomal 40S subunit, and utilization of one or more downstream AUG codons. Notably, the Met-27 AUG in the GR mRNA is surrounded by a stronger Kozak sequence (GtgAUGG) than the Met-1 AUG codon.

The fact that the ER22/23EK polymorphism is very close to both of the Met-1 and Met-27 translation initiation start sites led us to hypothesize that the change in nucleotide sequence (GAG AGG to GAA AAG) involved, might have consequences for the secondary structure of the GR mRNA. These changes might then influence the proportion in which the two initiation codons are used, resulting in altered rates of synthesis of GR-A and/or GR-B and possibly causing changes in the cellular GC sensitivity. Using the m-*fold* software (22, 23) for the prediction of secondary structures in nucleic acids, we did indeed find that the most stable secondary structures for the GR mRNA with the ER22/23EK polymorphism differed from those for the wt GR mRNA. Figure 8 shows an example of this, indicating that the mRNA containing the polymorphism results in a more stable structure [lower Gibbs free energy of formation (ΔG)] than the wt, apparently changing the choice of initiation codon. More indications that secondary mRNA structure may play a role in the choice of initiation codon is the observation (24) that from a GR mRNA containing the alternative exon 1A3 instead of the more common exon 1C, substantially more GR-B is translated. Also, Breslin *et al.* (25) found that in CEM-C7 cells the quantity of this 1A3 containing mRNA is increased 2.5-fold upon GC treatment of the cells.

The influence of the ER22/23EK polymorphism on synthesis of GR-A and GR-B proteins was investigated by expressing them independently from wt and polymorphic constructs and studying two parameters: the amount of specifically bound [^{3}H]-dexamethasone as a measure for the amount of GR protein (Figure 2), and the transcriptional activity of GR-A and GR-B in the presence of dexamethasone (Figures 3 and 4). These two experiments showed that, when translation is forced to start at AUG-1, using M27T plasmids, resulting in the synthesis of GR-A, approximately 15% more protein was expressed from the phGR-A-ER22/23EK, than from the wt construct phGR-A-wt (Figure 2). The expression of GR-B from phGR-B-ER22/23EK and phGR-B-wt was not affected by the polymorphism, which indicates that the ER22/23EK polymorphism only influences translation initiation from the first AUG start site (Met-1). The amount of total GR (GR-A + GR-B) expressed from phGR-wt and phGR-ER22/23EK did not differ (Figure 2), which means that the ER22/23EK polymorphism facilitates the expression of the less transcriptionally active GR-A, thereby reducing the expression of the more transcriptionally active GR-B.

Dose-response curves in cells in which both isoforms were synthesized showed that when the polymorphism was present (phGR-ER22/23EK), 14% less transcriptional activity was expressed than from the wt (phGR-wt) (Figure 3). The observed change in ratio in which GR-A and GR-B were synthesized is a direct effect of altered translation initiation and is not caused by differences in mRNA levels because the ER22/23EK polymorphism did not affect transcription efficiency and/or mRNA stability (Figure 7). Furthermore, the GR-A isoform has no dominant-negative effect on the transcriptional activity of GR-B or *vice versa* (Figure 5), and the ER22/23EK polymorphism itself does not affect GR signaling (Figure 6). Together, this indicates that the decrease in transcriptional activity of GR(ER22/23EK) is only caused by an increased GR-A/GR-B ratio, whereas total GR protein levels remain unaltered.

It has been suggested that GR-A and GR-B might be differentially expressed in a tissue-specific manner (26), which means that the impact of the ER22/23EK polymorphism also might vary among the different tissues.

In addition to translational variants of the GR, also splice variants exist: GR-α, GR-β, and GR-P (13, 14). The GR-α is ubiquitously expressed and is the foremost mediator of GC effects. The GR-β is much less abundant than GR-α, unable to bind ligand, and does not seem to possess transcriptional activity in itself (16). However, GR-β can inhibit activity of the α isoform (27, 28), although controversy remains (29, 30, 31). The GR-P splice variant is also not able to bind ligand and is thought to increase the activity of GR-α (14). Alternative translation initiation also occurs on GR-β and GR-P mRNA (26), and resulting isoform levels might also be influenced. These splice variants might play a role in the fine tuning of an individual's sensitivity to GCs and when discussing the decreased sensitivity in ER22/23EK carriers, also a possible influence of this polymorphism on splice variants must be considered.

Although the differences reported here are relatively small, it should be emphasized that *in vivo* lifelong exposure to a slightly decreased sensitivity to GCs,

as we see in ER22/23EK carriers, results in a better cardiovascular and metabolic health profile, as well as an increased chance of longevity. We postulate that a higher expression of the less transcriptionally active GR-A isoform, and thus a lower expression of the more transcriptionally active GR-B isoform, mainly cause this decrease in GC sensitivity. This shift in GR-A/GR-B expression ratio is evoked by the ER22/23EK polymorphism, possibly by changing the secondary structure of the mRNA of the GR, causing more translation initiation from the first AUG start site.

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Chapter 5

A Glucocorticoid Receptor Polymorphism affects Transrepression but not Transactivation

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Submitted

Abstract

Context: Glucocorticoids (GC) are extensively used in the treatment of inflammatory and auto-immune diseases. Their beneficial effects are thought to be mediated by their suppressive effects on gene expression. However their use is limited by serious adverse effects, presumably mediated by GC activation of gene expression.

Objective: The determinination of the haplotype of the glucocorticoid receptor (GR)-9β variant and its effect on GC transactivation and transrepression.

Design and methods: A cros-sectional study in 216 persons randomly selected from participants in The Rotterdam Study, a population-based cohort study in the elderly. Clinical and biochemical parameters of GC sensitivity were measured: weight, height, waist-hip-ratio, glucose, insulin, total cholesterol, high-density lipoprotein, and C-reactive protein. In a dexamethasone suppression test the response of serum cortisol concentrations was studied. Genotyping for four GR polymorphisms was performed. In leukocytes of ten controls and two persons homozygous for the GR-9β polymorphism, identified among 75 volunteers, the expression of two GC-sensitive genes, GC-induced leucine zipper (GILZ) and interleukin-2 (IL-2), was measured.

Results: The GR-9β polymorphism was present in two haplotypes, either alone, or in combination with the ER22/23EK polymorphism. Persons carrying the GR-9β haplotype without 22/23EK (n=53) revealed no significant differences compared to non-carriers (n=113) in their body mass index, waist-hip-ratio, fat spectrum, and insulin sensitivity, nor in their cortisol response to dexamethasone. However, their mean C-reactive protein levels were significantly higher (p=0.05). *Ex vivo*, GC-induced upregulation of GILZ mRNA did not significantly differ in GR-9β homozygotes, while the downregulation of IL-2 expression was decreased.

Conclusion: Persons carrying the GR-9β haplotype seem to have a decreased GC transrepression with normal transactivation.

5.1 Introduction

Glucocorticoids (GCs) are important regulators in a variety of processes such as growth, body composition, metabolism, and immune function (1). GCs exert their effects via the glucocorticoid receptor (GR), a ligand dependent transcription factor, which belongs to the superfamily of nuclear receptors. GCs have both stimulatory and inhibitory effects on gene expression (2). Their transrepressive effects are extensively used in the treatment of inflammatory and auto-immune diseases. However their practical use is limited by serious adverse effects, which are presumed to be mainly mediated by GC transactivation (3). While some patients are resistant to the anti-inflammatory effects of GCs but do show considerable side effects, others show a clear transrepressive response on the immune system to low dose GCs without any side effects. These differences are the result of the differences in GC sensitivity between individuals (4, 5). Four polymorphisms in the GR gene have been implicated in these interindividual differences (Figure 1). Of three polymorphisms, located in or near the transactivating domain, the associations with glucocorticoid sensitivity have been described extensively (6, 7). The ER22/23EK polymorphism (rs6189 and rs6190) is associated with a relative resistance to GCs. The 22/23EK carriers had higher cortisol levels after dexamethasone suppression testing (6). Their metabolic profile showed lower total cholesterol and low-density lipoprotein cholesterol levels, lower fasting insulin concentrations, an increased insulin sensitivity (8) as well as lower C-reactive protein (CRP) levels (6). The N363S and Bcl1 polymorphisms have been associated with increased GC sensitivity, decreased insulin sensitivity to dexamethasone and increased abdominal obesity

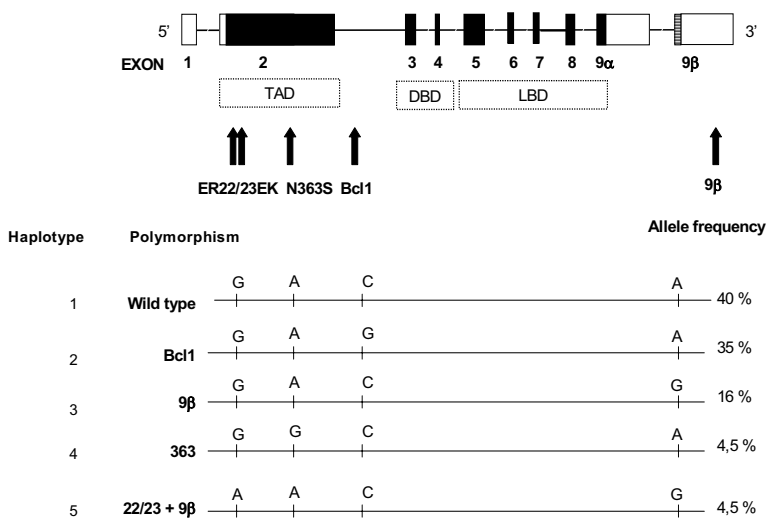

Figure 1 Schematic overview of the glucocorticoid receptor (GR) gene polymorphisms and haplotypes. Haplotypes are numbered in order of decreasing frequency. TAD = transactivating domain, DBD = DNA binding domain, LBD = ligand binding domain.

(6). The fourth polymorphism, GR-9β, is an A to G nucleotide substitution located in the 3' UTR of exon 9β, the terminal exon of the mRNA of the GR-α isoform (nucleotide 3669 in X03348; rs 6198). The A to G nucleotide substitution is located in an 'ATTTA' motif (to GTTTA). This 'ATTTA' motif is known to destabilize mRNA and decrease receptor protein expression *in vitro* (9). The GR-β splice variant has been reported to have a dominant negative effect on GR-α action (10, 11). *In vitro* data show that the GR-9β polymorphism leads to a more stable GR-β mRNA and possibly to a relative GC resistance and increased susceptibility to rheumatoid arthritis (12). However, in a low-dose dexamethasone suppression test, no differences in the cortisol levels after dexamethasone were found (13).

To explore the observed differential GC sensitivity in a cohort of healthy elderly subjects (4) we started an investigation on the haplotype and possible role of the GR-9β polymorphism on several endpoints of GC transactivation and transrepression.

5.2 Materials and Methods

5.2.1 Study population

This cross-sectional study was conducted as part of the Rotterdam Study, a prospective, population-based cohort study on determinants of disease and disability in elderly persons started in Rotterdam, The Netherlands in 1990 among 7983 participants (14). A total of 216 randomly selected persons participated in the present study. For 187 of the 216 participants the complete dataset, confounding variables and the GR genotype were available. Mean age was 67 years, 96 (51%) were women. The Medical Ethics Committee of the Erasmus MC approved the study. Informed consent and permission to retrieve information from treating physicians was obtained from all participants. For the *in vitro* experiments, a group of 75 healthy volunteers (laboratory workers) was genotyped, resulting in the identification of 2 persons homozygous for the GR-9β polymorphism and 10 controls homozygous for the reference allele.

5.2.2 Dexamethasone suppression test

The dexamethasone suppression test (DST) was performed as described previously (4). On day one venous blood for serum cortisol and insulin measurements was obtained between 8:00 and 9:00 h after an overnight fast. Participants were instructed to ingest a tablet of 1 mg dexamethasone (DEX) at 23:00 h on day one. On day two fasting blood for cortisol, insulin and DEX measurements was drawn by venipuncture at the same time as on day one. To check for compliance and possible abnormalities in the metabolism of DEX, the DEX concentration was also measured by radioimmunoassay.

Procedures for hormonal measurements of serum cortisol concentrations,

insulin, DEX and biochemical measurements of glucose, total cholesterol, high-density lipoprotein (HDL) and cholesterol were described previously (8). CRP levels were measured by highly sensitive kinetic nephelometry (Nephelometer BN200; Dade-Behring, Marburg, Germany). For CRP analysis subjects with possible acute inflammation as indicated by CRP levels higher than 10 mg/L were excluded.

5.2.3 Genotyping

DNA was isolated from peripheral blood leukocytes using standard techniques, dissolved in double-distilled water and stored at -20°C. PCR amplification and genotyping were performed using 5 ng genomic DNA for the Taqman allelic discrimination assay. Primer and probe sequences were optimized using the SNP assay-by-design service of Applied Biosystems. For details see http://store.appliedbiosystems.com. Reactions were performed on the Taqman Prism7900HT (Applied Biosystems, Foster City, CA, USA) in 384 wells format.

All participants were genotyped for the GR-9β polymorphism. The genotypes for the ER22/23EK, N363S and Bcl1 polymorphisms have been identified previously (8, 15, 16).

We used the genotype data for each of the 4 polymorphisms to infer the haplotypes present in the population using the program PHASE which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data (17).

5.2.4 GC-induced leucine zipper (GILZ) and Interleukin-2 (IL-2) expression assay

The expression of GILZ and IL-2 mRNA in response to DEX was measured using real-time quantitative PCR (Q-PCR) as described previously (18, 19) in peripheral blood mononuclear leukocytes (PBMLs) of 10 noncarrier controls (5 men and 5 women, aged 25 - 38) and 2 homozygous GR-9β carriers (both men, aged 27 and 25). All were healthy volunteers without a history of GC medication, identified from among 75 laboratory workers. Briefly, PBMLs were isolated using standard techniques, pre-cultured for 24 h and subsequently incubated for 4 h with a range of DEX concentrations (0 - 1000 nmol/L) and 10 µg/mL phytohemagglutinin (PHA). Cells were subsequently washed with NaCl (0.15 mol/l), total RNA was isolated, and cDNA was synthesized in a reverse transcription reaction. GILZ and IL-2 expression levels were determined in a real-time Q-PCR and normalized to the expression of the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) using the comparative treshold cycle method (20).

5.2.5 Statistical analysis

Data were analyzed using SPSS for windows, release 10.1 (SPSS, Chicago, IL, USA). Logarithmic transformations were applied to normalize variables. Because of their linkage disequilibrium, we analyzed GR-9β while excluding ER22/23EK carriers. Differences in means of continuous variables between G-allele carriers and non-

carriers of the GR-9β polymorphism were tested by ANCOVA using the general linear model procedure adjusted for age and sex. CRP was additionally adjusted for BMI because these are known to be correlated. The Pearson Chi-square test was used for differences between categorized variables and genotypes. Differences in the EC_{50} to DEX-induced up- and down-regulation of GILZ and IL-2 mRNA levels in homozygous GR-9β carriers compared to non-carriers were analyzed by the Student's *t*-test using the area under the curve. Results are reported as mean ± S.E.M. Two-sided P values of equal or less than 0.05 were considered to indicate statistical significance.

5.3 Results

For 187 of the 216 participants the complete dataset, confounding variables and the GR genotype were available. Mean age was 67 years, 96 (51%) were women. Figure 1 schematically depicts the GR gene, the location of all four polymorphisms, the specific nucleotide variation, haplotypes 1-5 and the allele frequencies. Three of the four polymorphisms were found to be mutually exclusive, only 22/23EK was always present in combination with the 9β G-allele. The distribution of genotypes for all GR gene polymorphisms was in Hardy-Weinberg equilibrium (p>0.05).

Comparison of means between noncarriers and the G-allele carriers of the GR-9β polymorphism (haplotype 3) revealed no significant differences for cortisol response in the DST, nor in BMI, WHR, insulin sensitivity, cholesterol, LDL and HDL. Only mean CRP levels were higher (2.8 mg/L) in 9β G-allele carriers (haplotype 3) compared to

Table 1 Genotypes of the study group in relation to the results of the 1-mg dexamethasone suppression test and metabolic parameters.

	non carriers	9β carriers		22/23EK carriers	
	mean (SEM)	mean (SEM)	p	mean (SEM)	p
Gender = female	54 (48%)	31 (59%)	0.2	11 (52%)	0.97
Age (years)	66.2 (0.6)	67.5 (0.8)	0.21	68.5 (1.5)	0.16
DST (1mg), n=	113	53		21	
fasting cortisol (nmol/L)	513 (13.6)	523 (17.3)	0.54	544 (41.8)	0.38
post dex cortisol (nmol/L)	26 (2.0)	27 (2.2)	0.7	53 (15.2)	0.012*
fasting CBG (pmol/L)	737 (19.7)	764 (24.7)	0.21	838 (76.8)	0.069
free fasting cortisol (nm/L)	37 (2.8)	30 (2.3)	0.31	28 (3.8)	0.15
free cort. post dex (nm/L)	1 (0.2)	0.8 (0.15)	0.85	2.5 (1.0)	0.023*
fasting glucose (mmol/L)	5.8 (0.7)	5.9 (0.2)	0.58	5.9 (0.2)	0.87
fasting insulin (mU/L)14	(0.7)	13 (1.1)	0.49	10 (1.4)	0.03*
BMI (kg/m^2)	26.6 (0.33)	25.8 (0.53)	0.18	26 (0.78)	0.40
WHR	0.93 (0.009)	0.90 (0.01)	0.61	0.93 (0.02)	0.96
total chol (mmol/L)	6.9 (0.11)	6.9 (0.17)	0.96	6.3 (0.22)	0.043*
LDL (mmol/L)	5.1 (0.11)	5.2 (0.16)	0.9	4.4 (0.22)	0.047*
HDL (mmol/L)	1.3 (0.04)	1.4 (0.05)	0.7	1.5 (0.12)	0.33
CRP (mg/L)	2.5 (0.21)	2.8 (0.31)	0.05*A	2.5 (0.55)	0.65

DST=dexamethasone suppression test, SEM=standard error of the mean. p-values are corrected for age and sexe, *:significant A:p corrected for age, sexe and BMI

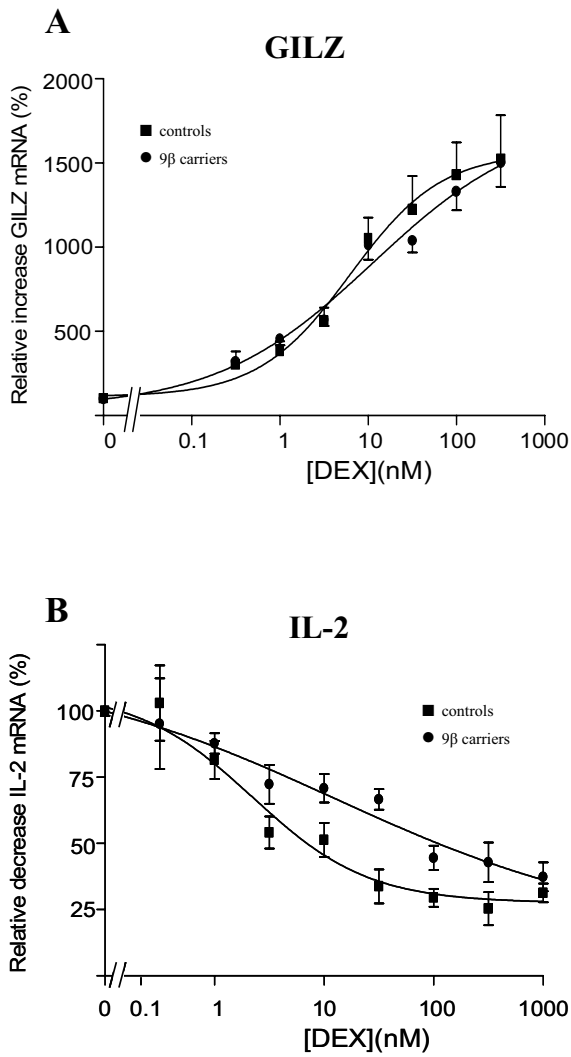

Figure 2 Relative responses to DEX of GILZ mRNA (A) and IL-2 mRNA (B) expression in PBMLs of noncarrier controls (haplotype 1, n=10, squares) and homozygous 9β carriers (haplotype 3, n=2, circles).
PBMLs of homozygous carriers of the 9β polymorphism (n=2) and healthy non-carrier controls (n=10) were incubated during 4h with PHA and the indicated concentrations of DEX, followed by mRNA isolation and quantification by real-time Q-PCR. Data are presented as the increase of GILZ mRNA (A) and decrease of IL-2 mRNA (B) relative to the values in the absence of DEX. Duplicate assays at different time points were performed for every subject. Duplicate DEX incubations and duplicate Q-PCR was performed for every sample. PHA was necessary to induce transcription of IL-2, but did not affect GILZ or GR mRNA expression levels (not shown). No systemic differences before stimulation were observed between both haplotypes for both mRNAs (not shown).

non-carriers (2.5 mg/L) (Table 1). This association was significant after correction for covariates age, sex and BMI. For comparison we added previously reported data (8) on the 22/23EK carriers (haplotype 5) showing significant differences in DST cortisol, insulin, cholesterol and LDL relative to the noncarriers of this allele.

In a previous report (19), we described the effects of haplotype 4 and 5 on the regulation of two endogenous GC-sensitive genes: transactivation of the GC-induced leucine zipper (GILZ) gene, and transrepression of the interleukin-2 (IL-2) gene. In haplotype 4 and 5 increased and decreased transactivation were found, respectively, while transinhibition was not affected. In order to carry out similar experiments with respect to the GR-9β polymorphism, we identified 2 homozygous 9β carriers and 10 noncarriers among 75 volunteers (laboratory colleagues). Figure 2 shows the reponse of these genes to DEX in PBMLs of homozygous carriers of the GR-9β polymorphism (haplotype 3) compared to controls. Upregulation of GILZ

mRNA did not significantly differ, while in the downregulation of IL-2 expression the mean EC_{50}, representing physiological conditions, was shifted to the right for GR-9β carriers (13 ± 1.4 nmol/l) compared to controls (5.2 ± 1.3 nmol/l, $p<0.05$), indicating reduced GC sensitivity in their transrepression (Figure 2). Expression levels of the GR-β splice variant mRNA were approximately 1000-fold lower than those of the GR-α mRNA, both in the GR-9β carriers and in controls. Furthermore, we did not find higher GR-β splice variant expression levels in homozygous 9β carriers than in controls (data not shown).

5.4 Discussion

Several polymorphisms in the GR gene have been found to be associated with glucocorticoid sensitivity. In this study five haplotype alleles were found (Figure 1): the reference allele (haplotype 1), Bcl1 (haplotype 2), 9β (haplotype 3), N363S (haplotype 4), and 9β+ER22/23EK (haplotype 5). Thus, the ER22/23EK is always accompanied by 9β, but not *vice versa*. This linkage disequilibrium for the 9β and ER22/23EK polymorphisms was not known in literature but it is important for interpretation of data on both the ER22/23EK and the GR-9β polymorphisms. We found a high allele frequency (16%) of 9β (haplotype 3) while the 9β + ER22/23EK (haplotype 5) is less frequent (4,5%) (8). Off course these haplotype results need to be confirmed in a larger cohort such as the total cohort of the Rotterdam Study. Both polymorphisms are thought to convey relative GC resistance. 22/23EK has been shown to result in lower GR-mediated transcriptional activation, due to a shift in translation of the GR mRNA from GR-B to GR-A. (7). This reduced GC sensitivity is thought to be the cause of the decreased level of cortisol suppression during the DST, lower BMI, lower WHR and healthier lipid profile in 22/23EK carriers compared to non-carriers. Possibly as a result of this healthier metabolic profile the CRP levels, as an indicator of atherosclerotic disease and the pro-inflammatory state, were found to be lower in a group of elderly men (6).

GR-9β on the other hand, has been reported to result in stabilization of GR-β mRNA, which has dominant negative effects on GR-α (9), resulting in a relative GC resistance leading to reduced immune suppression. This was supported by the higher frequency of this polymorphism found in rheumatoid arthritis patients (12) and the reduced risk for *Staphylococcus aureus* nasal carriage found in haplotype 3 carriers (E.L.T. van den Akker, manuscript submitted). In this study we did not find evidence for a reduced GC sensitivity of haplotype 3 at the level of cortisol suppression during the DST, nor in BMI, WHR, insulin sensitivity and lipid profile but CRP levels were significantly higher in carriers of the GR-9β polymorphism than in noncarriers. Thus, while haplotype 5 is associated with reduced GC-sensitivity through parameters supposedly influenced by GR-mediated transactivation,

haplotype 3 appears to be associated with reduced GC-sensitivity via a parameter of the immune system (elevated CRP levels) which is regulated by GR-mediated transrepression (21). Although the association with CRP needs reconfirmation in a larger study with more statistical power, this finding is in line with our *ex vivo* results of reduced transrepression of IL-2 in 9β homozygotes. The previously described associations of ER22/23EK polymorphism with cortisol resistance, are not the result of its linkage disequilibrium with the 9β polymorphism. We do not know why the GR-9β effect on CRP is not demonstrable in 22/23EK carriers. We hypothesize that the differential effects of these two haplotypes on GC sensitivity are a result of either tissue specificity or of the different pathways the GR uses for its effects. First, tissue specificity of different splice variants might influence their effect. At the mRNA level, the GR-9β polymorphism is expressed in the GR-β splice variant. The β splice variant expression is tissue specific and shows high expression in immune cells (11, 22). Therefore the GR-9β effects may be limited to tissues with high levels of GR-β expression such as immune cells. On the other hand, the role of the GR-β splice variants in other tissues may be limited as its expression is very low in most tissues and cell-types (23) and in the brain (24). Possible involvement of the GR-β splice variant in the regulation of the HPA-axis is unexplored, although the glucocorticoid resistance observed in ACTH-secreting tumors seems to be unrelated to the expression levels of GR-β (25). Even though the ER22/23EK polymorphism leads to decreased activity of the GR-α splice variant, the GR-α is more abundant than GR-β, and this might mask the effects of the GR-β polymorphism.

Alternatively, haplotypes 3 and 5 may lead to effects in different GR pathways. The effects of the GR on HPA-activity, BMI and lipids are exerted via the transactivating pathway. For transactivation GR ligand binding, dimerization and DNA binding are needed, while GR effects on inflammation are presumed to be mainly mediated by direct protein-protein interactions with AP-1 or NF-κB (26). The present investigation offers some indications that these are indeed separate processes. We previously described that haplotype 4 and 5 directly affected gene expression through transactivation, while transrepression seemed to be unchanged (19). In this study, we also studied this for haplotype 3. Transactivation was studied by measuring GC-mediated upregulation of GILZ mRNA (through DNA binding) and transrepression by measuring GC-mediated downregulation of IL-2 mRNA (via protein-protein interaction with NF-κB) (Figure 2). Transactivation was not influenced by haplotype 3 compared to controls, while transrepression of IL-2 was significantly reduced. The mean EC_{50} for IL-2 suppression was significantly higher in GR-9β homozygotes (13.0 ± 1.4 nmol/L) than in controls (5.2 ± 1.3 nmol/L, $p<0.05$). Thus haplotype 3 showed a reduced effect in transcriptional repression and this could lead to less GR-mediated immune suppression. While data from literature remain controversial on the effect of the GR-β splice variant on direct protein-protein interactions (via NF-κB)(27-29), our data support the hypothesis that the GR-9β polymorphism and the GR-β splice variant may have an important role

in human immune cells. Future studies on transrepressive effects of the GR such as inflammatory parameters and diseases as well as studies on the mechanistic background are needed. Understanding these differential effects on transactivation and transrepression are crucial for understanding differences in patients and their susceptibility to diseases and their response to steroid treatment.

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Chapter 6

Associations between Promoter Usage and Alternative Splicing of the Glucocorticoid Receptor gene

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Submitted

Abstract

The glucocorticoid receptor (GR) is widely expressed in various tissues throughout the human body. At least three different 3' splice variants of the GR have been reported: GR-α, which is functionally active, GR-β, a dominant negative inhibitor of GR-α function, and GR-P, which is thought to activate the function of GR-α. Expression of the GR gene is controlled by three different promoters: 1A, 1B and 1C. mRNA transcripts containing exon 1A are expressed from promoter 1A, while transcripts containing exons 1B or 1C are expressed form promoter 1B or 1C, respectively.

In this study, we explored if tissue-specific splicing of the 3' end variants of the GR is influenced by alternative promoter usage. cDNAs of different tissues and cell-lines were used to investigate which part of transcripts carrying the different exons 1A, 1B or 1C encodes for the splice variants GR-α, GR-β and GR-P. Our data demonstrate that expression of GR-α is preferentially regulated by promoter 1C and that for expression of GR-P promoter 1B is predominantly used.

This indicates that regulation of GR splice variants could partly occur through selective use of the multiple promoters and that this is another way to sensitize cells and tissues to the different activities of the GR isoforms.

6.1 Introduction

Glucocorticoids (GCs, cortisol and its synthetic analogs) can influence gene transcription via the glucocorticoid receptor (GR). Unliganded GR resides in the cytoplasm but translocates to the nucleus upon GC binding, where it acts as a factor regulating gene transcription (1). The GR encoding gene (NR3C1) is located on chromosome 5q31.3 and consists of 9 different exons (2). Transcription of the gene results in a broad range of mRNA transcripts, which can differ in their 5' and 3' ends (Figure 1) (3).

Alternative splicing generates three different 3' end mRNA transcripts, encoding the GR-α, GR-β and GR-P isoforms. The GR-α isoform (Figure 1) consists of 777-amino-acids and is the functional active isoform that mediates GC action. The GR-β transcript is generated by linking the end of exon 8 to the beginning of an alternative exon, exon 9β instead of exon 9α (Figure 1), resulting in a 742 amino-acid protein (2). This GR-β protein is not able to bind ligand and is reported to be a dominant negative inhibitor of GR-α action (4, 5). GR-β is expressed in almost every tissue and

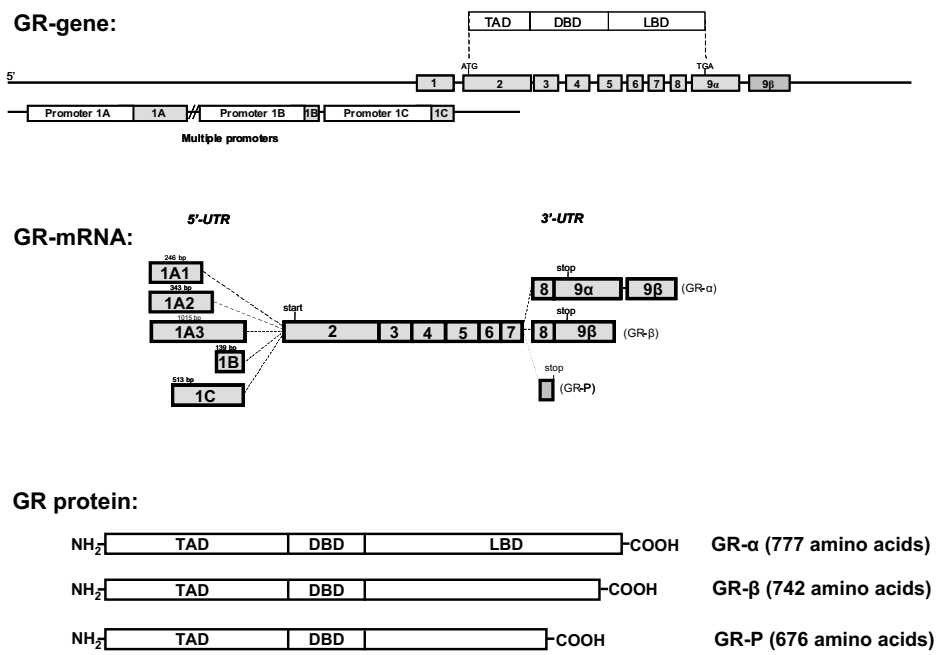


Figure 1 Organization and expression of the GR gene. The expression of the GR gene is controlled by three different promoters. Promoter 1A is located approximately 27 kbp upstream of the translation initiation start site in exon 2 and has been reported to be active in cells of the hematopoietic lineage. Promoters 1B and 1C are located much closer to the coding sequence of the GR gene and are thought to be responsible for its basal expression. 5'-end untranslated exons 1A (1A1, 1A2, 1A3), 1B and 1C are spliced to the same splice acceptor site in exon 2. 3' end alternative splicing results in mRNAs encoding three variants of the GR gene: GR-α, the functionally active protein, GR-β, a dominant negative inhibitor of GR-α function, unable to bind ligand, and GR-P, a truncated isoform, also unable to bind ligand but thought to increase the activity of GR-α.

in many cell-lines, albeit at a very low level (0-2%) compared to GR-α (4). Despite its wide tissue expression, controversy remains about its physiological relevance. A third 3' end mRNA variant only contains exons 2-7. The corresponding truncated GR protein of 676 amino acids lacks a large portion of the ligand binding domain, and is therefore also unable to bind ligand (6). GR-P is widely expressed in various tissues and cells, up to 55% of that of GR-α in several hematological malignancies (7, 8). The function of GR-P is also still not fully understood, but in contrast to GR-β it has been reported to enhance GR-α mediated gene transcription (8).

There are three alternative exons 1. In contrast to alternative splicing at the 3' end of the GR mRNA transcripts, alternative splicing at the 5' end does not affect the GR protein structure. The differences are restricted to the 5'-untranslated region (UTR), which is composed of exon 1 and the first 13 nucleotides of exon 2. These alternative exons 1, designated as exon 1A, 1B and 1C, are each preceded by their own promoter (Figure 1)(9, 10). Moreover, exon 1A contains three separate alternative splice sites, resulting in mRNA transcripts containing exons 1A1, 1A2, or 1A3, which means that at least 5 GR transcripts are expressed from three separate promoters (9), possibly resulting in cell type specific GR expression patterns. Transcripts containing exons 1B and 1C are ubiquitously expressed and are probably responsible for the basal GR expression (11). Transcripts containing 1A1 and 1A2 are marginally expressed, even less than GR-β, while mRNA transcripts containing exon 1A3 are expressed in cells of the hematopoietic lineage (12, 13). Footprint and functional analysis of the promoter regions reveal unique binding sites for several transcription factors, but only promoter 1A harbors a sequence resembling a GRE and could probably be autoregulated by the GR (13-15).

Heterogeneity in the 5' ends of mRNAs generated by alternative promoter usage seems to be a common feature among the members of the steroid hormone receptor family (16, 17). Furthermore, recent studies reported that promoter structure could modulate alternative splicing and suggest a physical and functional coupling between transcription and splicing (18-20). To determine whether splicing of the 3' end variants of the GR is influenced by alternative promoter usage, we explored whether correlations exist between the exon 1 variant (A, B or C) in the mRNA and 3' end splicing (α, β or P).

6.2 Materials and Methods

6.2.1 Cell culture

Epstein-Barr virus (EBV)-transformed lymphoblast cell-lines were established from PBMLs as described previously (21, 22). Cells were grown in RPMI-1640 medium supplemented with 15% FCS, 100 µg/mL penicillin and streptomycin under standard culture conditions.

6.2.2 cDNA

cDNAs were prepared as described previously (23). Different normal tissue samples were kindly provided by the Erasmus MC Tissue Bank. Thymic tissue samples were collected at operations. The tissue samples were taken postmortem or at operation, directly frozen and stored in −80°C until use. The protocols were in accordance with the Helsinki Doctrine on Human Experimentation. Informed consent was obtained. cDNAs prepared from tissues taken postmortem or at operation: lung (n=2), ileum (n=3), colon (n=2), rectum (n=2), thyroid (n=2), testes (n=2), adrenal (n=4), kidney (n=2), jejunum (n=2), pancreas (n=2), stomach (n=1). cDNAs prepared from cells of the hematopoietic lineage: T-cells (n=2), B-cells (n=2), macrophages (n=4), monocytes (n=2), dendritic cells (n=2), and thymocytes (n=2). cDNAs prepared from several untreated cell-lines: B-lymphoblasts (n=2), Jurkat (n=1), SaOs-1 (n=2), Mg63-1 (n=2), SV-HFO (n=2), PancI (n=1), Miapaca (n=1), Bon (n=1) and BxPc3 (n=1). cDNAs prepared form carcinoid tissue: liver tumor (n=1), pituitary tumor (n=1), lymph tumor (n=1), pheochromocytoma (n=1), renal tumor (n=1).

6.2.3 RT-PCR

To investigate if GR-α, GR-β and GR-P encoding transcripts are transcribed from all three different promoters: 1A, 1B and 1C, RT-PCR was performed by using 5' end (exon 1 specific) and 3' end (3' splice variant specific) primer combinations as indicated. These primers were also used for the quantitative real-time RT-PCR experiments and are shown in Table 1. PCR conditions, using the RT-PCR One Step Titan One Tube RT-PCR System (Roche, Almere, The Netherlands), were as follows. 250 ng RNA was incubated with a reaction mix containing 0.4 µl of enzyme mix, 0.2 mM dNTPs, 0.25 µM FW primer, 0.25 µM BW primer, 5 mM DDT, 1 u Protector RNAse and water in a total volume of 20 µl. Cycle conditions were: 30 min 50°C, then 10 cycles: 2 min 94°C, 10 sec 94°C, 10 sec 60°C, 2.5 min 68°C, then 25 cycles 10 sec 94°C, 10 sec 60°C, 2.5 min 68°C, with an additional 5 sec extension to the elongation time for each cycle. A PE-Applied Biosystem Gene Amp 9700 PCR-system was used. PCR products were examined on a UV-light source after electrophoresis on a 0.7% agarose gel containing ethidium bromide.

6.2.4 Quantification of the mRNA variants of the GR

Real-time Q-PCR analysis of mRNA was performed to investigate the amount of transcripts containing exon 1A, 1B, or 1C and the amount encoding GR-α, GR-β, or GR-P. The primers and probes used are shown in Table 1. For each splice variant 2.5 µl cDNA, corresponding to 50 ng total RNA was amplified in separate reactions in a total volume of 25 µl, containing 7.5 pmol of each primer and 5 pmol probe using the Universal Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) for PCRs amplifying the 5'-UTR and the qPCR Core-Kit (Eurogentec, Maastricht, The Netherlands) for PCRs amplifying the 3' splice variants. Correction for assay variability between the separate reactions for which the same cDNA was used was performed using the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) as described previously (24).

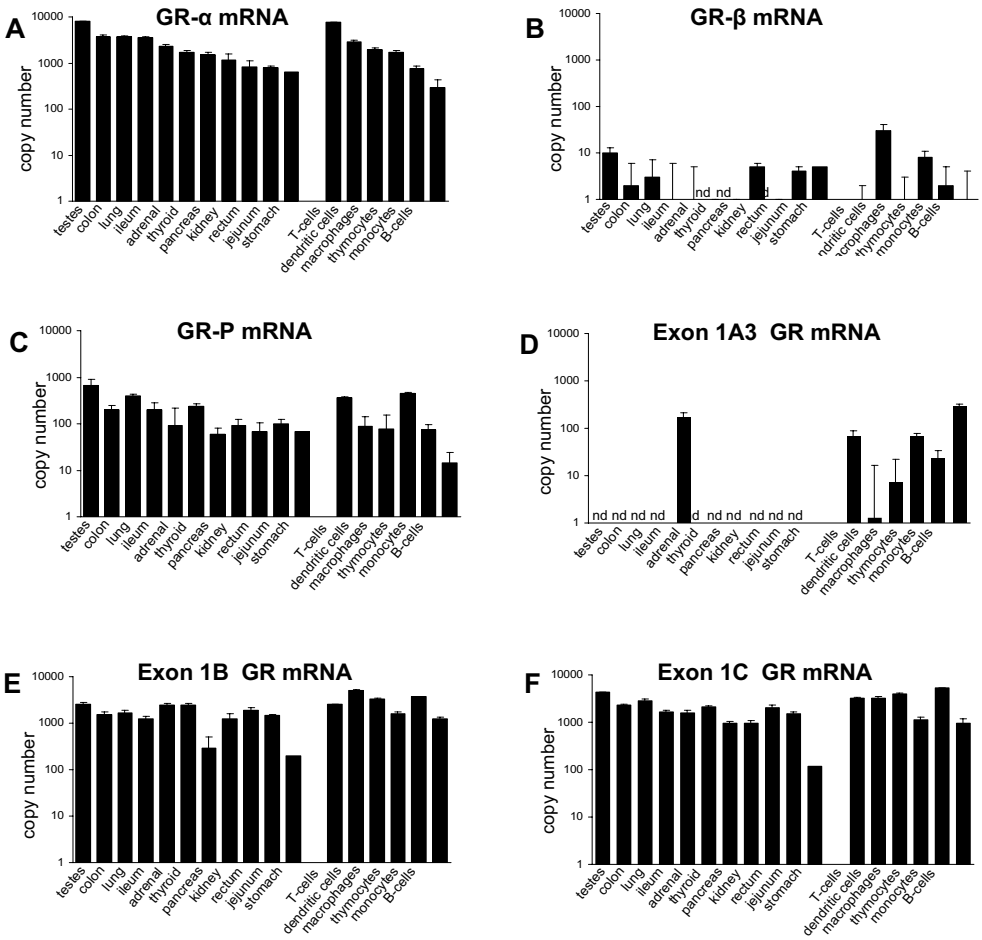


Figure 2 Quantification of mRNA molecules encoding the GR-α, GR-β, GR-P isoforms and carrying exons 1A, 1B or 1C in different tissues. Figure 2A shows the number of copies of GR-α mRNA/50 ng total RNA in descending order. Figures 2B, 2C, 2D, 2E and 2F represent GR-β, GR-P, exon 1A3, exon 1B and exon 1C mRNA expression, respectively, in the same tissues. nd = not detectable.

6.3 Results

6.3.1 Tissue distribution of GR exons 1 and 3' end splice variants

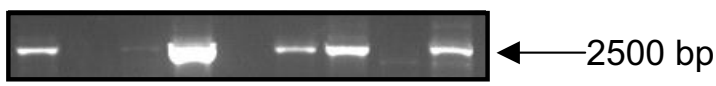
Human cDNAs, reverse transcribed from RNA isolated from different organ tissue biopsy samples and different cells of the hematopoietic lineage, were used to quantify expression levels of exon 1A, 1B, and 1C, and of GR-α, GR-β, and GR-P mRNA. Figure 2 shows that the different exons 1 and 3' end splice variants were differentially expressed in these tissues. The major GR mRNA encoded the GR-α isoform. Expression of GR-P was about 5-25 times lower than that of GR-α and GR-β mRNA represented less than 1%, if at all detectable. Approximately equal expression

levels of exon 1B and 1C were measured. mRNA containing exon 1 (1A3) was only detectable in adrenals and in cells of the hematopoietic lineage (T-cells, B-cells, dendritic cells, macrophages, thymocytes and monocytes). The expression of exons 1A1 and 1A2 was very low, even less than GR-β, and it was not further analyzed. The total amount of the different exons 1 and the total amount of 3' end splice variants are significantly correlated (Pearson r=0.81; p<0.001), indicating that both represent the amount of total GR present in the different tissues.

6.3.2 Correlation between the different exons 1 and the 3' end splice variants

Transcripts encoding GR-α, GR-β and GR-P could theoretically be formed from three different promoters: 1A, 1B, and 1C. Due to the long coding sequence (>2000 nucleotides) between exon 1 and exon 9, no quantitative real-time RT-PCR could be performed to investigate how expression of these 3' end splice variants is controlled by the different promoters. Therefore, we used semi-quantitative PCR analysis using RNA from a B-lymphoblast cell-line. Figure 3 shows that GR-α and GR-P mRNA could be expressed from all three promoters 1A, 1B and 1C. However, no combinations could be made with the GR-β specific primer, possibly due to low GR-β mRNA levels.

Furthermore, by calculating the percentage of each variant contributing to the total amount of GR mRNA presented in Figure 2, correlations were made between the different exons 1 and 3' end splice variants. Positive significant correlations were found between the contributions of exon 1B and the GR-P splice variant (Pearson r=0.66, p=0.004) and between exon 1C and the GR-α splice variant (Pearson r= 0.6, p=0.012) (Figure 4A). No positive correlations could be calculated between exon 1A and the 3' end splice variants and between GR-β mRNA expression and



PCR product:

1. GR-α mRNA containing exon 1A
2. GR-β mRNA containing exon 1A
3. GR-P mRNA containing exon 1A
4. GR-α mRNA containing exon 1B
5. GR-β mRNA containing exon 1B
6. GR-P mRNA containing exon 1B
7. GR-α mRNA containing exon 1C
8. GR-β mRNA containing exon 1C
9. GR-P mRNA containing exon 1C

Figure 3 Expression of different GR mRNA isoforms in B-lymphoblasts. PCR analysis was performed in B-lymphoblasts to investigate the expression of the indicated mRNA molecules. The specific amplification of these mRNA molecules was achieved by using the underlined primers shown in Table 1. In these B-lymphoblasts, GR-α as well as GR-P expression is driven from promoter 1A, 1B and 1C. No amplification of GR-β encoding mRNA was detected.

the various exons 1. The correlations presented in Figure 4B are based on 17 tissue samples and 13 additional cDNAs which were obtained from different cell-lines and carcinoid tissues to increase statistical power. These samples were obtained from various other studies and not included in Figure 2 because cDNA was created with non-comparable amounts of RNA. Similar correlations were found between exon 1B and GR-P (Pearson r=0.66, p<0.0001) and between exon 1C and GR-α (Pearson r=0.61, p=0.0004)(Figure 4B). The proportion of 1B usage strongly influences the GR-P splicing route, whereas the proportion of 1C usage favors the GR-α splicing route.

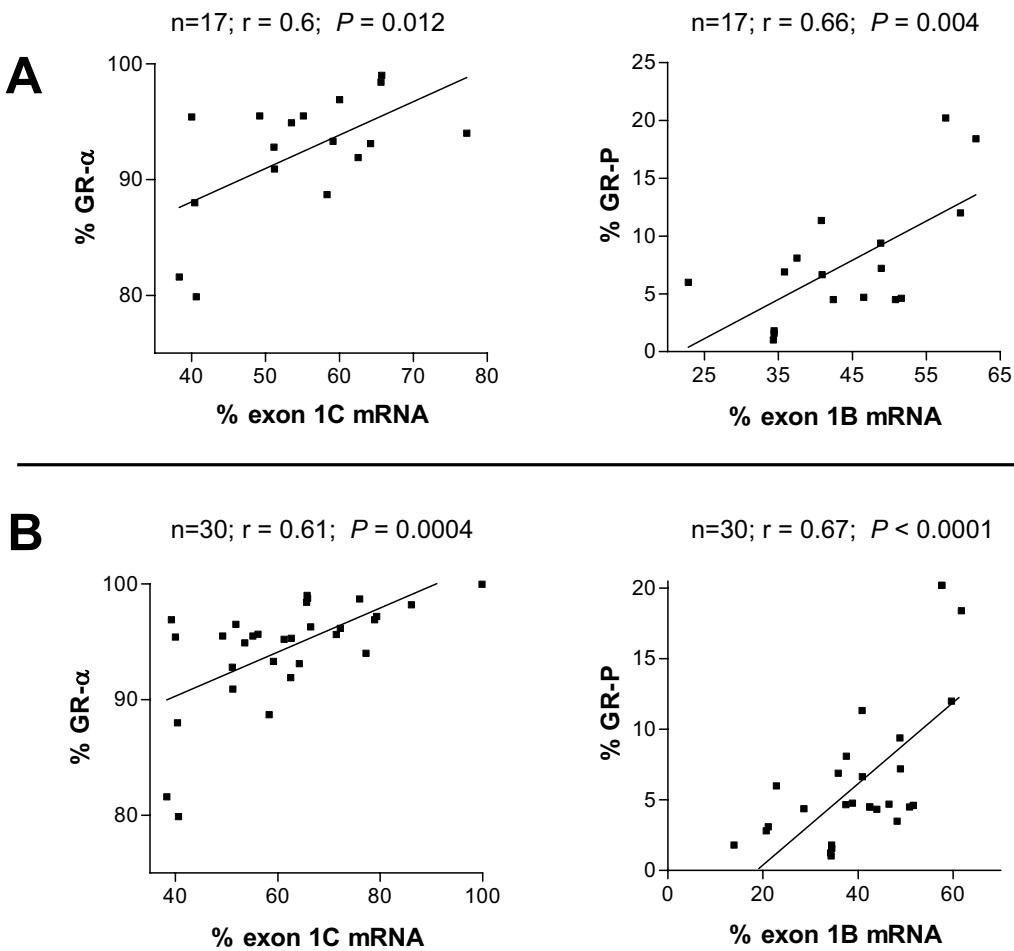


Figure 4 Positive significant correlations between contributions of exons 1 to the total amount of GR mRNA and mRNA encoding 3' end splice variants. Pearson correlations between the contribution of exon 1C and GR-α and exon 1B and GR-P to the total amount of mRNA expressed in the 17 tissues described in Figure 2 (A), and in the 17 tissues combined with 13 additional tissues and cell-lines to increase statistical power (B).

6.4 Discussion

In 60% of the human genes, processing of pre-mRNA by specific splice factors results in multiple mRNAs and hence, multiple proteins (19). The factors that influence the selection of splice sites are not understood very well. However, recent evidence reveals that transcription regulation and alternative splicing can be coupled events (25, 26). The promoter may affect alternative splicing through recruitment of factors with dual functions in transcription and splicing (i.e. factors that contain functional domains for both processes and hence link them). Studies in which promoter structure was changed, led to differences in alternative splicing of the transcript, which could not be explained by differences in promoter strength (25). Furthermore, a decreased processing rate of the RNA polymerase II or internal pauses could favor the inclusion of alternative exons, whereas a highly processive polymerase would favor exclusion of these exons. Elongation speed is regulated by the promoter and depends on the composition of the recruited transcription complex (27, 28), but also on the secondary structure of the pre-mRNA (29). When multiple promoters are involved in the transcription of a particular gene, occupation by promoter specific transcription factors might differentially influence the transcription/splicing coupling mechanism, leading to alternative splicing (20, 30).

The GR mRNA is expressed from multiple promoters. However, only a few studies have been reported on their characterization. Promoter 1A is mainly active in cells of hematopoietic origin and contains binding sites for nuclear factor-κB, interferon regulatory factor and GR (9). Promoters 1B and 1C however, are thought to play a role in the constitutive expression of GR. Constitutive or 'housekeeping' genes typically lack a TATA or CAAT box, but contain multiple GC regions that are potential sites for ubiquitous transcription factors such as SP-1. Four SP-1 binding sites have been identified in promoter 1B, whereas five were identified in promoter 1C (10, 31). Yin-Yang 1 (YY-1) binding sites are identified in promoter 1B (3 sites) and promoter 1C (1 site). YY-1 is a transcription factor that can act as an activator, a repressor, or an initiator of transcription, depending on the cellular context (11, 32). AP-1 and AP-2 sites are only identified in promoter 1C (11). Several transcription factors are reported to contain functional domains to recruit splicing factors (18) but if SP-1, YY-1, AP-1 and AP-2 can directly interact with the splicing machinery is unknown.

In this study, we measured 1A, 1B and 1C, but also GR-α, GR-β and GR-P containing transcripts by quantitative real-time RT-PCR and found associations between promoter usage and alternative splicing of the GR gene. The proportion of 1B usage strongly influences the GR-P splicing route, whereas the proportion of 1C usage favors the GR-α splicing route (Figure 4). However, a semi-quantitative PCR experiment in which 5' specific primers for the different exons 1 were combined with specific primers for the 3' splice variants showed that this is not an absolute effect, because PCRs with the possible primer combinations all resulted in amplification products

(Figure 3). Furthermore, no associations were found with transcripts including exon 9β, nor with those transcribed from promoter 1A, but this might be due to the low number of transcripts with these characteristics. The differences in amount of SP-1 and YY-1 transcription factor binding sites in promoters 1B and 1C, the presence of AP-1 and AP-2 binding sites in promoter 1C, or the abundance of transcription and splicing factors in the different tissues that are studied might result in a different composition of transcription complexes that are recruited on these promoters, and through associations with splicing factors, influencing the ratio in which GR-α and GR-P mRNA is expressed.

The cell-type specific use of different promoters in the GR gene, but also the different start sites and exons 1 resulting in differences in secondary structure of the pre-mRNAs might alter the elongation speed at which mRNAs are formed. This could also influence splicing regulation. These 5' differences are restricted to the non-coding region and do not influence the open reading frame, which starts in exon 2. If alternative promoter usage and splice site selection for GR expression are linked through either transcription factor recruitment or elongation speed by means of such a coupling remains to be proven. Any supporting evidence should first rule out the possibility that alternative splice variants are the consequence of important changes in pre-mRNA secondary structure resulting from the different exon 1 sequences.

Nonetheless, our data demonstrate that GR-α expression is related to the use of the 1C promoter and that expression of GR-P is related to the use of the 1B promoter. The regulation of GR splice variants through the multiple promoters and the cell type specific expression of transcription factors is another way in which cells and tissues can be sensitized to the actions of GCs and the functions of the different splice variants.

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Chapter 7

Strategies for the Characterization of Disorders in Cortisol Sensitivity

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Abstract

Context: The clinical presentation of abnormalities in glucocorticoid (GC) sensitivity is diverse, and therefore it is difficult to diagnose this condition.

Objective and Design: The objective of the study was to develop strategies for the characterization of GC sensitivity disorders.

Setting: The study was conducted in an outpatient clinic.

Patients: Nine patients with GC sensitivity disorders participated.

Interventions: Sequence analysis of the GC receptor (GR), determination of GR number per cell, GR ligand-binding affinity, and GR splice regulation were performed in freshly prepared peripheral blood mononuclear lymphocytes and Epstein-Barr virus-transformed lymphoblasts. Cellular GC sensitivity was determined *ex vivo* by measuring the effect of dexamethasone on GC-induced leucine-zipper and IL-2 mRNA levels and on cell proliferation.

Results: Differences in GR number per cell, GR affinity, GR splice variants, and effects on transactivation or transrepression of GC-sensitive genes were observed between patients and controls. Epstein-Barr virus transformation of lymphoblasts had no influence on GR affinity but increased the GR number 5-fold in healthy controls. In patients diagnosed as cortisol resistant, however, GR number after transformation was increased significantly less than 5-fold, whereas a higher GR number was observed in a patient suspected of cortisol hypersensitivity.

Conclusion: This study illustrates several strategies to define abnormalities in GC sensitivity by describing nine patients with affected GC sensitivity, all with a unique clinical course and background.

7.1 Introduction

Glucocorticoids (GCs) are key hormones in metabolic and immunological homeostasis and regulate many physiological processes (1). Cortisol concentration is tightly regulated by the hypothalamic-pituitary-adrenal (HPA)-axis feedback system and depends on neural and other stimuli (2, 3).

The extremes of variability in cortisol sensitivity can be divided in cortisol resistance (CR) and hypersensitivity (CH). So far, only one case of CH has been reported, diagnosed in a patient with Cushingoid manifestations, despite persistent hypocortisolemia (4). CR was first described (5) as an inherited disorder characterized by hypercortisolism without Cushingoid features. The negative feedback on the HPA axis is reduced, due to diminished GC sensitivity, resulting in higher cortisol secretion by the adrenal glands to keep balance between need and production. However, adrenal production of androgens and mineralocorticoids (MCs) is also increased, causing the symptoms of CR: hypertension, hypokalemia, disturbed spermatogenesis, and infertility in men and acne, hirsutism, male pattern of baldness, oligomenorrhea, and infertility in women (5, 6). In children, premature adrenarche was reported (7).

Decreased GC sensitivity is often caused by abnormalities in the GC receptor (GR) including decreased affinity for GCs (6, 7, 8), decreased receptor number (6, 8, 9), decreased receptor DNA binding (6, 10), receptor thermolability (11), impaired receptor translocation to the nucleus (12), or altered protein-protein interaction with coactivators (13). An increased concentration of the GR-β splice variant, a dominant-negative inhibitor of active GR-α, has also been reported to cause CR, but always as acquired rather than inherited (14, 15). A GR-P splice variant is thought to increase GR-α activity (15).

The molecular basis of CR has been elucidated in six kindreds and three sporadic cases as caused by mutations in the DNA- or hormone-binding domain of the GR gene. However, several years ago, we reported five patients diagnosed with clinical and/or biochemical CR, each with very diverse clinical presentations, without GR gene alterations (16).

For the present study, we invited nine patients with abnormal GC sensitivity. One patient hyperreacted to GC medication, whereas the others were diagnosed as CR. Of the latter group, three patients had been previously reported with mutations in the GR gene (17, 18, 19) and two patients without genetic GR alterations (16); the other three patients were recently diagnosed and have not been described previously. The aim of our study was to develop a strategy for the diagnosis of (inherited) disorders in GC sensitivity. This should also include techniques using materials from patients in whom current GC therapy cannot be interrupted, as well as opportunities to study cells more intensively, without the need for freshly isolated cells.

7.2 Materials and Methods

7.2.1 Patients

Patients 1–5 have been reported previously. In summary, patients were diagnosed with compensated CR characterized by increased cortisol secretion without Cushingoid features. They showed insufficient suppression of cortisol in a 1-mg dexamethasone (DEX) suppression test. Patient 1 presented with hypertension and oligospermia (17), and his CR was attributed to a heterozygous I559N mutation. The clinical symptoms of patient 2 were hypertension and hypokalemia (17, 18), caused by a heterozygous D641V mutation. Patient 3 presented with symptoms of hyperandrogenism, attributed to a 4-bp deletion (Δ4) identified at the 3' boundary of exon 6 and intron 6, removing a donor splice site in one allele, resulting in the transcription of unstable mRNA, consequently decreasing the amount of GRs by 50% (19). Patient 4 presented with hirsutism and menstrual irregularities, and patient 5 also developed acne, fatigue, and mood disorders, but no GR gene alterations were found explaining the clinical and biochemical CR in these two patients (16).

Patient 6, a 36-yr-old female patient, presented with fatigue, hypertension (systolic blood pressure, 225 mm Hg; diastolic blood pressure, 125 mm Hg), and a slight male pattern of baldness, without signs and symptoms of Cushing's syndrome, hirsutism, or menstrual irregularities [height, 172 cm; 0 SD score (SDS); weight, 66 kg]. In two overnight 1-mg DEX suppression tests, early morning cortisol was insufficiently suppressed [360 and 530 nmol/liter; normal range (N), <145 nmol/liter]. Urinary free cortisol [250–340 nmol/24 h (N, 40–200 nmol/24 h)], as well as early morning cortisol [1280 nmol/liter (N, <850 nmol/liter)], was elevated, accompanied by a slightly elevated plasma ACTH of 120 ng/ml (N, 30–100 ng/ml). Cortisol diurnal rhythm was present, albeit at a higher level. Plasma testosterone varied between 6.7 and 8.4 nmol/liter (N, 1–3 nmol/liter); dehydroepiandrosterone sulfate was 37–43 μmol/liter (N, 3–13 μmol/liter). Bone mineral density of the lumbar spine and hip were normal. The clinical presentation of the patient indicated elevated activity of the HPA axis without signs of Cushing's disease and was typical for CR.

Patient 7 developed renal insufficiency at the age of 40 yr after an unexplained glomerulonephritis. He was one of the first patients undergoing a postmortem donor kidney transplant in The Netherlands in 1972 at the age of 43 yr. Despite low immunosuppressive medication (prednisone, 7.5 mg/d; azathioprine, 100 mg/d), his renal function remained normal and is only slightly impaired today (creatine, 202–263 μmol/d). The 33 yr after transplantation were clinically largely uneventful. He has no other specific diseases. Blood pressure is normal. Because of this extraordinary clinical course, we suspected abnormal cortisol sensitivity. Despite long-term prednisolone medication, which could not be stopped, substantial serum concentrations of adrenal androgens were detected, which might indicate decreased GC sensitivity of the HPA-axis feedback system.

Patient 8, a 20-yr-old male patient, was diagnosed at birth with congenital

adrenal hyperplasia, and the underlying defect in his 21-hydroxylase gene was recently identified (M.A. Timmermans, F. H. de Jong, unpublished results). He was treated with GCs and MCs (final height, 167 cm; –2.4 SDS; weight, 63 kg). After puberty, he was admitted several times for an Addisonian crisis in relation to intermittent infections. He needed exceptionally high doses of GCs to overcome adrenal insufficiency, indicating GC resistance. Currently, 20 mg of hydrocortisone three times per day (N, 8–15 mg/m^2·d) or 0.5 mg of DEX four times per day are still insufficient to fully normalize serum ACTH, androstenedione, 17-OH-progesterone, and testosterone levels. Serum LH and FSH levels were fully suppressed, whereas serum TSH, free T_3, and free T_4 were normal. He is also treated with 0.625 mg of 9α-fludrocortisone three times per day (N, 0.05–0.2 mg/d) to reach a normal blood pressure (systolic, 120 mm Hg; diastolic, 70 mm Hg), without orthostasis or peripheral edema. Recently, bone mineral density of the lumbar spine and hip were found to be within normal values.

Patient 9, a 13-yr-old patient, presented with progressive obesity, some nausea, and tiredness. For asthma, she used low-dose inhalation GCs (budesonide 200 μg/d). Growth retardation was noticed (height 142 cm; –3.2 SDS) in combination with general obesity (weight 64.5 kg; +2.8 SDS) and striae. Blood pressure was normal (systolic, 95 mm Hg; diastolic, 63 mm Hg). Serum fasting cortisol level of less than 30 nmol/liter was too low (N, 200–600 nmol/liter) as well as the urinary free cortisol of less than 3 nmol/24 h (N, <500 nmol/24 h). Bone age was 3.5 yr retarded. Bone mineral density of the lumbar spine showed osteopenia (z-score, –2.5 SDS). These clinical features of Cushing's syndrome on low-dose steroid treatment in combination with the suppressed cortisol levels in blood and urine were considered typical for CH.

From all patients, informed consent was obtained, and the Medical Ethics Committee of Erasmus MC, The Netherlands, approved this study.

7.2.2 Whole cell DEX binding, [^{3}H]thymidine incorporation, and mRNA expression of GC-induced leucine zipper, IL-2, and GR

Blood (70 ml) was drawn into heparinized tubes by venipuncture. Peripheral blood mononuclear leukocytes (PBMLs) were isolated, and the number of GRs per cell (n), their dissociation constant (K_D), and the sensitivity of PBMLs to the inhibition of phytohemagglutinin (PHA)-stimulated incorporation of [^{3}H]thymidine by 100 nM DEX were determined, as described previously (6, 20). Expression of GC-induced leucine zipper (GILZ) and IL-2 mRNA levels in response to 100 nM DEX and expression of GR-α, GR-β, and GR-P splice variants were measured in a real-time quantitative PCR (Q-PCR), as described previously (21, 22).

7.2.3 Epstein-Barr virus transformation of B lymphocytes

Epstein-Barr virus (EBV)-transformed lymphoblast cell lines were established from PBMLs (23, 24). Cells were grown in RPMI 1640 medium supplemented with 15% fetal

calf serum, 100 µg/ml penicillin, and streptomycin at standard culture conditions.

7.2.4 Sequence analysis

The coding sequence of the GR gene including intron/exon boundaries was sequenced in all patients using primers as described previously (25).

7.3 Results

7.3.1 Analysis of GR characteristics and expression

Sequence analysis was performed on the nine exons and intronic flanking sequences of the GR in all patients. We have previously reported on the heterozygous mutations in patients 1 (I559N), 2 (D641V), and 3 (Δ4 bp). In patient 3 also, the earlier reported N363S singlenucleotide polymorphism (SNP) was found, enhancing GC sensitivity (26). In the other patients, several different SNPs were found, but only ER22/23EK, heterozygously present in patient 4, has been reported to decrease GC sensitivity (25, 26). Patient 7 had two heterozygous nucleotide changes in intron 8, 81 bases downstream of exon 8 (G to A) and 9 bases upstream of exon 9 (C to G). Codons 750 and 588 in, respectively, patients 7 and 8 were heterozygously mutated from, respectively, CCC to CCT and CAC to CAT, but this did not cause amino acid changes.

Subsequently, we performed radioligand-binding studies to determine the GR number per cell (n) and the K_D in PBMLs (Figure 1A) of patients and 14 healthy controls. Patient 3 (Δ4 bp) showed only half of the normal receptor number per cell, whereas patient 9, suspected of CH, showed an increased receptor number. Only patient 1, carrying the heterozygous I559N mutation, showed decreased affinity for DEX. The data of patients 5 and 7 were not included in Figure 1A because GC medication could not be stopped and subsequently interfered in these binding studies.

Expression of the GR was further analyzed by quantification of mRNA copies of the GR-α, GR-β, and GR-P splice variants using real-time Q-PCR. Figure 1B shows these levels measured in PBMLs of patients and controls. Patient 3 (Δ4 bp) shows approximately 50% of the normal amount of GR-α, GR-β, and GR-P mRNA copies. In patient 9, 70% more GR-α mRNA expression was measured, corresponding with GR number measured in PBMLs in the ligand-binding assay. Patient 7 showed 3-fold higher GR-β expression levels, although no differences for expression of GR-α and GR-P mRNA splice variants were found.

7.3.2 Cellular GC sensitivity

Liganded GR acts together with several cofactor complexes to regulate transcription of GC-responsive genes. Affected GC sensitivity was further investigated by measuring the expression of GILZ and IL-2, two endogenous GC-sensitive genes,

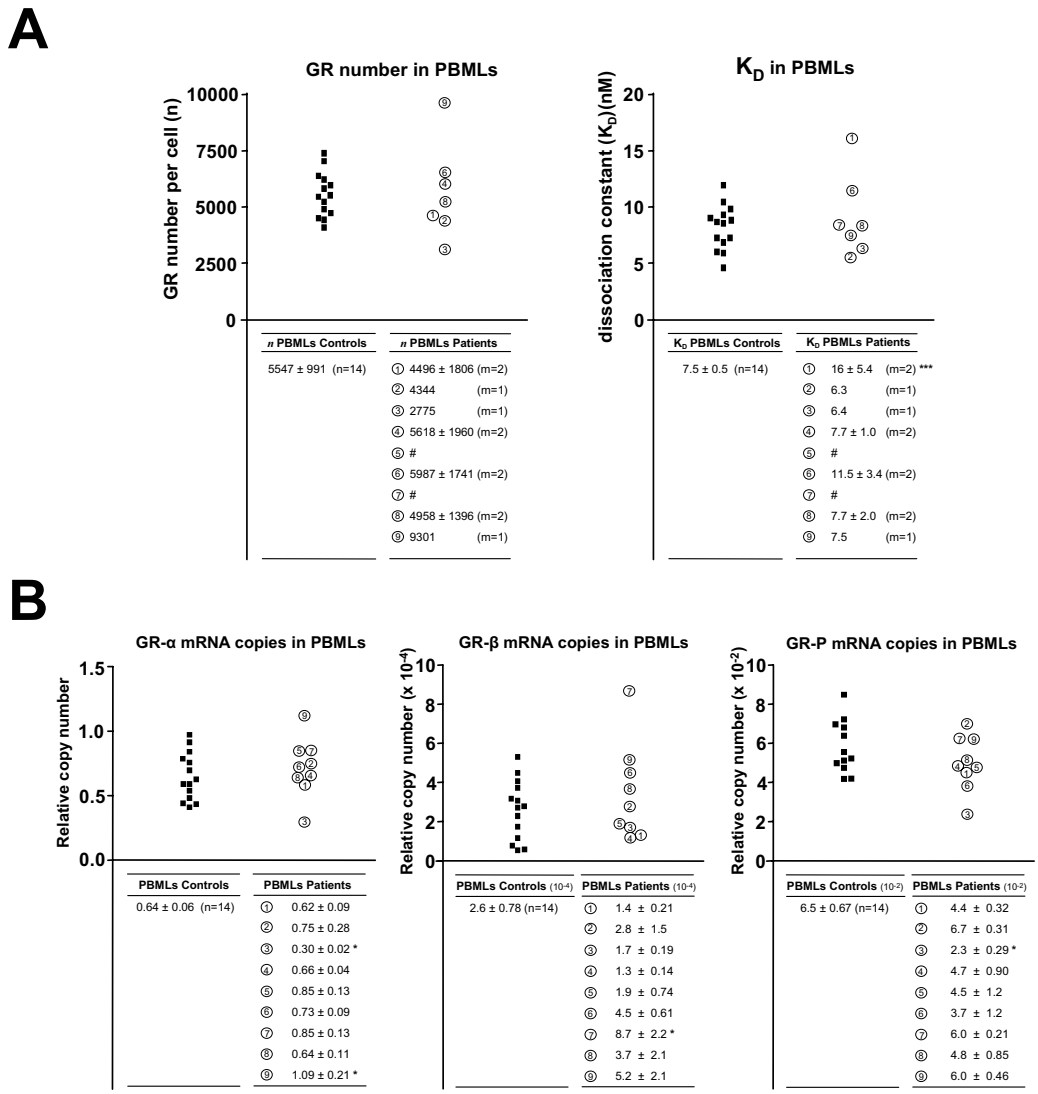

Figure 1 GR characteristics and GR mRNA copy numbers in PBMLs of patients and healthy controls. GR number per cell (n) and K_D (A) and relative copy numbers of GR-α, GR-β, and GR-P splice variants (B) in PBMLs of patients with affected GC sensitivity (circled numbers) and controls (■). From patients 5 and 7, no n or K_D could be obtained due to interference of DEX medication (indicated as #). Copy numbers were calculated relative to the levels of the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) by applying the formula $2^{[CT\ (HPRT)\ -\ CT\ (GR)]}$. For further details, see Livak and Schmittgen (32). Data represent means ± S.E.M., and the assay was performed in duplicate with duplicate measurements or as indicated (m). *, $p \leq 0.05$; ***, $p \leq 0.001$ by Student's *t* test.

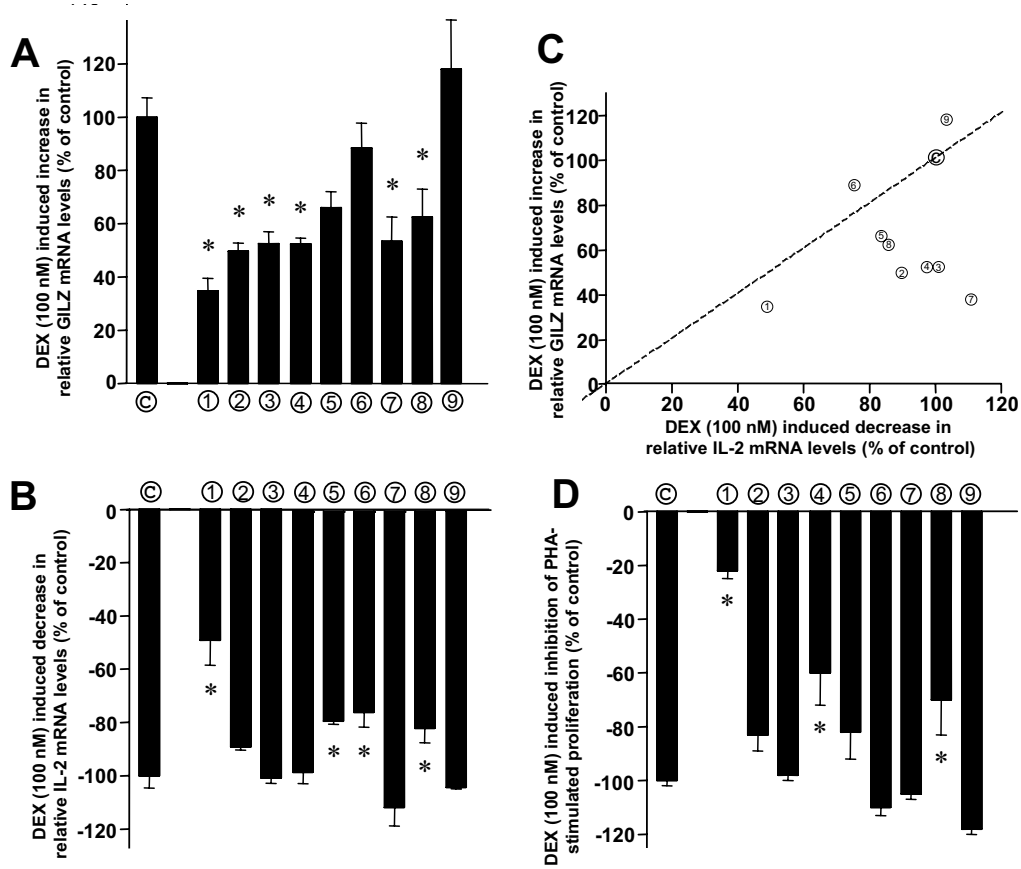

Figure 2 GILZ and IL-2 mRNA expression levels and repression of PHA-stimulated proliferation in PBMLs. Relative increase of GILZ (A) and decrease of IL-2 (B) mRNA levels induced by 100 nM DEX in PBMLs of patients (circled numbers) and controls (©). Cells were incubated for 4 h with PHA and with or without 100 nM DEX, followed by mRNA isolation and quantitation by real-time RT-PCR. Data are presented as the increase of GILZ (A) and decrease of IL-2 (B) mRNA relative to the values in the absence of DEX, which are also presented in C as GILZ *vs*. IL-2 response. DEX incubations were performed in duplicate, and duplicate real-time Q-PCR was performed for every sample. Levels for controls in arbitrary units were: 100 ± 8 (without DEX) and 1577 ± 115 (100 nM DEX) in the GILZ assay and 100 ± 5 (without DEX) and 28 ± 4 (100 nM DEX) in the IL-2 assay. These response levels were set to 100%. No systematic differences between patients and healthy controls were observed in the absence of DEX (data not shown). For all subjects, PHA treatment in the absence of DEX equally stimulated IL-2 mRNA levels 12- to 18-fold but did not affect GILZ or GR mRNA levels (data not shown). D, Relative inhibition of PHA-induced [^{3}H]thymidine incorporation by 100 nM DEX in PBMLs of patients and controls. The average response in the healthy controls was 100 ± 6 (without DEX) and 18 ± 3 (100 nM DEX) and was set to 100%. Data represent means ± S.E.M., and the assay was performed in duplicate with incubations in triplicate. The average response in the healthy controls was set to 100%. *, $p<0.05$ by Student's *t* test.

which could be strongly up- and down-regulated by GCs, respectively. Figure 2 shows the increase of GILZ (A) and decrease of IL-2 (B) in PBMLs of our patients when stimulated with PHA and 100 nM DEX, relative to levels in the absence of DEX. The average increase/decrease of GILZ/IL-2 mRNA in PBMLs of healthy controls was set to 100%. PHA, necessary to induce IL-2 gene transcription, did not affect expression of GILZ or GR mRNA levels. PHA induction, as well as basal expression levels of GILZ and IL-2 in the absence of DEX, was comparable between patients and controls (data not shown). Patients 2 (D641V), 3 (Δ4 bp), 4, and 7 showed less up-regulation of GILZ mRNA than the controls (50 ± 3%, 52 ± 5%, 52 ± 3%, and 53 ± 9%, respectively), whereas transrepression of the IL-2 gene was mainly unaffected. In patients 1 (I559N) and 8, transactivation of the GILZ, as well as transrepression of the IL-2 gene, was reduced (GILZ up-regulation and IL-2 repression compared with controls in patient 1, 35 ± 5% and 49 ± 9%, respectively; in patient 8, 62 ± 10% and 85 ± 6%, respectively). The same trend was observed in patients 5 and 6 (in patient 5, 66 ± 6% and 84 ± 5%, respectively; in patient 6, 88 ± 9% and 75 ± 6%, respectively). In patient 9, who overreacted to GC medication, more transcriptional regulation of the GILZ and IL-2 gene seemed to occur, but this was not significantly different from controls. In Figure 2C, the GILZ response is plotted against the IL-2 response. Patients 1, 5, 6, 8, and 9 lie close to the diagonal, indicating defects that equally affect transactivation and transrepression, whereas the marked GILZ defect without substantial alterations in IL-2 response puts patients 2, 3, 4, and 7 off the diagonal in the lower right section, clearly demonstrating that transactivation and transrepression are separable entities.

The PBMLs were also tested in a PHA-stimulated proliferation assay. The decrease in proliferation induced by 100 nM DEX is shown in Figure 2D and is related to the average decrease in the healthy control group set to 100%. Less suppression of proliferation was observed for patients 1 (23 ± 3%), 4 (60 ± 12%), and 8 (70 ± 15%), whereas more suppression was observed for patient 9 (115 ± 2%). Approximately the same trend was shown compared with the results of the IL-2-transrepression assay, only the outcomes for patients 4 and 6 did not correspond. In this proliferation assay, IL-2 gene repression certainly plays a role, but it is carried out over a much longer time scale (4 d, rather than 4 h), and the outcome is formed by the integration of many processes, including apoptosis.

7.3.3 EBV-transformed B lymphocytes

To obtain permanent cell lines, native B lymphocytes of patients and controls were transformed with EBV to obtain immortalized lymphoblast cell lines, and then GR number and ligand K_D were measured (Figure 3A). Ligand affinity was not influenced by viral transformation (Spearman's correlation: r=0.73; p=0.06). Patient 1 (I559N) showed decreased affinity for DEX. GR numbers in lymphoblasts were approximately five times higher than those measured in lymphocytes, even after correction for cell volume (data not shown). The increase in receptor number after EBV transformation

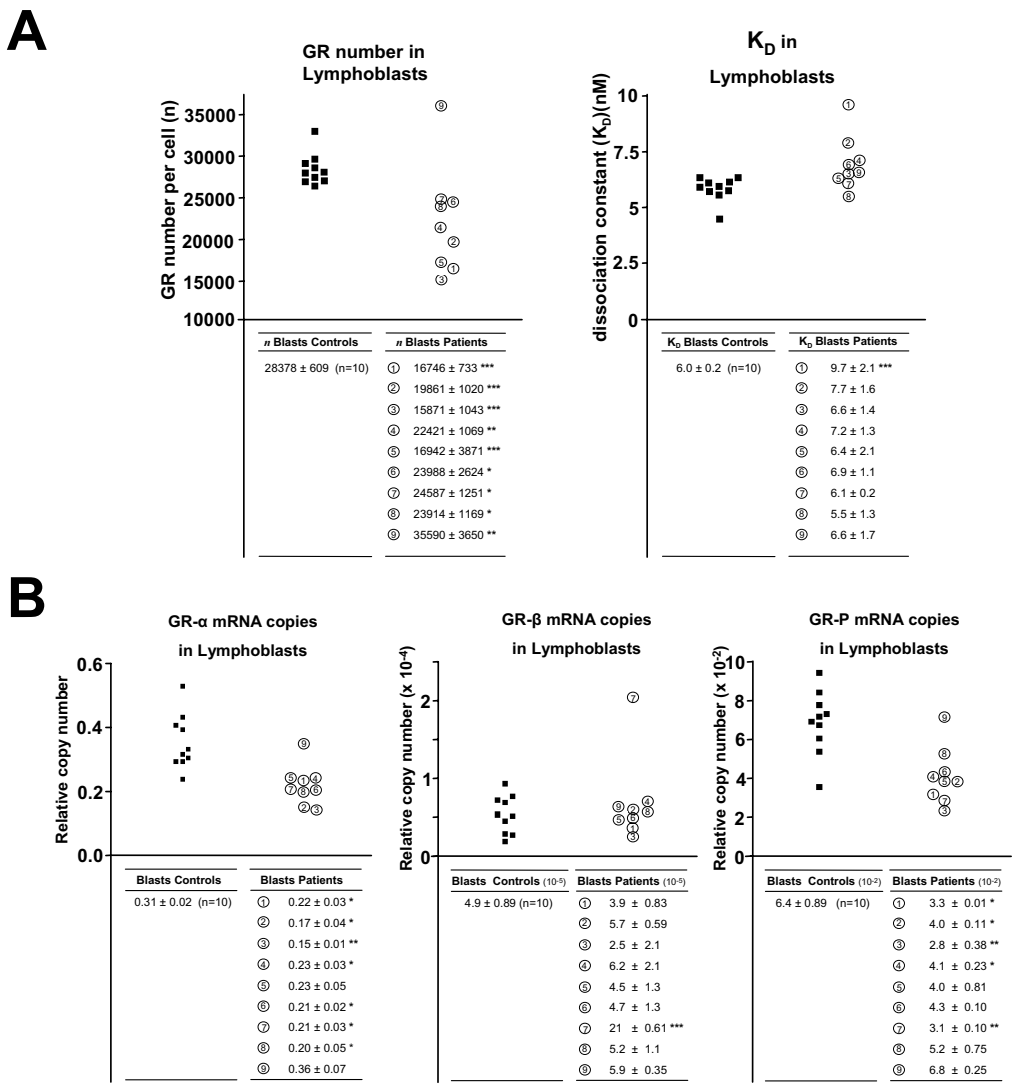

Figure 3 GR characteristics and GR mRNA copy numbers in lymphoblast cell lines of patients and healthy controls. GR number per cell (n) and K_D (A) and relative copy numbers of GR-α, GR-β, and GR-P splice variants (B) in lymphoblasts of patients with affected GC sensitivity (circled numbers) and controls (■). Copy numbers were calculated relative to the levels of the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) by applying the formula $2^{[CT\ (HPRT)\ -\ CT\ (GR)]}$. For further details, see Livak and Schmittgen (32). Data represent means ± S.E.M., and assays were performed at least three times in duplicate, exactly 3 months after transfection. *, p≤0.05; **, p≤0.01; ***, p≤0.001 by Student's *t* test.

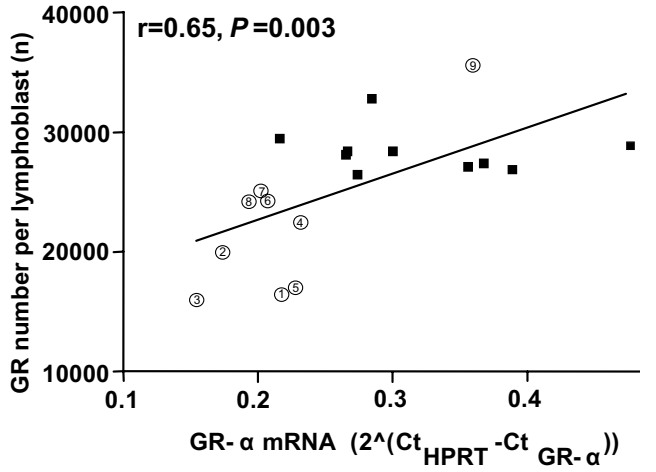

Figure 4 Correlation analysis (Spearman's correlation) between GR number and relative GR-α mRNA copy numbers in lymphoblast cell lines of patients and healthy controls.

in patients diagnosed as CR, however, was significantly less, whereas the receptor number in the patient who overreacted to GC treatment was significantly higher, than in controls (Figure 3A). Quantifying GR-α and GR-P mRNA expression levels also showed that GR expression is affected after viral transformation (Figure 3B). Figure 4 shows the correlation in lymphoblasts between GR number determined in the radioligand-binding assay and the number of GR-α mRNA copies, further indicating that GR concentrations in affected patients were different from those measured in controls. GR-β levels, however, seemed not to be affected by viral transformation, but this might be obscured by the higher variability in quantifying these very low expression levels. Interestingly, GR-β expression in patient 7 was more than 4-fold higher than in the other subjects, which was also shown, although to a lesser extent, in PBMLs (Figure 1B).

7.4 Discussion

We have used three different approaches to study altered GC sensitivity: 1) analysis of the GR characteristics (n and K_D), its coding sequence, and its expression (including mRNA splice variants); 2) examination of GC sensitivity *ex vivo* by measuring responses of endogenous GC sensitive genes (GILZ and IL-2) and the inhibition of mitogen stimulated proliferation; and 3) obtaining permanent cell lines, free of systemic influences, and preceding therapy by transforming B lymphocytes with EBV. The supplementary table summarizes the described patients, genotype, clinical presentation, biochemical phenotype, and a summary of the data obtained from this study.

Supplementary table: Patients with affected glucocorticoid sensitivity

Patient (male/ female) age	Sequence analysis GR gene	Clinical Presentation		Biochemical Phenotype		Present study						
						DEX (100 nM) response on:			GR-characteristics			
									PBMLs		EBV-blasts	
						GILZ mRNA Induction (*ex vivo*)	IL-2 Induction (*ex vivo*)	PHA-stimulated proliferation	Copy number	affinity for ligand	Copy number	Affinity for ligand
1 (male) 51 yrs	Heterozygous I559N	Hypertension Oligospermia	CR	Affinity for ligand ↓ Transactivation (*in vitro*) ↓ Transrepression (*in vitro*) ↓ Transdominance (*in vitro*)	*Ref:* (17) (27) (28)	Decreased	Decreased	Decreased	Normal	Decreased	Decreased	Decreased
2 (male) 52 yrs	Heterozygous D641V	Hypertension Hypokalemia	CR	Affinity for ligand ↓ Transactivation (*in vitro*) ↓ Transrepression (*in vitro*) → Nuclear translocation ↓	(17) (18) (27) (28)	Decreased	Unaffected	Unaffected	Normal	Normal	Decreased	Normal
3 (female) 48 yrs	4-bp deletion in exon-intron 6, N363S SNP	Hyperandrogenism	CR	Copy number GRα: 50% of control	(19)	Decreased	Unaffected	Unaffected	50% of controls	Normal	Decreased	Normal
4 (female) 48 yrs	ER22/23EK SNP	Hyperandrogenism	CR		(16)	Decreased	Unaffected	Decreased	Normal	Normal	Decreased	Normal
5 (female) 42 yrs		Hyperandrogenism Acne, Fatigue, Mood-disorders	CR		(16)	Decreased	Decreased	Unaffected	n.d.	n.d.	Decreased	Normal
6 (female) 36 yrs		Male pattern of baldness, Fatigue, Hypertension	CR			Decreased	Decreased	Unaffected	Normal	Normal	Decreased	Normal
7 (male) 76 yrs	intronic flanking sequences: Heterozygous G → A, +81 bp exon 8 Heterozygous C → G, -9 bp exon 9	33 years clinically uneventfull after kidney transplantation despite low immunosuppressive medication	CR			Decreased	Unaffected	Unaffected	n.d.	n.d.	Decreased	Normal
8 (male) 20 yrs		Need for exceptionally high doses of GCs in order to overcome congenital adrenal hyperplasia diagnosed at birth	CR			Decreased	Decreased	Decreased	Normal	Normal	Decreased	Normal
9 (female) 13 yrs		Cushing's syndrome on low dose steroid treatment in combination with suppressed cortisol levels in blood	CH			Unaffected	Unaffected	Decreased	Increased	Normal	Increased	Normal

n.d. = not determined

The process through which the I559N and D641V mutations in patients 1 and 2 impair the physiological mechanisms of GC action at the molecular level is multifactorial and involves impaired ligand-binding ability, decreased intrinsic transcriptional activity, and abnormal interaction with certain coactivators (27, 28). Furthermore, the D641V mutation showed aberrant nucleocytoplasmic trafficking and the I559N mutation exerted a dominant-negative effect on GR-α activity by hampering nuclear translocation (28). The *ex vivo* results presented in this paper were in line with the results found in these *in vitro* studies (27, 28): transactivation and transrepression was affected in patient 1. Due to the dominant-negative effect of the GR (I559N), only 30–40% transactivating activity was measured. Transrepression in patient 2, however, was normal, and only decreased transactivational activity was observed. In a previous *in vitro* study, we also observed decreased effects on transactivation of GR (D641V), with no effects on transrepression (27). An explanation for this discrepancy between patients 1 and 2 may be that transactivation occurs through a mechanism characterized by GR interaction with specific DNA sequences, the GC response elements (GREs), whereas transrepression involves interaction of GR with other transcription factors in the absence of specific DNA binding (29).

Patient 3, expressing only half of the normal number of GRs, might demonstrate the strong relationship between GR number and GC sensitivity. Transactivation is 50% reduced (Figure 2A), whereas maximal transrepression was unaffected (Figure 2B and 2C). This might indicate that GR action through transactivation might be more GR-concentration dependent than through transrepression. Malchoff and Malchoff (30) already speculated that alterations of the promoter region or factors modulating gene expression, leading to fewer GRs, could cause CR.

Patients 4, 5, and 6 had hypercortisolism without Cushingoid features, insufficient suppression of early morning serum cortisol concentrations in reaction to 1-mg DEX, variable degrees of androgen overproduction, and fatigue. Cellular sensitivity at the level of transactivation of GILZ was significantly reduced in patients 4 and 5 (Figure 2) and on transrepression of IL-2 also in patients 5 and 6 (Figure 2). GR expression levels and characteristics were normal (Figures 1 and 3), suggesting that the condition of the patients was not caused by reduced GR expression, as might be the result of mutations in the promoter region of the GR gene. However, GR mRNA copy numbers and DEX binding after EBV transformation was lower, which might suggest a defect in GR synthesis or regulation that only becomes apparent in these lymphoblasts. Possible pathophysiological bases of CR in these patients could also be formed by alterations in cellular trafficking or in interactions with other nuclear cofactors. The ER22/23EK SNP in patient 4 is reported to slightly decrease GC sensitivity (26) but could not be exclusively responsible for the severe reduced sensitivity as described in this study. At present, hyperactivity of the HPA axis was normalized by low doses of DEX.

Patient 7, who only needed low immunosuppressive medication for a postmortem donor kidney transplant, was suspected of increased immunosuppressive function of

the HPA axis. Sequence analysis of the GR gene revealed (among other mutations) a heterozygous C to G mutation in the pyrimidine tract of the exon 9α splice acceptor. Splice site analysis (https://splice.cmh.edu) predicted that the strength of the acceptor splice site is slightly weakened, possibly resulting in skipping of exon 9α in favor of exon 9β. However, this is not an absolute effect, because at the level of the mRNA, another heterozygous mutation in this patient (P750P in exon 9α) was also found to be present. But quantitative RT-PCR did show that the GR-β expression was three to four times higher than in controls, both in PBMLs and in lymphoblasts (Figures 1B and 3B). In the GILZ assay, cells from this patient showed a significantly reduced response, indicating reduced transactivating capacity, whereas in the IL-2 assay, the response was similar to that measured in controls. Our hypothesis is that in this patient, the reduction of transactivating capacity, possibly due to increased expression of GR-β, results in CR at the level of GRE-mediated GR action (also involved in the feedback sensitivity of the HPA axis), whereas the immunosuppressive function (not GRE mediated) is not affected. As a result, the immune system is exposed to higher compensatory cortisol concentrations and is subsequently relatively suppressed. Increased GR-β levels have frequently been associated with acquired GC resistance in various disease states (e.g. asthma, rheumatoid arthritis); however, increased GR-β levels in this particular patient may have resulted in positive effects.

In patient 8, transactivation and transrepression activities were decreased (Figure 2), whereas GR characteristics were normal (Figure 1). Extraordinarily high doses of both GCs and MCs were needed to overcome his 21-hydroxylase deficiency. However, this was still insufficient to fully compensate and normalize adrenal function because 17-OH-progesterone, androstenedione, testosterone, and ACTH plasma levels remained elevated. Cofactors influencing both the GR and the MC receptor (MR) activity could be involved, but then, androgen and thyroid receptor function might also be impaired because many coactivators are involved in the functioning of more than one nuclear receptor. Plasma TSH and T_3 were within the reference range, indicating normal thyroid function. Furthermore, plasma LH and FSH were fully suppressed by the elevated testosterone, indicating that androgen receptor function is not impaired either. It is not clear whether cofactors exist that specifically interact with the GR and MR, without influencing other nuclear receptors. Recently, differences between splice variants of steroid receptor coactivator-1 have been reported that strongly interact with GRs and MRs in a promoter-, receptor-, and ligand-dependent way (31). Disturbances in splicing regulation or tissue-specific expression of these cofactors could have dramatic influences on the cellular GC sensitivity, whereas other nuclear receptor activities might hardly be affected.

The increased sensitivity in patient 9 is mainly due to an increased receptor number (Figures 1 and 3) and slightly increasing cellular sensitivity (Figure 2). Cofactors inducing GR expression or alterations in the promoter region of the GR could be responsible for this. After stopping steroid medication that was used to

treat her asthma, the Cushingoid features disappeared.

Viral transformation had no influence on GR quality because similar receptor affinities (K_D) were found for native and transformed cells (Figures 1 and 3) but increased GR number 5-fold. Interestingly, in lymphoblasts of patients diagnosed as CR, induction was less, whereas in the patient diagnosed as CH, a higher GR number than in controls was measured (Figure 3). Tomita *et al.* (24) already reported that CR patients (from the D641V kindred) showed diminished induction during viral transformation. The molecular mechanism explaining this phenomenon, however, is still unknown. We hypothesize that during viral transformation, autoregulation of the GR occurs that might be impaired or enhanced by CR or CH, respectively. Although this phenomenon limits our possibilities to study the GR in its signaling context, due to noncomparable GR concentrations, the abnormalities in GR up-regulation in lymphoblasts of patients might be an additional indicator of altered GC sensitivity. Plotting GR number per lymphoblast against GR mRNA copy number (Figure 4) grouped all patients diagnosed as CR into the lower left sector, regardless of the molecular basis of the defect. Compared with measuring GR characteristics and GC response in freshly prepared cells, EBV transformation is more laborious. However, measuring GR up-regulation during EBV transformation seemed to be the most powerful tool to differentiate CR and CH from controls. The other markers (GILZ, IL-2, and proliferation) are easier to obtain, but as individual markers, they are less powerful because they sample distinct aspects of GC sensitivity (transactivating, transrepressing capacity but also proliferation processes, including apoptosis), which can differ strongly between patients.

In conclusion, for the appropriate diagnosis of CR or CH, a careful interpretation of clinical presentation is essential, but it is subsequently also important to quantify these syndromes biochemically. To do this, we have investigated GR characteristics and GC response using freshly isolated PBMLs and permanent cell lines. The results of these approaches are illustrated in this study by describing nine patients with suspected abnormalities in GC sensitivity, all with a unique clinical course and background.

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Chapter 8
General Discussion

The glucocorticoid (GC) receptor (GR) is the key-modulator of the GC signaling pathway. In the ligand activated form, the GR acts as a transcription factor, influencing gene expression in virtually every cell-type of the body. The consequent *de novo* synthesis of new proteins, but also the downregulation of other proteins influences many processes in the body (1).

Cortisol, the endogenous GC in humans is secreted by the adrenals under the influence of the hypothalamic-pituitary-adrenal axis (HPA-axis) that is activated by the circadian rhythm, but also by physical and psychological stress. The balance between production and need is maintained by the inhibitory feedback of cortisol on the hypothalamus and pituitary (2).

Synthetic GCs are used therapeutically for numerous indications. However, due to their broad physiological effects across many systems, side effects of GC therapy can be extensive and limit the clinical utility of GCs as a drug (3).

One of the main urgent questions at this moment is to develop insights into the cause of the differences in the response between individuals to therapeutically applied GCs. Some patients respond to low doses, with or without side effects, while others do not respond at all. This thesis discusses a number of possible explanations for these differences in GC sensitivity and is focused on genetic, but also on transcriptional and translational aspects of the GR gene. An important tool in these studies has been a newly developed bioassay, measuring cellular GC sensitivity *ex vivo,* based on GR action at the transcriptional level by studying GC-regulated mRNA expression.

8.1 A novel bioassay to measure cellular GC sensitivity ex vivo

There are only a few bioassays available that allow us to study variability in GC sensitivity and these are summarized in Table 1 (4-8). New techniques for quantifying mRNA expression provided us with possibilities to develop a novel bioassay to measure cellular GC sensitivity, based on GC responsive gene expression, which is described in chapter 2. Alterations in gene expression were usually studied by using Northern blots or nuclear transcription assays, but these methods are not very sensitive and semi-quantitative at best. Quantitative real-time RT-PCR allowed us to quantify changes in mRNA transcript levels very specifically, and we used it to investigate the inter-individual differences in GR-mediated gene regulation. We developed assays to measure GC sensitivity based both on GC-transactivation and GC-transrepression. As a prototypical gene whose expression is upregulated by GCs, we chose GILZ (9). Transactivation of this gene occurs via direct interaction of the GR with different GC response elements (GREs) in the promoter region (9). Similarly, IL-2 was chosen as an example of a gene that is strongly downregulated by GCs. This cytokine is, like other genes involved in the inflammatory response, regulated

by the NF-κB and AP-1 transcription factors, having their own binding sites in the promoter region of the IL-2 gene. These transcription factors could be inhibited by liganded GR through direct protein-protein interaction (10).

These mRNA transcription studies demonstrated that in the healthy population a wide variation in interindividual sensitivity to GCs exists, while intraindividual sensitivity is rather stable. This intraindividual stability over time was also reported in several other studies and has also been described to be independent of age and sex (11, 12). We also found (chapter 2) that the maximal expression of GILZ and IL-2 induced by pharmacological doses of GCs (E_{max}) is a more reliable variable to categorize individuals into GC-sensitive and GC-resistant groups than the variable that shows the concentration of half-maximal expression (EC_{50}). Therefore, in chapter 3 and 7, E_{max} values were used instead of EC_{50} values to explore changes in GC sensitivity caused by polymorphisms (chapter 3) and disturbed GC sensitivity in disease states in which GC sensitivity is affected (chapter 7).

Table 1: Bioassays to study variability in GC sensitivity

* Skin blanching assay (5)
GCs on skin cause vasoconstriction. The resulting white spot should correlate with the GC sensitivity of the exposed individual.

* DEX suppression assay (6)
The degree of DEX-induced suppression of endogenous cortisol levels via the strong HPA-axis feedback system is an index of an individual's GC sensitivity.

* Proliferation assay (7)
Mitogen stimulated proliferation of T-lymphocytes, which could be measured by incorporation of [^{3}H]-thymidine, could be dose-dependently inhibited by DEX. E_{max} and EC_{50} values could serve as an index of an individual's GC sensitivity.

* Cytokine production assay (8)
The release of the proinflammatory cytokines IL-6 and TNFα from monocytes and macrophages, measurable by RIA's could be dose-dependently inhibited by DEX. E_{max} and EC_{50} values could serve as an index of an individual's GC sensitivity.

* FKBP51 mRNA expression assay (4)
The dose-dependently activated expression of FKBP51 mRNA by GCs could be quantified by real-time RT-PCR and the resulting E_{max} and EC_{50} values could serve as an index of an individual's GC sensitivity.

These novel expression assays offer several advantages compared to the proliferation assay that also measures cellular sensitivity. The most important one is that measuring the regulation of GC responsive genes is a primary, direct result of GC action, while the outcome of the proliferation assay is formed by the integration of many processes, including apoptosis, due to a much longer time-scale (4 days, rather than 4 h in the expression assay). Furthermore, in the expression assay both the suppressive and stimulatory action of GCs could be explored. It is generally thought that transactivation is the predominant mechanism by which GCs exert many of their metabolic and cardiovascular actions, and that these actions are mainly responsible for their side effects (13), while the intended beneficial effects are exerted through both activation and inhibition of gene expression by GCs, which can be either immunostimulatory or immunosuppressive (14, 15). Conventional

GCs do not discriminate between desired and adverse effects and understanding the transcriptional basis of the different physiological (side) effects is important because it may be possible to develop more selective agents, either steroids or other agents, which mainly target the inflammatory process.

Recently however, various studies reported that GILZ also has immunosuppressive activity (16-19). The immunosupressive effect of activated GR, leading to downregulation of inflammatory genes is described to occur at different levels (see Figure 1). GILZ could directly repress the transcriptional activity of NF-κB and AP-1 by inhibiting their binding to DNA (16, 17), but could also interact more upstream in the inflammatory pathway by sequestering the MAPK-kinase-kinase Raf, consequently impairing AP-1 activation (18) (Figure 1). This interference of GILZ with processes determining inflammation indicates that GC-mediated downregulation of

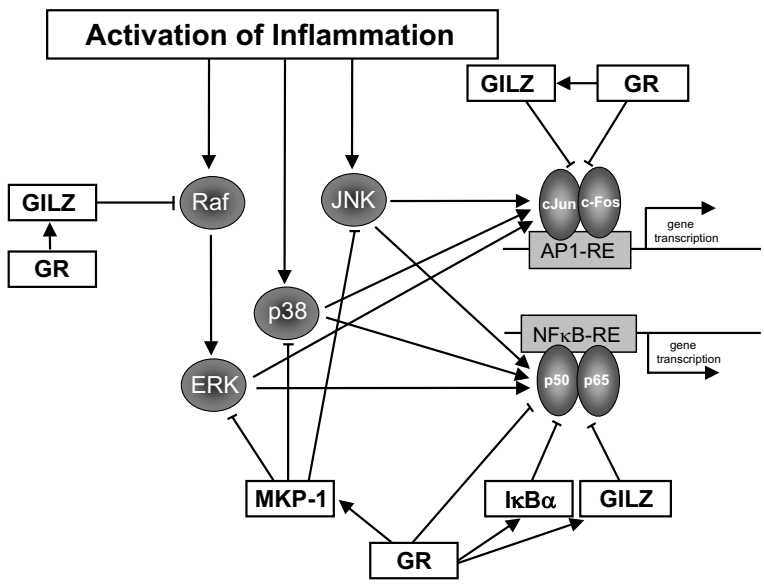

Figure 1 The immunosupressive effect of activated GR, leading to downregulation of inflammatory genes. Liganded GR can suppress inflammation in different ways. Firstly, liganded GR interacts with AP-1 complexes (generally c-Jun/c-Fos heterodimers) and prevents their transcriptional activity. Similar tethering is reported to occur with NF-κB. Secondly, liganded GR regulates expression of mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) by GRE-mediated upregulation and the attenuation of proteasomal degradation. MKP-1 inactivates MAPKs such as p38, c-Jun N-terminal kinase (JNK), and extracellular regulated kinase (ERK), which are key-activators in the pathways that stimulate inflammation. Thirdly, NF-κB is sequestered in the cytoplasm with an inhibitor, IκBα, whose production could be induced by GCs. The upregulation of MKP-1 and IκBα indicates that inflammation is not only repressed by direct protein-protein interaction, but that also GRE-mediated *de novo* protein synthesis is involved. GC-inducible leucine zipper (GILZ) has immunosuppressive activity by directly inhibiting the activity of AP-1 and NF-κB, or indirectly by inhibiting the activity of Raf.

IL-2 and upregulation of GILZ expression is more interlaced than we initially thought. Nevertheless, we consider these GC responsive genes as reliable, independent parameters to distinguish transactivation from transrepression. Regulation of mRNA levels by DEX has already been measured after 4 hours stimulation, and is therefore considered as a primary effect. Furthermore, in the expression experiments in chapter 2, 3, 5 and 7, no correlation between GILZ and IL-2 expression were found, indicating that induction of GILZ and repression of IL-2 did not interfere. Extending our panel of GC-responsive genes would be desirable to further explore inter-individual differences in GC sensitivity, but the response of a number of candidate genes, which have not been further specified in this thesis, was not sufficient to be useful.

8.2 Functional polymorphisms in the GR-gene modulate GC sensitivity

The large variation in interindividual sensitivity to the action of GCs described in chapter 2 is partly the result of minor genetic variations (mostly single nucleotide polymorphisms (SNPs)) in genes of various proteins that are involved in GC signaling. The GR gene itself is the most obvious candidate to study this genetic basis. Currently (January 2006) 387 polymorphisms in the GR gene are described in the SNP database (dbSNP (www.ncbi.nlm.nih.gov)) and we decided, based on frequency, possible functionality, and previous data in the literature, to explore the *TthIII*I, a restriction fragment length polymorphism (RFLP) in the promoter region, ER22/23EK, two linked single nucleotide polymorphisms of which the second results in an aspartic acid to lysine amino-acid change, N363S, a SNP involving an asparagine to serine amino-acid change, *Bcl*I, an RFLP in intron 2, and 9β, a polymorphism lying in the 3' untranslated region of exon 9β (Figure 9, Chapter 1).

Initially dexamethasone (DEX)-suppression tests *in vivo* were performed to investigate if a different response in sensitivity to GCs could be detected between the carriers and noncarriers of these polymorphisms. Carriers of the N363S (20) and the *Bcl*I (21) polymorphisms showed and increased response to DEX, indicating that these polymorphisms increase the sensitivity to the effects of GCs (20, 21), while in carriers of the ER22/23EK polymorphism a decreased response to DEX was reported, indicating relative resistance to GCs (22). In chapter 5, we described that the response to DEX in carriers of the 9β polymorphism did not differ between carriers and noncarriers, just as was found for the *TthIII*I polymorphism (23).

These findings provided a rationale for exploring the hypothesis that there would be an association between GR polymorphisms and other parameters that are affected by GCs, such as body composition and metabolic parameters (insulin and lipids). As expected, associations were found between the N363S and the *Bcl*I polymorphisms and several measures of increased GC response (more body fat, a stronger insulin

response to dexamethasone and less lean-body mass) (20, 21), while the ER22/23EK polymorphism was associated with measures of decreased GC effects (lower insulin levels, lower cholesterol levels and beneficial changes in body composition). The 9β polymorphism did not seem to affect the *in vivo* response to DEX in the 1-mg overnight DST. However, it was shown to be associated with decreased sensitivity to GCs but primarily concerning GC regulation of the immune system (less GR-mediated immunosupression) (Chapter 5). The *TthIII*I polymorphism could not be associated with any anthropometric parameter, nor with glucose and insulin concentrations or lipid levels, but it was shown to be in linkage disequilibrium with the ER22/23EK polymorphism (23).

An important feature of association studies is that there should be a biological plausibility and testing the effects of a polymorphism *in vitro* can help to distinguish between functional and non-functional polymorphisms. In chapter 3 and 5 we describe that the ER22/23EK, N363S and 9β polymorphisms could influence GR action. In chapter 3 we describe how these polymorphisms exert their effects on transcriptional regulation *in vitro* by testing their impact on transactivating capacity on a GRE promoter and on transrepression of NF-κB activity, while in chapter 3 and 5 the influence of these polymorphisms on upregulation of GILZ and downregulation of IL-2 mRNA expression were investigated *ex vivo*. We concluded that transactivation is reduced in ER22/23EK and increased in N363S carriers, while no effect on transrepression of both polymorphisms was detected compared with the control group. This is consistent with association studies performed, which showed that there is reduced sensitivity to GCs in carriers of ER22/23EK and increased sensitivity to GCs in carriers of N363S *in vivo*. Upregulation of GILZ in homozygous 9β carriers was shown to be unaltered compared to the control group, while diminished transrepression of the IL-2 gene was observed, which explains the associations found on immunosupression and not on transactivation. This also includes the DEX response, because feedback on the HPA-axis is mainly thought to occur via nGREs (chapter 5)(24, 25).

The location of the polymorphism in a gene is important with respect to function. For example, the N363S polymorphism changes asparagine to serine, which creates a potential phosphorylation site that might be relevant for DNA binding by the GR (26, 27). However, this serine residue is not in a known consensus site for phosphorylation (28). Moreover, the effects of GR phosphorylation are not very clear and the molecular mechanism how the N363S polymorphism exerts its function remains to be elucidated. By contrast, we elucidated the molecular mechanism through which the ER22/23EK polymorphism reduces sensitivity to GCs and this is described in chapter 4. Yudt *et al*. (29) reported that at least two methionine initiation codons in the GR mRNA, AUG-1 and AUG-27, result in two translation variants, GR-A (94 kDa), which is the full length GR of 777 amino acids and GR-B (91 kDa) of 751 amino acids. The latter protein was reported to have a stronger transactivating effect in transient transfection experiments (29). In chapter 4, we showed evidence

Table 2 The influence of genetic GR gene polymorphisms on *in vivo* and *ex vivo* GC sensitivity and phenotype.

GR gene	GR protein	*In vitro*	*In vivo*	Phenotype
ER22/23EK (GAGAGG → GAAAAG)	GR-A ↑ GR-B ↓ Chapter 4	*Trans*activation ↓ *Trans*repression = Chapter 3	Response to 1 mg DEX ↓ Change in cortisol after 1 mg DEX ↓	-Healthier metabolic profile (insulin↓, total and low-density lipoprotein cholesterol↓ and C-reactive protein levels↓) -Beneficial effects on body composition -Risk of dementia and cerebral white matter lesions ↓ -Longevity ↑
N363S (AAT → AGT)	Phosphorylation state ↑ ? or ↑ Interaction with co-factors ?	*Trans*activation ↑ *Trans*repression = Chapter 3	Response to ¼ mg DEX = Change in cortisol after ¼ mg DEX ↑	-More body fat -Less lean mass -Hypersensitive insulin secretion -Increased cholesterol levels -Higher risk of depression
9β (ATTTA→ GTTTA)	Stability GR-β ↑	*Trans*activation = *Trans*repression ↓ Chapter 5	Response to 1 mg DEX = Change in cortisol after 1 mg DEX =	-More active immune system Chapter 5 -Worse cardiovascular profile

that the sensitivity to GCs in carriers of ER22/23EK is lower because more of the longer, less transcriptionally active GR-A isoform is formed and that the reduction in transactivating capacity of the GR in carriers of ER22/23EK is explained by the change in the ratio GR-A:GR-B. We suggested that this polymorphism influences translation by altering the secondary structure of GR mRNA, forcing more GR-A to be initiated from AUG-1 at the expense of initiation from AUG-27. Transinhibition seems to be unchanged because the isoforms are equally potent at inhibiting the transactivating activity of NF-κB. The location of the 9β polymorphism is in the 3' untranslated region and does therefore not affect the structure of the GR protein. The molecular mechanism through which the 9β polymorphism exerts its effect was described by De Rijk *et al*. (30). *In vitro* data showed that the 9β polymorphism leads to more stable mRNA encoding GR-β (30, 31), a dominant negative inhibitor of the functional active GR-α (32). We postulated in chapter 5 that increased GR-β levels decrease GR mediated repression of inflammation, directly on NF-κB and AP-1 activity. We have not further investigated the functional effects of the intronic *Bcl*I polymorphism. Often, intronic polymorphisms are considered to be non-functional because they do not change the coding sequence. However, they might still be involved in the splicing process by, for example, changing the sequence of so-called intronic splicing silencers and enhancers, and through other mechanisms that are important for gene expression (33). Alternatively, a non-coding polymorphism could be linked to another functional polymorphism. An example is the *TthIII*I polymorphism in the promoter region of the GR gene. Originally, this polymorphism with allele frequency of 38% has been reported to be associated with increased diurnal cortisol levels (34), However, the ER22/23EK polymorphism (allele frequency of 3%) is without exception linked to the *TthIII*I polymorphism. The *TthIII*I polymorphism seemed not to be functional on its own and the associations with decreased GC sensitivity are

fully attributed to the ER22/23EK polymorphism. This indicates that the effects of the ER22/23EK polymorphism are strong enough to be also detectable in the much larger group of all *TthIII*I carriers (allele frequency 38%).

The main outcomes on *in vivo* and *ex vivo* GC sensitivity, their (putative) mechanism of action, as well as the phenotypic consequences of the ER22/23EK, N363S and 9β polymorphisms are summarized in Table 2.

8.3 Splice variants that modulate GR sensitivity

Differences in the 3'-splicing process could also contribute to individual differences in GC sensitivity. At the time it was cloned, two variants of the GR were found (35). The normal, hormone binding variant, GR-α, that uses exon 9α and GR-β that uses exon 9β through using an alternative splice site in exon 9. Due to its specific C-terminal part of 15 amino acids, GR-β is not able to bind ligand (36). A second alternative splice variant is called GR-P and is the result of failure to correctly remove intron 8, resulting in the introduction of a premature stop codon. This variant is also not able to bind ligand (37, 38). We analyzed the mRNA expression of these 3' variants, GR-α, GR-β, and GR-P in the 9 patients described in chapter 7 and tried to correlate the expression pattern with their GC sensitivity disorders. GR-α was increased in a patient who showed a hypersensitive response to GC medication (patient 9), while increased GR-β expression levels were measured in a patient diagnosed as GC resistant (Patient 7). Expression of GR-α, and GR-P mRNA was reduced to 50% in the patient heterozygous for a Δ4bp deletion at an exon-intron boundary (patient 3), but no abnormal expression patterns were detected in the other patients.

8.3.1 GR-β

A large amount of data exists on the subject of the GR-β splice variant, but is not always consistent. This inconsistency resulted in 1999 in two articles in Trends in Endocrinology and Metabolism, describing, from two viewpoints, the arguments for and against the existence of a functional role for GR-β (39, 40). The conflicting data extend to all aspects of GR-β. The mRNA expression levels reported for GR-β vary from virtually zero (41-44) to levels comparable with that of the GR-α mRNA (32, 45). Immuno-histochemical detection of the GR-β protein produced very mixed results (46-51), and in transient transfection experiments some authors found considerable dominant negative effects of co-transfected GR-β (32, 45, 49, 52, 53), while others found none (48, 50, 54, 55). A summary of the literature dealing with this type of experiment is presented in Table 3. Most of the reports showing dominant-negative effects of GR-β described these effects only for transactivation (Table 3), but there is one article also describing reduced transrepression by GR-α in the presence of co-

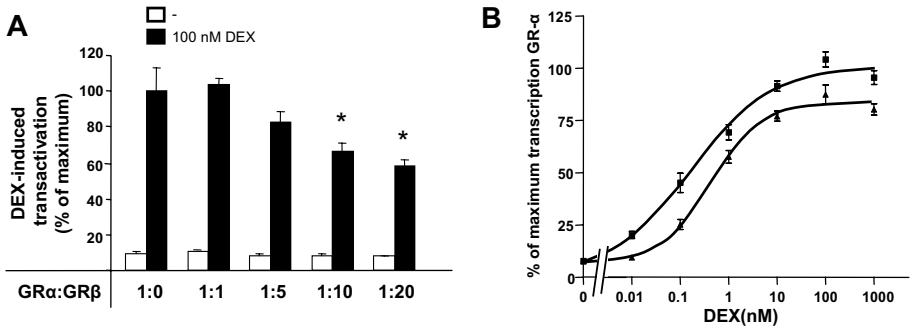

Figure 2 The transdominant negative effect of GR-β on the GR-α-mediated transactivation of the GRE-LUC gene. COS-1 cells were transfected with constant amounts of the GR-α coding plasmid pcDNA3GRα (7.5 ng), constant concentrations of the reporter plasmids GRE-LUC (50 ng) and CMV-RL (2 ng) and **(A)** increasing concentrations (0-150 ng) or **(B)** constant concentrations (60 ng) of pcDNA3GRβ. In addition, the pcDNA3 plasmid was co-transfected to obtain a constant concentration of expression plasmid. Five hours after transfection, cells were treated **(A)** with 100 nM (filled bars) or without (open bars) DEX or **(B)** with increasing concentrations of DEX as indicated. Data are expressed relative to the activity of GR-α without co-transfected GR-β, which was set to 100%. Data represents the mean ± S.E.M. of four transfections carried out in triplicate. *, $p<0.05$.

transfected GR-β (49). Furthermore, the dominant negative effect of GR-β was only measurable when the concentration of the GR-β expression plasmid was at least 5 times higher than that of the GR-α expression plasmid, with one exception (53), where dominant negative effects were already observed when GR-α and GR-β were equally expressed. This was also achieved with other GCs than DEX. The extent of the effect of GR-β on GR-α varied highly between the studies. Bamberger *et al.* (32) showed almost complete repression of GR-α activity when five times more GR-β was expressed than GR-β, while a reduction of only 30% was achieved in the study of Gougat *et al.* (52). These discrepancies and the wide range of GR-β action in the studies presented in Table 3 are difficult to explain. However, as can be seen that there is much variation in the experimental methods used. Experimental factors that might influence the results are: the transfection method, the duration of the transfection, the promoter used to drive the expression plasmid, the 5'- and 3'-untranslated regions of the GR cDNA clones, the cell type used and squelching of co-factors by the excessive amounts of expression plasmid used.

As shown in Table 3 and in contrast to earlier experiments in our laboratory (54) we have also been able to demonstrate a (modest) inhibitory effect of GR-β on GR-α mediated transactivation, albeit only at GR-β concentrations that were 10 to 20 times higher than GR-α (Figure 2A). This discrepancy may be explained by the use of a different transfection agent. These results are not described elsewhere in this thesis.

Despite all this confusion, there is a considerable amount of work describing

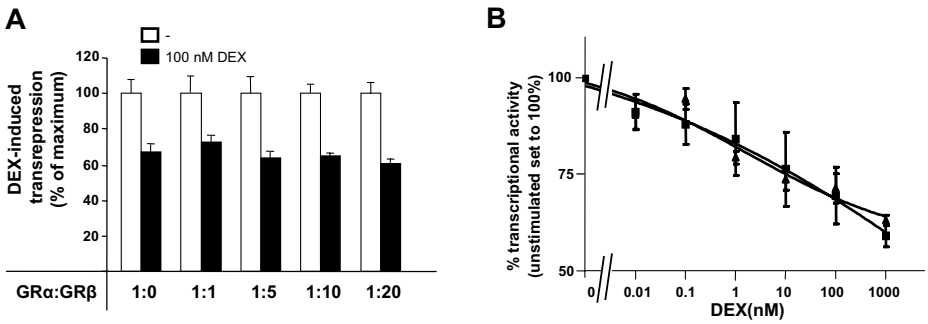

Figure 3 No effect of GR-β on the GR-α-mediated transrepression of the NF-κB-RE-LUC gene. COS-1 cells were transfected with constant amounts of the GR-α coding plasmid pcDNA3GRα (4.0 ng), constant concentrations of the reporter plasmids 5xNF-κB-RE-LUC (50 ng), p65 (10 ng) and CMV-RL (2 ng) and (A) increasing concentrations (0-150 ng) or (B) constant concentrations (60 ng) of pcDNA3GRβ. In addition, the pcDNA3 plasmid was co-transfected to obtain a constant concentration of expression plasmid. Five hours after transfection, cells were treated (A) with 100 nM (filled bars) or without (open bars) DEX or (B) with increasing concentrations of DEX as indicated. The activity of GR-α without DEX treatment for each condition was set to 100% and represents the mean ± S.E.M. of four transfections carried out in triplicate. *, $p<0.05$.

the association of (increased) GR-β levels with GC insensitive states of various diseases (47, 56-62), mainly diseases with a strong autoimmune component. The general conclusion from these studies was that GR-β hampered GR-α in its immune-suppressive action, resulting in increased activity of the immune system. Having found similar associations ourselves (association of 9β polymorphism with less immune suppression in chapter 5, and GC resistance in patient 7 described in chapter 7 possibly due to increased GR-β levels), we set out to investigate if we could also show interference of GR-β with the GC-mediated suppression of NF-κB directed gene expression, a process generally assumed to be the most important immune-suppressive pathway of GC-action (63, 64). As shown in Figure 3, we could not detect effects of GR-β on GR-α mediated transinhibition of an NF-κB response element in this experimental setup (not further described in this thesis). NF-κB activity was induced by co-transfecting p65 plasmid and not by inducing inflammation endogenously. However, some studies stimulated inflammation endougenously (50, 52, 55) and were also not able to detect any effect. Table 3 shows that only Oakley *et al.* (49) detected a dominant negative effect of GR-β but this study included no internal control to correct for variations in transfection efficiency. Without such control a change in transfection efficiency may be misinterpreted as a regulation of NF-κB activity.

In vivo, GR-β is widely, but only marginally expressed compared to GR-α, questioning its inhibitory influences on total cellular GC sensitivity. Further research to this 3' splice variant is warranted to fully understand its function and to judge if GR-β is a critical factor playing a role in determining interindividual differences in GC sensitivity in health and disease.

Table 3 Studies on effect of GR-β on GR-α-mediated transactivation and transrepression

Author Year (Ref)	Experimental Setup					Transdominance GR-β	
	Cell Type	Transfection Method	Expression Plasmids (µg)	Reporter Plasmids (µg)	Experimental Media T.P. / I.P. Post DEX	on Transactivation (% effect at (GR-α:GR-β))	on Transrepression
Bamberger *et al.* 1995 (32)	COS-7	DOTAP*	pRS-hGRα (1µg) pRS-hGRβ (0-15 µg) pRSV-erbA (to 23 µg)	pSV-β-gal (2 µg) pMMTV-LUC (5 µg)	0-48h/24-48 h 100 nM DEX	+ (-90%, (1:10))	n.d.
Oakley *et al.* 1996 (45)	HeLa-S_3	Calcium Phosphate Precipitation	Endogenous GRα pCMV-hGRβ (1-3 µg) pCMV- (1-3 µg)	pGRE$_2$CAT (5.0 µg)	0-40h / 16-40h 100 nM DEX	+ (large, endogenous)	n.d.
Hecht *et al.* 1997 (48)	COS-7	DOTAP*	pMT-hGRα (0.2 µg) pRS-hGRβ (0-1.6 µg) PRS-hGRβΔSalI	pMMTV-SALP (1.5 µg)	0-6h / 24-48 h 1 µM DEX	-	n.d.
Bamberger *et al* 1997 (55)	PBMLs	Electro-poration	pRS-hGRα (5 µg) pRS-hGRβ (25 µg)	pGL3-GRE-tk81-LUC (10 µg)	0-12h / 12-20h 100 nM DEX	-	
	Jurkat	Electro-poration	TPA/ionomycin – Induced endogenous IL-2 pRS-hGRα (5 µg) pRS-hGRβ (0-25 µg) pRSV-erbA (0-25 µg)	pIL-2RE-LUC (10 µg)			-
De Lange *et al* 1999 (54)	COS-1	Calcium Phosphate Precipitation	pRS-hGRα (0.1 µg) pRS-hGRβ (0-1.0 µg) pRS- (0-1.0 µg) pTZ (up to 2 µg)	pMMTV-LUC (1 µg) GRE-LUC (1 µg)	0-24h / 0-24h 100 nM DEX	-	n.d.
Oakley *et al.* 1999 (49)	COS-1 COS-7 CV-1	Calcium Phosphate Precipitation	pRSV-hGRα (0.1 µg) pCMV-hGRα (0.1 µg) pCMV-hGRβ (0-1 µg) pCMV- (0-1 µg)	pMMTV-LUC (0.1 µg)	0-18h / 0-18h 100 nM DEX	+ (-60%, (1:10))	
	COS-1		pCMV-p65 (2.5 µg) pCMV-hGRα (2.5 µg) pCMV-hGRβ (0-25 µg) pCMV- (0-25 µg)	pMHC-CAT (2.5 µg)			+

Brogan *et al.* 1999 (50)	COS-7	Calcium Phosphate Precipitation	pcDNA3-hGRα (0.05 µg) pcDNA3-hGRβ (0-1.0 µg) pcDNA3- (0.5-1.05 µg)	pGRE$_2$-GAL (15 µg)	0-6h / 20-44h 150 nM DEX	-	
			TPA induced – Endogenous AP-1 pcDNA3hGRα (0.1 µg) pcDNA3hGRβ (0-1.7 µg) pcDNA3- (0-1.7 µg)	pTRE$_4$-GAL (0.4 µg)			-
			TNFα induced – Endogenous NF-κB pcDNA3hGRα (1 µg) pcDNA3hGRβ (1-10 µg) pcDNA3- (1-10 µg)	pNRE$_5$-GAL (4 µg)			-
Gougat *et al.* 2002 (52)	COS-1	Transferrin-* Polylysine	pRS-hGRα (0.1 µg) pRS-hGRβ (0-0.5 µg) pRSV-erbA (0-0.5 µg)	pHH-LUC (0.6 µg) pCMVβ-GAL (0.25 µg)	0-20h / 0-20h 100 nM DEX	+ (-30%, (1:5))	
	A549	Transferrin-* Polylysine	Endogenous GRα pRS-hGRβ (0.5 µg) pRSV-erbA (0-0.5 µg)	pHH-LUC (0.6 µg) pCMVβ-GAL (0.25 µg)		+ (-20%, (1:5))	
			TPA induced – Endogenous AP-1 Endogenous GRα pRS-hGRβ (0.5 µg) pRSV-erbA (0-0.5 µg)	Coll-LUC (0.6 µg) pCMVβ-GAL (0.25 µg)			-
			TNFα induced – Endogenous NF-κB Endogenous GRα pRS-hGRβ (0.5 µg) pRSV-erbA (0-0.5 µg)	3xIgκ-Cona-LUC (0.6 µg) pCMVβ-GAL (0.25 µg)			-
Fruchter *et al.* 2005 (53)	HepG2/ C3A	Lipofec-tamine 2000*	pRS-hGRα (? µg) pRS-hGRβ (? µg) pRSV-erbA (? µg)	pMMTV-LUC (? µg) pSV40-β-GAL (? µg)	0-6h / 30-46h 100 nM Dexamethasone Hydrocortisone Prednisolone Methylpredn. Triamcinolone Betamethasone	+ (-70% (1:1)) + (-62% (1:1)) + (-50% (1:1)) + (-22% (1:1)) + (-96% (1:1)) + (-50% (1:1))	n.d. n.d. n.d. n.d. n.d. n.d.
Russcher *et al.* (unpublished)	COS-1	FuGENE6*	pcDNA3-GRα (7.5 ng) pcDNA3-GRβ (0-150 ng) pcDNA3- (0-100 ng)	pGRE-LUC (50 ng) pCMV-RL (2 ng)	0-24h / 5-24h 0-1uM DEX	+ (-34% (1:10)) (-42% (1:20))	
			p65 (10 ng) pcDNA3-GRα (4.0 ng) pcDNA3-GRβ (0-150 ng) pcDNA3- (0-100 ng)	NF-κB-RE-LUC (50 ng) pCMV-RL (2 ng)			-

T.P. = transfection period I.P.-Incubation period with DEX n.d.=not determined *cationic liposome-mediated
+ = transdominant negative effect; - = no transdominant negative effect

8.3.2. GR-P

Not much is known about GR-P, except that it is expressed in many hematological tumors, but also in normal hematologic cells (41, 65), but otherwise not much attention is paid to this splice variant. However, studies in our laboratory strongly suggest that GR-P could modulate the effects of GCs in a cell type specific manner, by enhancing the activity of GR-α (65), but no further information is yet available. To investigate if and how GR-P could regulate cellular GC sensitivity, we tried to use the recently developed technique RNA interference (RNAi) to selectively silence the expression of the this splice variant, but these experiments were not very successful. We were not able to silence GR-P (nor GR-α or GR-β) in a selective and efficient manner without disturbing the expression of the other splice variants or other genes and had to conclude that silencing these variants, expressed from the same gene is a challenge. Indeed, a number of recent papers (e.g. (66)) indicate that the RNAi-technique may be more complicated than initially thought. However, these preliminary experiments could be used to follow the improvements of this technique and to develop protocols to successfully implement RNAi in our research to investigate factors that determine interindividual differences in GC sensitivity.

8.4 Modulation of GC sensitivity by the use of different promoters

Transcription of the GR gene not only results in mRNA transcripts that differ at their 3', but also in their 5' ends. The diversity in the 5' end, in contrast to the 3' end GR mRNA transcripts (GR-α, GR-β, and GR-P), does not affect the GR protein structure, since the differences are restricted to the untranslated region (UTR), which is composed of exon 1 and the first 13 nucleotides of exon 2. The expression of transcripts containing alternative exons 1A1, 1A2, and 1A3, which are splice variants of each other, is driven by promoter 1A, whereas alternative exons 1B and 1C are driven by promoter 1B and 1C, respectively (67). Promoters 1B and 1C are used in virtually all human cells and tissues and are responsible for the basal expression of GR, while promoter 1A is mainly used in cells of hematopoietic origin and is thought to play a role in GR autoregulation. In chapter 6, we described the relation between the 3' splice variants and the 5' variants of GR mRNA transcripts and hypothesized that the regulation of 3' end splicing could be associated with the use of the different promoters and exons 1. GR-α encoding transcripts are relatively more dependent on expression driven from promoter 1C than from 1B, while expression of GR-P is more controlled by promoter 1B. However, a semi-quantitative PCR experiment in which 5' specific primers for exons 1A, 1B and 1C were combined with 3' specific primers for exons 9α, 9β and P showed that this is not an absolute effect, because PCRs with the possible primer combinations all resulted in amplification products. Future experiments could focus on the relation between the 3' splice variants and

the 5' variants of GR mRNA transcripts and may confirm our findings described in chapter 6 that the regulation of 3' end splicing may be related to the use of the different promoters and exons 1. These experiments could include investigations how to influence transcription from either the 1B or 1C promoter by modulating the activity of certain specific transcription factors and, even more drastic, the consequences when a promoter sequence would be erased by making 1B and/or 1C knock-out cell-lines. Also polymorphisms in these promoter sequences could reduce or enhance promoter activity by abolishing or creating binding sites for transcription factors or transcription enhancer sites. The use of these different promoters and exons 1 might lead to new insights on how GCs regulate GR expression.

8.5 The extremes of variability in GC sensitivity

In most cases individual differences in GC sensitivity are only revealed upon GC treatment: some patients develop side effects on low dose GC treatment, whereas others fail to respond at all to high dosages.

However, in a small number of patients generalized GC resistance can lead to complaints. Cortisol resistance is characterized by a compensatory increase in HPA-axis activity leading to hypercortisolism which counterbalances the decreased GC sensitivity. This hypercortisolism is not accompanied by Cushingoid features, but does involve hyperandrogenism and/or hypermineralocorticoidism due to adrenal overactivity (7). Extreme hypersensitivity is characterized by signs and symptoms of Cushingoid syndrome in the absence of high endogenous cortisol levels (7). Absolute resistance to GCs is not compatible with life. GR knockout mice die at birth from severe respiratory disorders (68). In humans, the spectrum of clinical manifestations is broad (sometimes even asymptomatic, only showing biochemical changes) (69) and GC sensitivity disorders as described in chapter 7 might give strong insight into the physiological importance of hormonal actions of GCs and may provide clues to unknown important functions of these hormones.

In chapter 7, we characterized 9 patients with GC sensitivity disorders by studying the cellular effect of GCs at the level of mRNA expression of the GILZ and IL-2 gene, but also by analyzing GR expression and characteristics. We have also immortalized cells by transfecting B-lymphocytes with Epstein-Barr virus. This viral transformation leads to an upregulation of GR levels. However, in cells from patients diagnosed as GC resistant less upregulation of GR was shown than in cells from healthy controls. This phenomenon provided us a new strong tool to discriminate patients with GC sensitivity disorders from individuals without these disorders.

In four patients, the molecular basis for their GC resistance has been ascribed to mutations in the hormone binding domain of the GR gene. These mutations activated the pathophysiological mechanisms as summarized in the supplementary

table of chapter 7, leading to clinical manifestations of GC resistance. Three of these patients had been described before and the molecular cause of their GC resistance is very well understood. The GILZ and IL-2 mRNA expression in PBMLs of these three patients served as standard to explore disorders in the other patients.

In cells of a fourth patient (chapter 7; patient 7), we measured elevated GR-β levels, which might be caused by alterations in his GR gene sequence inducing altered splicing of exon 9 (9β instead of 9α). The consequent increase in GR-β levels might lead to decreased GC sensitivity. However, higher GR-β levels are always described to be associated with acquired GC resistance rather than with generalized GC resistance. Furthermore, this patient would probably be the first in whom increased GR-β levels resulted in beneficial effects rather than in GC resistant disease states. However, as also discussed in a previous paragraph, the physiological meaning of GR-β is not entirely clear yet and further research is needed to confirm this molecular explanation for the GC sensitivity disorder in this patient.

The other five patients that have been described in chapter 7 were also diagnosed with biochemical and clinical affected GC sensitivity, all with a very diverse clinical course and background. However, in these patients, no mutations in their GR gene were found that could have explained their condition. Alternative explanations could be formed by mutations in the GR promoter regions or in genes encoding proteins involved in the pre-ligand binding complex (e.g. heat shock protein 90), but neither changes in mRNA or protein expression levels, nor changes in ligand binding affinity were observed in these patients. Furthermore, New *et al.* (70) postulated that alterations in co-regulators of GR activity could also be responsible, however, many of them are involved in the functioning of more than one nuclear receptor and no differences in response to other steroid hormones in these patients have been found. Further research is needed to investigate if their affected GC sensitivity could be explained by abnormalities concerning these co-factors and their complex formation. A strong technique that could analyze and quantify the composition of these complexes could be the matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) technique.

8.6 Concluding remarks

As now insight is gained which factors (e.g. genetic factors, but also differential regulation of GR gene transcription/translation) could be responsible for inter-individual differences in GC sensitivity, a basis for further research is provided.

This thesis focused on the N363S, ER22/23EK, and 9β polymorphisms in the GR gene, which are shown to affect GC sensitivity *in vivo* and *in/ex vitro* and result in a wide variety of phenotypic signs (chapters 3, 4, and 5). Furthermore, studies described in chapter 4, 5, 6, and 7 have demonstrated that also transcriptional and

translational variants of the GR could modulate GC sensitivity. However, also the genetic background and expression of enzymes responsible for GC bioavailability, systemic absorption and pharmacokinetic handling are factors that could result in interindividual differences in GC sensitivity. GC action involves many proteins, from their synthesis to their eventual excretion, and in order to complete the picture of GC sensitivity it is clear that, apart from the GR, these other players need to be considered as well.

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Chapter 9

Summary
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Summary

As described in **chapter 1**, the major glucocorticoid (GC) in most mammals is cortisol and necessary for the correct functioning of almost every part of the body. Like other steroids, cortisol is generated in the adrenal glands by a process referred to as steroidogenesis. Secretion and the regulation of the amount of cortisol in the bloodstream is tightly controlled by the hypothalamic-pituitary-adrenal axis. Cortisol exerts its effects by binding to the GC receptor (GR). The GR modulates target gene transcription and hence protein expression via direct interaction with the DNA or indirectly via cross-talk with other DNA-bound transcription factors.

Various different GR isoforms exist in cells throughout the human body. The use of different promoters, alternative mRNA splicing, alternative translation, and posttranslational modifications results in a broad diversity of GR isoforms expressed from one single gene.

Synthetic GCs are used therapeutically for numerous indications. However, due to their broad physiological effects across many systems, side effects of GC therapy can be extensive and limit the clinical utility of GCs as a drug.

One of the main urgent questions at this moment is to develop insights into the cause of the differences in the response between individuals to therapeutically applied GCs. Some patients respond to low doses, with or without side effects, while others do not respond at all. This thesis discusses a number of possible explanations for these differences in GC sensitivity and is focused on genetic, but also on transcriptional and translational aspects of the GR gene. An important tool in these studies has been a newly developed bioassay, measuring cellular GC sensitivity *ex vivo*, based on GR action at the transcriptional level by studying GC-regulated mRNA expression.

In **chapter 2** we introduce this newly developed bioassay. As a prototypical gene whose expression is upregulated by GCs, we chose GC-induced leucinezipper (GILZ), while interleukin-2 (IL-2) was chosen as a model for genes that are downregulated by GCs. These transcription studies demonstrated that in the healthy population a wide variation in interindividual sensitivity to GCs exist, while intraindividual sensitivity in time is rather stable. The maximal expression of GILZ and IL-2 induced by pharmacological doses of GCs (E_{max}) is a more reliable marker to categorize individuals into GC-sensitive and GC-resistant groups than the variable that shows the concentration of half-maximal expression (EC_{50}).

In **chapter 3**, we describe the use of the expression bioassays to explore the influence of minor genetic variations in the GR gene. In our laboratory, we studied, among others, the ER22/23EK and N363S polymorphisms on cellular GC sensitivity. ER22/23EK significantly reduced transactivating capacity of the GR, while the presence of the N363S polymorphism led to increased transactivating capacity. No effects of both polymorphisms were found on transrepression. We concluded that the presence of these and other GC-sensitivity modulating polymorphisms may have

consequences for the use of GCs in a clinical setting.

Chapter 4 deals with the molecular mechanism of how the ER22/23EK polymorphism decreases the receptor's sensitivity to GCs. We investigated the influence of this polymorphism on the synthesis of GR-A and GR-B, two translational isoforms of the GR, of which the latter is more transcriptionally active. Different experiments showed that, when the ER22/23EK polymorphism is present, approximately 15% more GR-A protein was expressed, while total GR levels (GR-A + GR-B) were not affected. The transcriptional activity in GR(ER22/23EK)-carriers is decreased because more of the less transcriptionally active GR-A isoform is formed, which is probably caused by altered mRNA secondary structure.

Chapter 5 focuses on a third polymorphism in the GR gene. This polymorphism, 9β, is in the 3' untranslated region of the GR. The effects and associations of the 9β polymorphism are completely different from those found for the ER22/23EK polymorphism, while both polymorhisms are reported to decrease cellular GC sensitivity. No effects were found on body-mass index, waist-hip-ratio, fat spectrum and insulin sensitivity, nor in cortisol response on dexamethasone. However, the mean C-reactive protein levels in GR-9β carriers were significantly higher. When performing the expression bioassays to explore GC-mediated mRNA regulation, no effects on transactivation could be measured, while transrepression was decreased by the 9β polymorphism. This indicates that persons carrying the GR-9β haplotype, in contrast to ER22/23EK carriers, seem to have a decreased GC transrepression with normal transactivation.

In **Chapter 6** we describe how the expression of GR-α, GR-β and GR-P splice variants could partly be regulated by the use of multiple promoters. The GR gene is controlled by three different promoters: 1A, 1B and 1C. mRNA transcripts containing exon 1A are expressed from promoter 1A, while transcripts containing exons 1B or 1C are expressed from promoter 1B or 1C, respectively. In cDNAs of different tissues and cell-lines were used to investigate which part of GR transcripts that carry the different exons one, 1A, 1B or 1C encodes for the splice variants GR-α, GR-β and GR-P. Data in chapter 6 demonstrate, however not absolutely, that GR-α is more favorably expressed from the 1C promoter and that expression of GR-P is favored when the 1B promoter is used and that this is another way how cells and tissues can be sensitized to the different activities of the GR isoforms.

Extremes in sensitivity to cortisol are harmfull and **Chapter 7** is a description of our investigations of 9 patients with serious GC sensitivity disorders and offers several strategies for the characterization. Differences in GR number per cell, GR affinity, GR splice variants and effects on transactivation or transrepression of the GC sensitive genes GILZ and IL-2 were observed between these patients and healthy controls. To obtain permanent cell-lines of these patients, we immortalized B-lymphocytes by transforming them with EBV. This transformation had no influence on GR-affinity, but increased GR number 5-fold when lymphocytes of healthy controls were transformed. However, lymphocytes of patients diagnosed as cortisol

resistant showed a significantly less than 5-fold upregulation, while a higher GR number was observed in a patient suspected of cortisol hypersensitivity. Exploring GC sensitivity disorders as described in chapter 7 might give strong insight into the physical importance of hormonal actions of GCs and may provide clues to unknown important functions of these hormones.

Chapter 8 contains a general discussion about the findings described in this thesis. Broader insight is gained which factors are responsible for interindividual differences in GC sensitivity, with an important role for genetic variants, translational and transcriptional isoforms of the glucocorticoid receptor. Furthermore, plans for further research are proposed.

Samenvatting

In **hoofdstuk 1** is beschreven dat het meest voorkomende glucocorticoïd in zoogdieren en mensen cortisol is, wat een onontbeerlijk hormoon is om vele processen in het lichaam goed te laten functioneren. Net als andere steroïden wordt cortisol geproduceerd in de bijnieren door een proces wat steroïdogenese wordt genoemd. De afgifte en regulatie van de hoeveelheid cortisol in de bloedbaan wordt nauwkeurig gecontroleerd door de hypothalomo-hypofyse-bijnier as. Na het verlaten van de bloedbaan kan cortisol de cel binnendringen om daar aan zijn receptor te binden. In geactiveerde vorm transloceert deze glucocorticoïdreceptor naar de kern waar het de transcriptie kan beïnvloeden van tal van genen die coderen voor eiwitten die een rol spelen in de fysiologische processen.

In de cellen van het menselijk lichaam komen verschillende glucocorticoïdreceptoren voor. Het gebruik van verschillende promotoren, alternatieve mRNA splicing, alternatieve translatie en post-translationele modificaties leiden tot een grote diversiteit van glucocorticoïdreceptoren isovormen. Deze glucocorticoïdreceptoren isovormen worden allemaal gecodeerd door het glucocorticoïdreceptor gen, waarvan er per cel slechts twee (één maternale en één paternale) aanwezig zijn.

Naast het natuurlijke glucocorticoïd cortisol zijn er ook synthetische glucocorticoïden ontwikkeld. Deze synthetische glucocorticoïden worden gebruikt als medicijn om veel chronische, immuungerelateerde ziektebeelden te behandelen. Omdat glucocorticoïden zo'n brede functie hebben in het lichaam gaat de behandeling helaas vaak gepaard met erg veel bijeffecten, die kunnen variëren van matig tot zeer ernstig. In de gezonde populatie bestaat er een grote variatie in gevoeligheid voor GCs en hierdoor is de manifestatie van bijeffecten van glucocorticoïd behandeling in iedere patiënt anders. Sommige mensen ontwikkelen al ongewenste bijeffecten bij erg lage doses glucocorticoïden, terwijl anderen nagenoeg geen last ondervinden. Dit proefschrift gaat in op de vraag waarom er in de populatie zo'n verschil in gevoeligheid voor glucocorticoïden bestaat en richt zich hierbij vooral op genetische oorzaken, maar ook op transcriptionele en translationele aspecten van het glucocorticoïdreceptor gen.

Een belangrijk middel om meer meer inzicht te krijgen in de variatie in glucocorticoïd gevoeligheid is de ontwikkeling geweest van een nieuwe bioassay. Deze bioassay om per individu de cellulaire gevoeligheid voor glucocorticoïden te kunnen bepalen is beschreven in **hoofdstuk 2** en is gebaseerd op glucocorticoïd gemedieerde regulatie van genexpressie. Het glucocorticoïd-induceerbare leucine zipper (GILZ) gen is uitgekozen als model voor genen waarvan de expressie wordt geïnduceerd door de werking van glucocorticoïden, terwijl het interleukine-2 (IL-2) gen uitgekozen is als prototype gen voor genen die worden geremd door glucocorticoïden. Deze bioassay laat zien dat er inderdaad in de normale populatie een grote variatie in gevoeligheid voor glucocorticoïden bestaat. De intraindividuele

gevoeligheid, dus de gevoeligheid in één bepaald persoon daarentegen is behoorlijk stabiel. Hoofdstuk 2 laat verder zien dat de maximale expressie van GILZ en IL-2, geïnduceerd door farmacologisch hoge doses glucocorticoiden (E_{max}) een veel betrouwbaardere parameter is om individuen in glucocorticoïd gevoelige en ongevoelige groepen in te delen dan de parameter die de half maximale expressie voorstelt (EC_{50}).

In **hoofdstuk 3** hebben we de expressie bioassays gebruikt om de effecten van verschillende kleine genetische varianten van de glucocorticoïd receptor op de cellulaire gevoeligheid voor glucocorticoïden te analyseren. Twee genetische varianten die veelvuldig in ons laboratorium zijn onderzocht zijn het ER22/23EK en het N363S polymorfisme. Het ER22/23EK polymorfisme verlaagt de transactiverende capaciteit van de GR terwijl de aanwezigheid van het N363S polymorfisme tot een toename van de transactiverende capaciteit leidt. Beide polymorfismen blijken geen effect te hebben op de transinhiberende capaciteit van de receptor. We concluderen dat de aanwezigheid van deze en andere polymorfismen die een invloed hebben op glucocorticoïd gevoeligheid gevolg kan hebben voor het gebruik van synthetisiche glucocorticoïden als medicijn.

Hoofdstuk 4 beschrijft het moleculaire mechanisme waarmee het ER22/23EK polymorfisme de gevoeligheid voor glucocorticoïden kan beïnvloeden. We onderzochten de invloed van dit polymorfisme op de synthese van GR-A en GR-B. Dit zijn twee translatie isovormen van de glucocorticoïdreceptor waarvan GR-B transcriptioneel een hogere activiteit heeft dan GR-A. Deze twee isovormen hebben we onafhankelijk tot expressie gebracht van constructen met en zonder het polymorfisme en verschillende experimenten laten zien dat wanneer de ER22/23EK polymorfisme aanwezig is ongeveer 15% meer GR-A gevormd wordt bij gelijkblijvende totale hoeveelheid receptor (GR-A + GR-B). De glucocorticoïd gevoeligheid in individuen die drager zijn van het ER22/23EK polymorphisme is dus lager omdat deze mensen in verhouding meer van het minder transcriptioneel actieve GR-A isovorm aanmaken.

Hoofdstuk 5 beschrijft een derde polymorfisme in het glucocorticoïdreceptor gen waarnaar we in ons laboratorium veel onderzoek hebben gedaan. Dit polymorphisme, het 9β polymorfisme verlaagt ook de cellulaire gevoeligheid voor glucocorticoïden, maar de effecten en associaties van dit polymorfisme zijn compleet anders dan die gevonden zijn voor het ER22/23EK polymorfisme. In tegenstelling tot het ER22/23EK polymorfisme zijn er geen effecten gevonden op de body-mass-index, waste-hip-ratio, vetverdeling en insulinegevoeligheid. Echter, de gemiddelde C-reactieve eiwit niveau's, een indicator voor ontstekingen, zijn in dragers van het 9β polymorfisme significant hoger. Als een expressie bioassay wordt uitgevoerd met materiaal van 9β dragers zien we geen effect op GILZ mRNA expressie, maar wel een verminderde remming van IL-2 expressie. Dit betekent dat in tegenstelling tot ER22/23EK dragers, individuen die drager zijn van het 9β polymorphisme een verminderde glucocorticoïd gemedieerde gentransrepressie laten zien terwijl het vermogen om genen te

transactiveren niet veranderd is.

In hoofdstuk 6 beschrijven we hoe de expressie van bepaalde splice varianten van het glucortcoïdreceptor gen, GR-α, GR-β en GR-P gedeeltelijk gereguleerd kunnen worden door het gebruik van verschillende promotoren. Expressie van het glucocorticoïdreceptor gen staat namelijk onder controle van 3 verschillende promotoren, die 1A, 1B en 1C genoemd worden. mRNA transcripten die exon 1A bevatten, worden tot expressie gebracht onder invloed van promotor 1A, terwijl transcripten die de exonen 1B of 1C bevatten tot expressie worden gebracht onder invloed van respectievelijk promotor 1B of 1C. In experimenten die beschreven zijn in dit hoofdstuk werden cDNAs van verschillende weefsels en cellijnen gebruikt waarmee onderzocht werd of het gebruik van een specifieke promoter ook verantwoordelijk is voor de expressie van een bepaalde splice variant. Resultaten in hoofdstuk 6 laten zien, hoewel niet absoluut, dat GR-α meer tot expressie wordt gebracht onder invloed van promotor 1C terwijl GR-P meer tot expressie wordt gebracht onder invloed van promotor 1B. Ook op deze manier vindt dus regulatie plaats in gevoeligheid van verschillende weefsels en cellen voor de werking van glucocorticoïden.

In hoofdstuk 7 zijn negen patiënten beschreven met glucocorticoïd gevoeligheidsstoornissen en biedt verschillende strategieën om deze stoornissen biochemisch en klinisch te kunnen karakteriseren. Verschillen in glucocorticoïd-receptor aantal per cel, glucocorticoïdreceptor affiniteit, glucocorticoïdreceptor splice varianten en effecten op glucocorticoïd gemedieerde transactivatie en transrepressie zijn vastgesteld in deze patiënten. Om permanente cellijnen van deze patiënten te verkrijgen werden B-lymfocyten getransformeerd met EBV zodat geïmmortaliseerde cellen ontstonden. EBV transformatie van B-lymfocyten bleek geen invloed op de glucocorticoïdreceptor affiniteit te hebben, maar wel op het glucocorticoïdreceptor aantal, want deze nam vijfvoudig toe. Echter, patiënten die gediagnostiseerd zijn als cortisol resistent lieten een toename zien die minder was dan vijfvoudig, terwijl juist een hoger glucocorticoïdreceptor aantal werd vastgesteld in een patiënt die gediagnostiseerd was als glucocorticoïd hypergevoelig. Het onderzoeken van deze glucocorticoïd gevoeligheidsstoornissen, zoals beschreven is in hoofdstuk 7, kan inzicht geven in het fysiologische belang van de werking van glucocorticoïden en kan een belangrijke bron vormen om eigenschappen te ontdekken van deze hormonen, die tot nu toe onbekend zijn.

In **Hoofdstuk 8** worden de bevindingen die beschreven zijn in dit proefschrift bediscussieërd. Er is meer inzicht verkregen welke factoren verantwoordelijk zijn voor interindividuele verschillen in glucocorticoïd gevoeligheid. Hierin spelen de genetische, maar ook de transcriptionele en translationele varianten van de glucocorticoïdreceptor een belangrijke rol. Ook zijn plannen voor verder onderzoek voorgesteld.

Abbreviations

AC,	acetonide
ACE,	angiotensin-converting–enzyme
AR,	androgen receptor
BW,	backward
CV,	coefficient of variation
CH,	cortisol hypersensitivity
CMV,	cytomegalovirus
CR,	cortisol resistance
DEX,	dexamethasone
DP,	dipropionate
DST,	DEX suppression test
E2,	17β-estradiol
EBV,	Epstein-Barr virus
FW,	forward
GC,	glucocorticoid
GILZ,	GC-induced leucine zipper
GR,	GC receptor
GRE,	GC response element
h,	human
HPA,	hypothalamic-pituitary-adrenal
IGF-1,	insulin-like growth factor 1
nGRE,	negative GRE
HPRT,	hypoxanthine phosphoribosyltransferase
KD,	dissociation constant
LUC,	luciferase
MC,	mineralocorticoid
MPA,	6α-methyl-17α-hydroxyprogesterone acetate
MR,	MC receptor
mu,	mutant
NF-κB,	nuclear factor-κB
PBML,	peripheral blood mononuclear lymphocyte
PHA,	phytohemagglutinin
PR,	progesterone receptor
Q-PCR,	quantitative polymerase chain reaction
RFLP,	restriction fragment length polymorphism
SDS,	SD score
SNP,	single nucleotide polymorphism
TBS-T,	Tris-buffered saline with Tween 20
wt,	wild type
WHR	waist-hip-ratio
BMI	body mass index

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Annekee: tijdens mijn periode als promovendus werd je mijn vrouw, maar ook intenser betrokken bij al mijn promotieperikelen. We vonden het samen niet leuk als ik veel moest "schrijven", maar we vierden ook samen feest (met een gebakje) als er weer een artikel was geaccepteerd. Voor je liefde, intense betrokkenheid, overweldigende steun en ruimte die je me geeft ben ik je heel erg dankbaar!

Mijn promotor en ook mijn rector magnificus, prof.dr. S.W.J. Lamberts: Uw oneindig enthousiasme voor het onderzoek, stimulatie om verder te gaan, vertrouwen dat het allemaal goed zou komen en uw steun betreffende mijn verdere carrière hebben erg veel voor mij betekend. Uw allure heeft mij altijd het gevoel gegeven een bijzondere promotor te hebben!

Mijn copromotor en dagelijkse supervisor, Jan Willem Koper: "Weet je het wel zeker dat je in Rotterdam wilt wonen?", was één van de dingen die je me vroeg tijdens het sollicitatiegesprek. Misschien had je wat dit betreft wat weinig vertrouwen in iemand uit het provinciestadje Groningen, maar ik heb het uitstekend naar m'n zin (gehad) in het Rotterdamse! Jouw persoon heeft daar zeker aan bijgedragen. De goede begeleiding, je positief kritische invloed aangaande de mansucripten en de luxe dat de deur voor ieder wissewasje open stond zijn allesbepalend geweest voor een succesvolle periode op het lab Inwendige Geneeskunde!

Mijn opleider Klinische Chemie, prof.dr. Jan Lindemans: Hartelijk dank dat je deel uit wilde maken van de "leescommissie". Oud en nieuw komen zo prachtig samen en ik zie als aankomend "KC" uit naar een goede toekomst!
Prof.dr.ir. J. Trapman: Hartelijk dank dat u bereid was zitting te nemen in de kleine commissie en de snelle beoordeling van het manuscript.
Dr. Albert Brinkmann: Hartelijk dank voor je vakkundige beoordeling van mijn manuscript. Samen met prof.dr. Frank de Jong was je onmisbaar in het proces om de studies gepubliceerd te krijgen. Jullie jarenlange ervaring met steroïden, jullie opbouwende commentaar op mijn manuscripten en jullie inbreng tijdens de maandelijkse werkbesprekingen in de D-vleugel waren enorm insprirerend, waarvoor mijn hartelijke dank!

Mijn paranimfen, Liesbeth van Rossum en Erica van den Akker. Het was een voorrecht om met zulke intelligente mensen te hebben mogen samenwerken. Liesbeth: In het dankwoord van jouw proefschrift liet je weten dat ik je doorzettingsvermogen heb

geleerd. Wat ik van jou geleerd heb is dat ik trots mag zijn op mijn resultaten, en dat is denk ik nog veel belangrijker! Erica: We leerden elkaar eigenlijk pas beter kennen toen we na de verhuizing op één kamer kwamen te zitten. We hadden de meest serieuze gesprekken, maar ook de wildste hypotheses aangaande de werking van de GR, en vanwege onze verschillende achtergronden vulden we elkaar goed aan. Ook de gesprekjes over andere dingen dan alleen werk maakten de maandag, dinsdag en donderdag altijd erg gezellig.

Mijn overige "GR-collega's": Pauline, Richard, Antje, Marieke, Marieke, Ellen en Manon. Ongelofelijk eigenlijk dat zoveel mensen geboeid kunnen zijn door cortisol en haar receptor en ook bijzonder dat we allemaal van een andere kant dit onderwerp konden belichten!

Gardi en Rita: Gardi, jij hebt mij, toen nog in de L-vleugel, geleerd hoe ik EBV-cellijnen kon opzetten. Rita, jij was altijd bereid om me met raad en daad bij te staan als het opstarten van sommige patiënten wat moeizamer ging. Ook als ik te lui was om helemaal naar jullie lab te komen, was jij het die me opbelde om te zeggen dat ik snel nieuw medium moest komen geven om te voorkomen dat m'n cellen zouden verhongeren. Hartelijk dank voor jullie zorgen!

Alle collega's van het lab Inwendige Geneeskunde: Bedankt voor jullie gezelligheid en collegialiteit. Met name wil ik de mensen van het hypofyselab noemen: Marlijn, Diana, Christiaan, Joost, Sander, Ellen, Peter, Michel, Rebecca, Marjolein, Bedette, Carlotta, Patrick, Giovanni, Katy, Joris, Rosario, Diego en Leo! Virgil bedankt voor je vriendschap! Joop, bedankt voor de gezellige, goede gesprekken en motivatie! De dames van het secretariaat, Carine , Nel en Lenie: Jullie service en jullie gezelligheid waren grandioos!

Op mijn "dedication" pagina staat een dictaat van Desiderius Erasmus, de man naar wie ons Medisch Centrum is vernoemd. Het dictaat gaat echter nog verder: "-wat zo verborgen is omzichtig naar boven te halen, wat zo diepzinnig is met het verstand te vatten, wat zo veelomvattend is in het geheugen op te slaan, wat zo onontbeerlijk is voor het welzijn van de gehele mensheid aan allen aan te bieden: dat moet toch iets zijn dat groter is dan de mens zelf en zonder meer van God!"

Curriculum Vitae

De schrijver van dit proefschrift werd op 27 juni 1978 geboren te Dordrecht. In 1996 behaalde hij het atheneum diploma (ongedeeld) aan het Lambert Franckens College in Elburg. In september 1996 begon hij aan de studie Scheikunde aan de Rijksuniversiteit Groningen, met als differentiatierichting Biochemie. Op 28 juni 2001 werd het doctoraal diploma behaald, na 3 afstudeeronderzoeken. Het eerste onderzoek van 8 maanden werd uitgevoerd op de afdeling Inwendige Geneeskunde, disciplinegroep Hematologie van het Academisch Ziekenhuis Groningen onder leiding van dr. K.U. Birkenkamp en prof.dr. E. Vellenga, getiteld: "De rol van de transcriptiefactor nuclear factor kappaB (NF-κB) bij de spontane apoptose van acute myeloïde leukemie (AML) cellen". Het tweede onderzoek van 8 maanden werd uitgevoerd in het laboratorium Celbiologie, afdeling Biochemie van de Rijksuniversiteit Groningen onder leiding van dr. L. Bosgraaf en prof.dr. P.J.H. van Haastert, getiteld: "Het identificeren en karakteriseren van eiwitten die een rol spelen in de chemotaxis van de amoebe *Dictyostelium discoideum*". Het derde onderzoek betrof een literatuurstudie van 2 maanden onder leiding van Dr. M. Fraaije met als titel: "Rifampicine en Isoniazide in de strijd tegen Tuberculose". In september 2001 werd aangevangen met het in dit proefschrift beschreven onderzoek op de afdeling Inwendige Geneeskunde van het Erasmus MC, Universitair Medisch Centrum Rotterdam, in het kader van het NWO-project: "Factors determining Glucocorticoid Sensitivity in Men", onder leiding van prof.dr. S.W.J. Lamberts en dr. F.J.W. Koper. In januari 2006 startte hij met zijn opleiding tot klinisch chemicus, opleider prof.dr. J. Lindemans bij de Afdeling Klinische Chemie in het Erasmus MC.

List of Publications

1. *Leonard Bosgraaf, Henk Russcher, Janet L. Smith, Deborah Wessels, David R. Soll, and Peter J.M. Van Haastert*
A novel cGMP signalling pathway mediating myosin phosphorylation and chemotaxis in Dictyostelium
The EMBO Journal. Vol. 21 No. 17 pp. 4560-4570, 2002

2. *Leonard Bosgraaf, Henk Russcher, Helena Snippe, Sonya Bader, Joyce Wind, and Peter J.M. Van Haastert*
Identification and Characterization of Two Unusual cGMP-stimulated Phosphodieserases in Dictyostelium
Molecular Biology of the Cell. Vol.13, 3878-3889, November 2002

3. *Pauline Smit, Henk Russcher, Frank H. de Jong, Albert O. Brinkmann, Steven W.J. Lamberts, Jan W. Koper*
Differential regulation of synthetic glucocorticoids on gene expression levels of glucocorticoid-induced leucine zipper and interleukin-2
J Clin Endocrinol Metab. 2005 May;90(5):2994-3000

4. *Henk Russcher, Elisabeth F.C. van Rossum, Frank H. de Jong, Albert O. Brinkmann, Steven W.J. Lamberts, Jan W. Koper*
Increased expression of the glucocorticoid receptor-A translational isoform as a result of the ER22/23EK polymorphism
Mol Endocrinol. 2005 Jul;19(7):1687-96

5. *Henk Russcher, Pauline Smit, Erica L.T van den Akker, Elisabeth F.C. van Rossum, Albert O. Brinkmann, Frank H. de Jong, Steven W.J. Lamberts, Jan W. Koper*
Two polymorphisms in the glucocorticoid receptor gene directly affect glucocorticoid-regulated gene expression
J Clin Endocrinol Metab. 2005 Oct;90(10):5804-10

6. *Elisabeth F.C. van Rossum, Henk Russcher, Steven W.J. Lamberts*
Genetic Polymorphisms and Multifactorial Diseases: Facts and Fallacies
The Example of the Glucocorticoid-related Genes
Trends Endocrinol Metab. 2005 Dec;16(10):445-50

7. *Henk Russcher, Pauline Smit, Elisabeth F.C. van Rossum, Erica L.T. van den Akker, Albert O. Brinkmann, Loek J.M. de Heide, Frank H. de Jong, Jan W. Koper, Steven W.J. Lamberts*
Strategies for the Characterisation of Disorders in Cortisol Sensitivity
J Clin Endocrinol Metab. 2006 Feb;91(2):694-701

8. *Erica L.T. van den Akker, Henk Russcher, Elisabeth F.C. van Rossum, Albert O. Brinkmann, Frank H. de Jong, Anita Hokken, Huibert A.P. Pols, Jan W. Koper, Steven W.J. Lamberts*
A Glucocorticoid Receptor Polymorphism affects Transrepression but not Transactivation
Submitted

9. *Henk Russcher, Virgil A.S.H. Dalm, Frank H. de Jong, Albert O. Brinkmann, Leo J. Hofland, Steven W.J. Lamberts and Jan W. Koper*
Associations between Promoter Usage and Alternative Splicing of the Glucocorticoid Receptor Gene
Submitted