Factors Determining Glucocorticoid Sensitivity in Man

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Factors Determining Glucocorticoid Sensitivity in Man

Factoren die van invloed zijn op glucocorticoïd gevoeligheid in de mens

PROEFSCHRIFT

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Prof.dr. S.W.J. Lamberts

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Pauline Smit

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Promotor:	Prof.dr. S.W.J. Lamberts
Overige leden:	Prof.dr. J. Lindemans Prof.dr. F.H. de Jong Prof.dr. A.J. van der Lelij
Copromotor:	Dr. F.J.W. Koper

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Chapter 1

General Introduction

1.1 Steroidogenesis

The adrenal glands lie in the retroperitoneum above or medial to the upper poles of the kidneys. They are comprised of an outer cortex and an inner medulla, which are embryologically and functionally distinct. The medulla synthesizes the cathecholamines epinephrine and norepinephrine. The adrenal steroids are synthesized in the adrenal cortex. The cortex is composed of three layers: an outer zona glomerulosa (producing aldosterone), a zona fasciculata (producing cortisol and androgens), and an inner zona reticularis (producing cortisol and androgens). All steroid hormones which are produced by the adrenal cortex are derived from cholesterol, which is converted into steroids with glucocorticoid, mineralocorticoid (MC), or androgen activity by means of a series of enzymatic steps (Fig. 1) (1, 2).

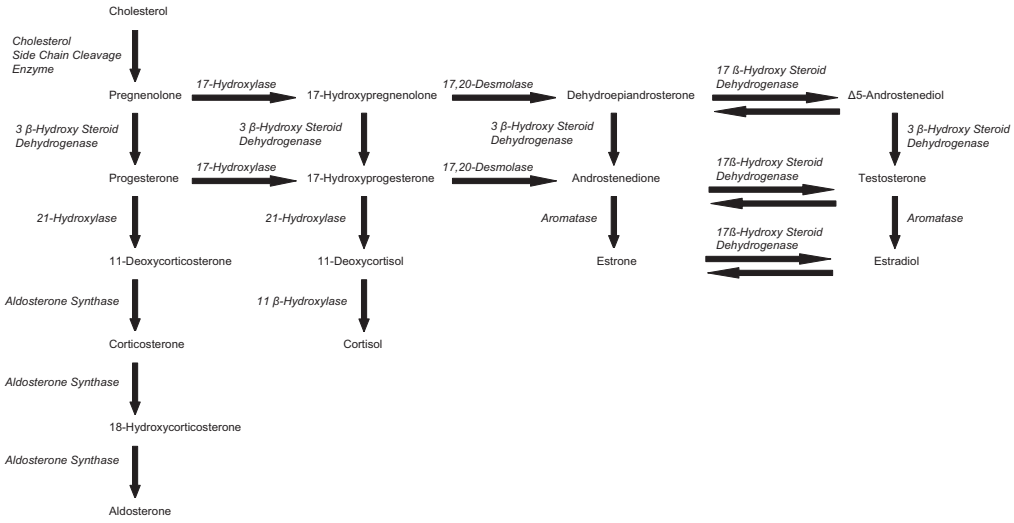

Figure 1. Diagram of the biosynthetic pathways and enzymes involved in the synthesis of adrenal steroids.

1.2 The Hypothalamic-Pituitary-Adrenal-axis

The secretion of glucocorticoids (GCs) is under control of the hypothalamic-pituitary-adrenal (HPA)-axis. A simplified model of the HPA-axis is shown in Figure 2. In response to stress and a circadian rhythm paralleling the activity cycle, the hypothalamus releases corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) (3).

Both CRH and AVP stimulate the release of adrenocorticotrophic hormone (ACTH) from the pituitary, which is synthesized as part of a large precursor, pro-opiomelanocortin (POMC) (4, 5). CRH is probably the most potent ACTH secretagogue (6). AVP is a weak stimulator of ACTH secretion, but it potentiates the action of CRH (7). In response to ACTH, the zona fasciculata cells of the adrenal cortex synthesize and secrete GCs, as well as adrenal androgens. The principal MC is aldosterone. In humans, cortisol is the main GC produced by the adrenal cortex. It exerts a strong negative feedback both at the level of the hypothalamus and the pituitary, completing a negative feedback-loop (8). There are several mechanisms involved in this feedback-action of GCs. In the corticotropic cell, GCs have inhibitory effects on both POMC gene transcription and secretion of ACTH (5). In the paraventricular nuclei of the hypothalamus, GCs decrease mRNA levels of CRH and AVP (9-11). Finally, GCs block the stimulatory effect of CRH on POMC gene transcription (5).

The activity of the HPA-axis is under the influence of a circadian rhythm. Peak levels of ACTH occur prior to or at the time of awakening and decline during the day, reaching a nadir during the late evening/early morning. In humans, this circadian rhythm is independent of posture, and persists with bed rest and in the absence of the adrenals, indicating an endogenous periodicity, synchronized by environmental phenomena such as the light-dark cycle (12).

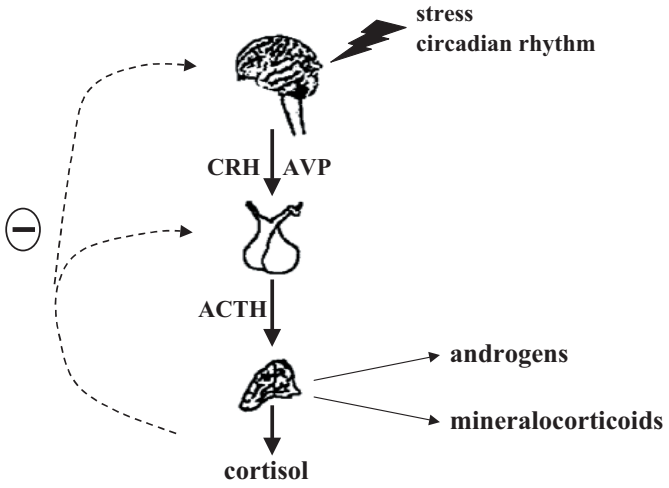

Figure 2. A simplified overview of the regulation of GCs by the HPA-axis. Under the influence of a circadian rhythm and different forms of stress, the hypothalamus secretes CRH and AVP. This increases the release of ACTH from the pituitary. In reaction, the adrenal glands secrete cortisol along with other adrenal steroids with mineralocorticoid and androgen activity. Cortisol has a negative feedback action both at the level of the hypothalamus and the pituitary.

Stress factors such as severe illness, infections, trauma, anesthesia, and surgery, but also psychological stress lead to activation of the HPA-axis, which is demonstrated by increased ACTH and cortisol production (8, 13-20). This activation is essential for the adaptation to stress and contributes to maintenance of homeostasis (8, 20).

1.3 Effects of Glucocorticoids

GCs exert a wide variety of functions throughout the human body, including mediation of the stress response, regulation of lipid and glucose metabolism, immunosuppressive and anti-inflammatory actions, vascular effects, increase of bone resorption, as well as effects on the brain and development and function of numerous organs.

1.3.1 Effects on Glucose and Lipid Metabolism

The overall effect of GCs on glucose metabolism is to reduce glucose utilization and to increase glucose production (21-23). GCs inhibit glycogenolysis by activating glycogen synthetase and inactivating glycogen phosphorylase (21, 22). Furthermore, GCs increase gluconeogenesis by increasing lactate and pyruvate availability and stimulating the release of glucogenic amino acids from peripheral tissues such as skeletal muscle. They also directly activate gluconeogenetic enzymes such as glucose-6-phosphatase and phosphoenol pyruvate carboxy kinase (23). Finally, GCs inhibit glucose uptake and utilization by peripheral tissues, partly by a direct inhibition of glucose transport into the cell, causing insulin resistance (24, 25).

GCs acutely activate lipolysis in adipose tissue, in this way providing free fatty acids as energy supply for gluconeogenesis (26, 27). GCs also have long-term effects. Increased exposure to GCs with aging may contribute to an increase of visceral fat accumulation. In addition, overactivity of the HPA-axis is related to increased vascular risk, including hypertension and obesity (28, 29). GCs negatively affect body composition, with a redistribution of body fat and deposition of adipose tissue on abdomen and trunk, and also muscle atrophy (30). Longterm exposure to high levels of GCs induces loss of muscle mass and the inhibition of growth (31, 32). As body composition plays an important role in lipid metabolism and insulin sensitivity, it affects the risk on cardiovascular disease (33).

1.3.2 Anti-inflammatory and Immunological Effects

The immunosuppressive effects of GCs are routinely used in the treatment of chronic inflammatory or immune diseases (e.g. inflammatory bowel disease, asthma). However, severe side effects (including diabetes mellitus (DM) and osteoporosis) are associated with GC treatment, limiting its therapeutic usefulness (34, 35).

GCs act on the immune system by both suppressing and stimulating a large number of pro-inflammatory or anti-inflammatory mediators. In many ways, GCs lead to termination of inflammation by enhancing the clearance of foreign antigens, toxins, microorganisms, and dead cells. They do so by enhancing opsonization and the activity of scavenger systems, and by stimulating macrophage phagocytic ability and antigen uptake (36-39). At the same time, they prevent inflammation from overshooting by suppressing the synthesis of many inflammatory mediators, such as several chemokines, prostaglandins, leukotrienes, proteolytic enzymes, free oxygen radicals, and nitric oxide.

Also a great number of cytokines, including tumor necrosis factor (TNFα) and interleukin-2 (IL-2), is broadly downregulated by GCs. Interestingly, some anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-ß) are instead upregulated by GCs (40-44).

1.3.3 Effects on Bone and Mineral Metabolism

Many factors contribute to bone loss during GC therapy, such as underlying disease, malnutrition, vitamin D insufficiency, and low body weight. Bone loss occurs particularly in the first few months of GC treatment and affects cancellous rather than cortical bone, with reduced bone formation and increased bone resorption (45, 46). The risk of fracture increases rapidly in patients with GC therapy. The existence of a threshold is not well defined but the risk of fracture is increased in patients with higher doses of GCs (47, 48). Other effects include altered production of gonadal sex hormones, inhibition of intestinal calcium absorption, and enhancement of renal excretion of calcium (49-52).

1.3.4 Effects on the Brain

GCs have numerous biological effects on the brain, affecting neurotransmission, metabolism, neuronal structure and maturation, and survival. Chronically high levels of GCs are harmful and have been associated with neuro-psychiatric disorders,

cognitive impairment, structural deterioration and neuroendocrine dysfunction (53). Especially the hippocampus, a key locus for memory, cognition and neuroendocrine control and known to be among the first tissues to be degenerated in Alzheimer's disease (AD), is sensitive to GCs (54, 55).

1.3.5 Effects on Growth and Development

The growth-retarding effect of GCs is well known in children, due to a direct effect of GCs on chondrocyte function and an indirect effect on growth through inhibition of the growth hormone/insulin growth factor 1 axis (56-58). Long-term GC administration may also result in myopathy (59). Therefore, GCs reduce two critical mechanical challenges (muscle forces and changes in bone length) that normally stimulate bone mineral accrual during development (60). GCs accelerate the development of a number of systems and organs in fetal and differentiating tissues, though the mechanisms are unclear. As GCs are generally inhibitory, these stimulatory effects may be due to GC interactions with other growth factors (61). Examples of these development-promoting effects are increased surfactant production in the fetal lung and the accelerated development of hepatic and gastrointestinal enzyme systems (62-64).

1.4 Signaling Pathways of Glucocorticoids

GCs are thought to diffuse freely across the cell membrane because of their lipophilicity. Once in the cytoplasm, they interact with the glucocorticoid receptor (GR) which mediates most, if not all, of the GC-induced actions (65). The GR is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors. The general structure of the members of this family shows a centrally located DNA binding domain (60-70 amino acids), flanked on the carboxy-terminal by an approximately 250-amino acid ligand-binding domain (LBD). These two domains are very similar for the different ligand-activated transcription factors. However, the variably sized, non-homologous amino-terminal domain is much less conserved. (66-68).

Prior to ligand binding, the GR primarily resides in the cytoplasm associated with two molecules of heat shock protein 90 and several immunophilins such as FKBP51 (Fig. 3). Upon ligand binding, the GR undergoes a series of conformational alterations,

leading to its dissociation from the cytoplasmic chaperones and translocation to the nucleus. Once inside the nucleus, readily formed GR homodimers recognize and interact with specific sequences called GC-response elements (GREs) in target gene promoters, leading to the enhancement of gene transcription. Alternatively, ligand-bound GR can also bind to negative GREs (nGREs) on promoter regions of target genes to suppress transcription (70).

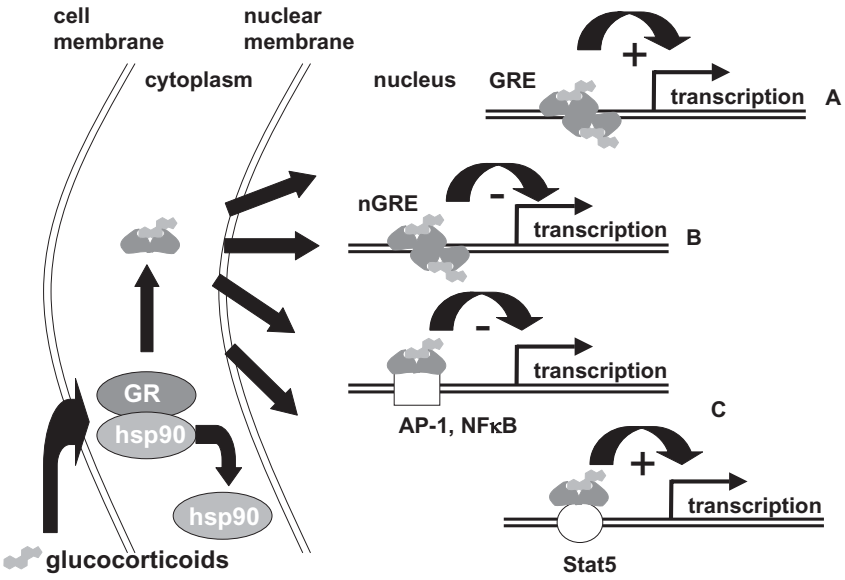

Figure 3. Signaling pathways of GR-mediated transcriptional regulation. Following binding to GCs and translocation to the nucleus, the GR readily dimerizes and modulates target gene transcription via: direct interaction with (A) GREs and (B) nGREs; and (C) direct protein-protein interaction. The resulting modulation of target gene transcripts leads to altered protein expression. *Adapted from Reichardt et al.* (69).

In addition, transcriptional modulation by the GR can be achieved through its cross-talk with other transcription factors such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and signal transducer and activator of transcription (STAT). It is thought that GR interaction with NF-κB and/or AP-1, and the subsequent suppression of their target genes, is the major mechanism by which GCs exert their anti-inflammatory action (70). However, recently Galon *et al.* showed a more selective and complex picture of the actions of GCs, in which GCs also can have both immunostimulatory and immunosuppresive actions at the same time, as well as opposite regulation of molecules implicated in inflammatory and immune responses (43).

1.5 Differences in Glucocorticoid Sensitivity

Within the normal population, there exists a considerable interindividual variation in GC sensitivity. Whereas some patients develop side effects on relatively low doses of GCs, others appear to be less sensitive to GCs, as they do not show an adequate improvement in response to treatment even on high doses (71). Some patients are even resistant to the anti-inflammatory effects of GCs while at the same time showing side effects known to reflect normal sensitivity to GCs, including suppression of the hypothalamic-pituitary-adrenal (HPA) axis (71, 72). The extremes of variability in GC sensitivity can be divided into cortisol resistance (CR) and hypersensitivity syndromes.

1.5.1 Cortisol Resistance Syndrome

An extreme example of systemic decreased GC sensitivity is CR syndrome, in which the relative insensitivity of all target tissues to GCs leads to clinical signs and symptoms. This syndrome was described for the first time in 1976 by Vingerhoeds *et al.* (73), who reported a patient with hypercortisolism without the signs and symptoms of Cushing's disease. In these patients, the negative feedback is reduced at both the level of the hypothalamus and the pituitary, leading to an increase in the secretion of CRH and ACTH and subsequent higher serum cortisol levels. However, the circadian rhythm is intact and the clinical signs of hypercortisolism such as muscle weakness, obesity, moon facies and osteoporosis are absent. The increased ACTH secretion, however, also results in overproduction of MCs and androgens, causing the symptoms of CR: hypertension, hypokalemia, abnormal spermatogenesis and infertility in men, and in women acne, hirsutism, male pattern baldness ("geheimratsecken") and manifestations affecting the reproductive system such as oligomenorrhoea and infertility (73-75). In children, also precocious pseudopuberty was reported (76). However, these clinical features were not present in all patients with CR, and even within families the clinical presentation varied. This makes this syndrome difficult to diagnose (71, 74, 77).

Until now, approximately 10 patients have been diagnosed with CR syndrome (78-85). Most patients carried a mutation of defect in the LBD domain of the GR, only one patient had a mutation in the DNA-binding domain (86). In the other cases however, the mechanism(s) causing CR syndrome is unknown.

1.5.2 Cortisol Hypersensitivity Syndrome

An extreme example of systemic GC hypersensitivity is the syndrome of cortisol hypersensitivity (CH). Iida *et al.* reported a patient who presented with clinical features of Cushing's syndrome in spite of hypocortisolemia (87). More recently, Newfield *et al.* described a patient with serious symptoms of Cushing's syndrome at peripubertal age, but with normal cortisol levels (88). So far, the molecular mechanism of CH syndrome has not been elucidated yet (87-89).

1.6 Factors Determining Glucocorticoid Sensitivity

GC signaling is a complex process, in which distinct pathways are involved that can influence GC sensitivity. Also, other mechanisms such as the local conversion of GCs play a role in the intracellular bioavailability of GCs.

1.6.1 Glucocorticoid Receptor Expression

The level of cellular GR expression is closely correlated with the magnitude of the GR-mediated response (90). This has been demonstrated *in vivo* in transgenic mouse expressing GR anti-sense RNA, in which the level of GR was reduced, leading to signs of CR (91). This was also illustrated by a patient with a microdeletion and nonexpression of one of the two GR alleles, lowering GR expression to 50% of normal levels and compensated cortisol resistance (82).

1.6.2 Glucocorticoid Receptor Affinity

The potency of the GR as a transcriptional regulator also correlates with its hormone-binding affinity, which is determined by a number of factors. Point mutations within the coding region for the GR LBD causing amino acid substitutions can lead to altered hormone-binding affinity of the GR. Almost all such mutations are known to cause reduction of either GR-binding affinity (80) or ligand/receptor complex stability (92), and are associated with various clinical syndromes of cortisol resistance. Further, it is important that prior to ligand binding, the GR is maintained in the cytoplasm in a ligand-friendly high affinity conformation (70, 93). Proper assembly and folding of the GR/heat shock protein (hsp) complex are therefore essential for normal hormone-receptor interaction.

1.6.3 Polymorphisms in the Glucocorticoid Receptor Gene

A polymorphism is a location of the genome that varies in sequence between individuals and is present in a considerable number of individuals in a population, traditionally with an allelic frequency of at least 1% (94). There are different kinds of polymorphisms, of which the single nucleotide polymorphism (SNP) is the most common (95). In dbSNP (http://www.ncbi.nlm.nih.gov/SNP), currently 387 SNPs are shown for the human GR sequence.

For several GR polymorphisms functional associations have been reported: the N363S polymorphism (rs 6195), the *Bcl*I restriction fragment length (RFLP) polymorphism, the ER22/23EK polymorphism (rs 6189 and rs 6190), the *TthIII*I RFLP (rs 10052957), and the exon 9β polymorphism (rs 6198) (Fig. 4).

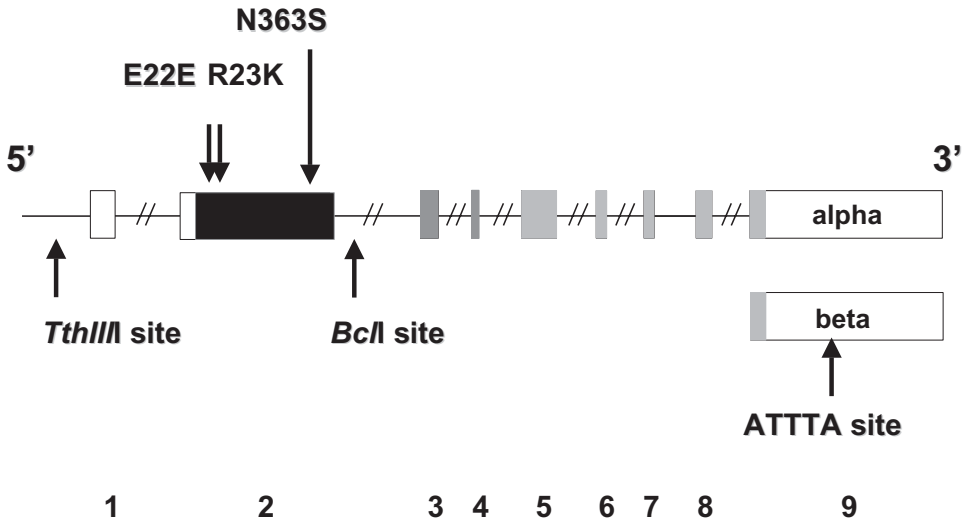

Figure 4. Schematic overview of the GR gene with the N363S polymorphism, the *Bcl*I RFLP polymorphism, the ER22/23EK polymorphism, the *TthIII*I RFLP polymorphism, and the exon 9β polymorphism. RFLP, restriction fragment length polymorphism.

The N363S Polymorphism

The N363S polymorphism, located in codon 363, causes an AAT→AGT nucleotide change, resulting in an asparagine to serine amino acid change. This polymorphism is asscociated with a higher GC sensitivity *in vivo* (96). Studies on the effects of this polymorphism on body composition parameters such as body mass index (BMI) and

waist-to-hip ratio (WHR), are still quite controversial (96-104). The exact mechanism underlying the increased GC sensitivity associated with this polymorphism is still unknown. It is possible that the N363S variant modulates the phosphorylation state of the GR as it introduces another serine residu (although not in a known phosphorylation site context) and might alter its interactions with other transcription factors (105).

The BclI Polymorphism

The *Bcl*I polymorphism was first described by Murray *et al.* (106), who reported an RFLP, consisting of a short fragment of 2.3 kb and a large fragment of 4.5 kb. The exact nucleotide alteration was identified several years later: a C→G substitution, 646 nucleotides downstream from exon 2, resulting in fragments of 2.2 kb and 3.9 kb (107). This polymorphism is thought to be associated with GC hypersenstivity, however, contrasting data have been reported on the effects on body composition (107-116). The molecular mechanism behind the *Bcl*I polymorphism has not been clarified yet. It is possible that this intronic polymorphism is linked to another polymorphism in the promoter region of the GR gene, which could result in increased expression of the GR or a variant in the 3'-untranslated region which increases mRNA stability. Another possibility could be the linkage to another gene near the GR gene. It is also possible that the point mutation in the *Bcl*I site influences the splicing process, although it is not located near a regulatory splice site (117).

The ER22/23EK Polymorphism

The ER22/23EK polymorphism consists of two linked, single-nucleotide polymorphisms in codons 22 and 23 in exon 2. The first mutation in codon 22 does not result in an amino acid change (GAG→GAA, both coding for glutamic acid). However, the mutation in codon 23 (AGG→AAG) causes an amino acid change from arginine to lysine (118).The polymorphism is associated with a relative GC resistance and a healthier metabolic profile, with lower cholesterol levels and increased insulin senstivity. At young age, the ER22/23EK polymorphism is also associated with a beneficial body composition, while in the elderly, it is associated with a lower risk of dementia and a better survival (117, 119, 120). Recently, Russcher *et al.* (121) identified the mechanism behind this polymorphism.

The TthIII Polymorphism

The *TthIII*I restriction fragment length polymorphism (RFLP) was originally reported by Detera-Wadleigh *et al.* (122). Rosmond *et al*. (123) found this polymorphism to be associated with elevated diurnal cortisol levels in a population of 284 Swedish men. No associations with anthropometry, glucose, and insulin metabolism or lipid spectrum were found.

Recently, van Rossum *et al.* (107) identified the exact location of the *TthIII*I RFLP; 3807 bp upstream of the GR mRNA start, causing a C/T change. No associations with sensitivity to DEX, baseline cortisol, insulin, glucose or cholesterol levels were found. However, it was shown that the *TthIII*I RFLP is linked to the ER22/23EK polymorphism.

The Exon 9β Polymorphism

Previously, De Rijk *et al.* (124) reported an A to G substitution in an "ATTTA motif" in exon 9β. This polymorphic ATTTA (to GTTTA) sequence is located in a region encoding the 3'untranslated region (UTR) of the mRNA of the GR-β splice variant. Introduction of this polymorphism in GR-β mRNA *in vitro* increased mRNA stability as well as protein expression. They also found this 9β polymorphism to be associated with rheumatoid arthritis. It was suggested that this polymorphism results in an increased expression and stability of GR-β *in vivo*, consequently leading to GC resistance (124, 125).

1.6.4 Glucocorticoid Receptor Splice Variants

Three different 3'-splice variants of the GR gene have been reported, namely GR-α, GR-β and GR-P (Figure 5). The α isoform is the functional receptor and is encoded by exon 2 to 9α (126). The GR-α protein is located in the cytoplasm in the absence of GCs, but migrates to the nucleus upon binding with GCs (127). The β isoform is the result of the alternative splicing of exon 9β instead of exon 9α, resulting in 15 different amino acids encoded by 9β. As a consequence, this receptor cannot bind GCs (126). At mRNA level, GR-β expression accounts for 0.2-1% of the total GR expression (128). GR-β has a dominant negative effect on GR-α, and increased expression of GR-β has been reported in steroid resistant states (128-134). Recently, it was shown that patients with Cushing's disease have a higher expression of the GR-β splice variant compared to controls (135).

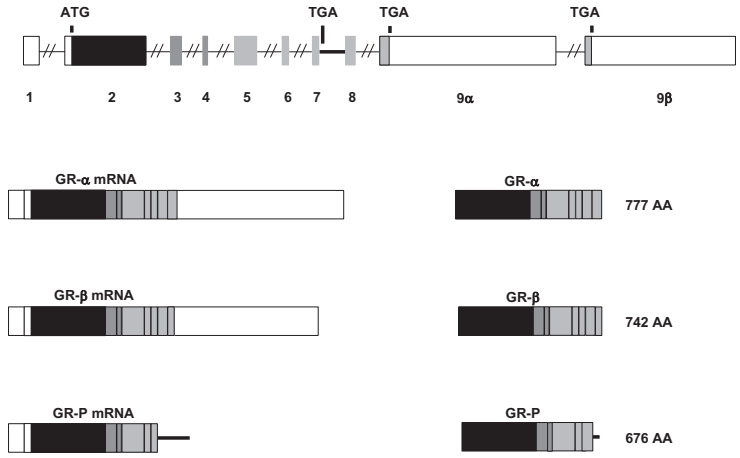

Figure 5. Schematic overview of the GR gene and its three splice variants GR-α, GR-β and GR-P.

The GR-P splice variant is encoded by exons 2-7 plus several basepairs from the subsequent intron region. As a consequence, this isoform lacks most of the LBD and therefore cannot bind GCs. GR-P expression accounts for up to 10-20% of total GR mRNA (135, 136). The effects of this isoform *in vivo* are still unknown, *in vitro* however, it was demonstrated that transfection of GR-P increases the activity of GR-α (137).

Several other GR splice variants have been detected in tissues and in certain cancer cell lines, e.g. the GR-γ transcripts where a 3-bp sequence from the intron separating exons 3 and 4 is retained, yielding an inframe single amino acid insertion between the two zinc-fingers in the DNA binding domain (138, 139).

1.6.5 Alternative Initiation of Glucocorticoid Receptor Translation

In addition to GR splice variants, also two translational isoforms of the GR have been described (140). The translation from the first initiator AUG codon (Met-1) results in a longer isoform, GR-A. However, this start codon lies within a weak Kozak translation initiation concensus sequence, leading tot leaky ribosomal scanning and translation initiation from the next downstream AUG codon (Met-27), resulting in GR-B. The GR-B isoform thus lacks the first 26 amino acids of the full-length GR. *In vitro,* both GR-A and GR-B exhibit similar ligand-dependent translocation from the cytoplasm to the nucleus. However, the GR-B isoform is nearly twice as efficient in GRE-mediated

transactivation compared to GR-A (140).

Recently, Russcher *et al.* (121) showed that, when the GR ER22/23EK polymorphism is present, approximately 15% more GR-A protein was expressed, whereas total GR levels (GR-A + GR-B) were not affected. Therefore, the transcriptional activity in carriers of this polymorphism is decreased. The formation of more GR-A is probably caused by altered secondary mRNA structure, due to the presence of this polymorphism.

1.6.6 Posttranslational Modifications of the Glucocorticoid Receptor

Mature GR proteins are covalently modified by various processes which further modulate the transcription regulation activity. Several posttranslational modification processes have been discovered which seem to affect GR function: sumoylation, nitrosylation, phosphorylation, and ubiquitination. Sumoylation seems to affect GR activity (141-143). Nitrosylation at cysteine residues on GR decreases ligand binding and may disable GCs from exerting anti-inflammatory effects during fatal septic shock (144). On the other hand, both phosphorylation and ubiquitination enhance the transactivation activity of the GR (145-149).

1.6.7 11β-Hydroxysteroid Dehydrogenase Activity

The cellular actions of GCs are largely mediated through binding to the GR. In mammalian tissues, the two isoenzymes of 11β-hydroxysteroid dehydrogenase (11β-HSD) have a pivotal role in the prereceptor regulation of GC action, catalysing the interconversion of hormonally active GCs and their inactive 11-keto forms.

11β-Hydroxysteroid Dehydrogenase Type 1

11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) is mainly expressed in liver and adipose tissue. The gene encoding 11β-HSD1 (*HSD11B1*) is located on chromosome 1q32.2, and encodes a 34-kDa protein that resides within the endoplasmic reticulum (150, 151). The enzymatic activity of 11β-HSD1 is bi-directional, possessing both dehydrogenase (cortisol to cortisone) and oxo-reductase (cortisone to cortisol) components. In intact cells or organs *in vivo*, it acts predominantly as an oxo-reductase. In the last decade, there has been an exponential increase in research focusing on 11β-HSD1, as this isoenzyme may be involved in obesity and its complications (152-154). It is thought that 11β-HSD1 also might be involved in cortisone reductase deficiency

(CRD), as individuals with CRD excrete virtually all GCs as cortisone metabolites (tetrahydrocortisone) with low or absent cortisol metabolites (tetrahydrocortisols) (155).

In rodents, Masuzaki *et al.* (156) showed that alterations in the activity of 11β-HSD1 have functional significance for body composition. Targeted overexpression of 11β-HSD1 in adipocytes caused a threefold accumulation of visceral adipose tissue, which was caused by lipid accumulation and an increase in adipocyte size. They also demonstrated increased corticosterone levels in adipose tissue in the 11β-HSD1 transgenic mouse. In humans, however, there is much controversy about the role of 11β-HSD1 on body composition (150, 157, 158)

Central obesity is strongly associated with insulin resistance which is considered to play an important pathogenetic role in the development of type 2 diabetes mellitus (DM) (159). The inhibition of 11β-HSD1 has been proposed for the treatment of DM and central obesity (160-162). Indeed, 11β-HSD1-null mice have enhanced hepatic and adipose insulin sensitivity and are protected from weight gain and hyperglycemia when on a high-fat diet (163, 164). In cross-sectional studies in humans, 11β-HSD1 activity and variability in HSD11B1 have been associated with obesity, diabetes, and glucose tolerance (150, 165, 166). However, Kerstens *et al.* (167) found no relationship between 11β-HSD setpoint and insulin sensitivity in healthy subjects and patients with type 2 DM.

11β-HSD1 is highly expressed in the cerebellum, hippocampus, and cortex (168, 169). In two small, randomized, double-blind, placebo-controlled cross-over studies, administration of the 11β-HSD inhibitor carbenoxolone (3 times a day 100 mg) improved verbal fluency after 4 weeks in 10 healthy elderly men (aged 55-75 yr) and improved verbal memory after 6 weeks in 12 patients with type 2 DM (170). Furthermore, de Quervain *et al.* (171) showed that a specific haplotype of HSD11B1 was associated with a 6-fold increased risk of AD.

11β-Hydroxysteroid Dehydrogenase Type 2

11β-Hydroxysteroid dehydrogenase type 2 (11β-HSD2) is a high-affinity NAD-dependent, unidirectional dehydrogenase that converts cortisol to cortisone (151, 172). The *HSD11B2* gene, located on chromsome 1q32.2, encodes a 44-kDa protein which shares only 14% sequence homology with 11β-HSD1 (173, 174). As the MC receptor (MR) has equal affinity for aldosterone and cortisol, 11β-HSD2 protects the MR from

occupation by the higher circulating concentrations of cortisol by its inactivation to cortisone (174). Therefore, the expression of 11β-HSD2 is mainly restricted to MC target tissues, such as kidney, sweat glands, salivary glands, and colonic mucosa, where it colocalizes with the MR and is involved in salt and water balance (175, 176). Mutations in *HSD11B2* give rise to the syndrome of apparent MC excess (AME), a rare, inherited form of hypertension (177).

1.6.8 Hexose-6-phosphate Dehydrogenase Activity

The oxo-reductase activity of 11β-HSD1 requires NADPH. There is evidence that hexose-6-phosphate dehydrogenase (H6PDH) is the only source of NADPH within the lumen of the endoplasmatic reticulum and therefore it is crucial for the oxo-reductase activity of 11β-HSD1 (155, 178, 179). H6PDH, encoded by *H6PD*, is present in most tissues, but the highest expression levels are found in liver and adipose tissue, major sites of 11β-HSD1 oxo-reductase activity.

H6PDH is an autosomally-linked, microsomal glucose dehydrogenase, distinct from the cytosolic sex-linked glucose-6-phosphate dehydrogenase (G6PDH). H6PDH has a broad substrate specificity, including glucose-6-phosphate (G6P) and galactose-6-phosphate (Gal6P), and other hexose-6-phosphates such as 2-deoxyglucose-6-phosphate (2-deoxyG6P), and also simple sugars such as glucose (180, 181). It has dual nucleotide specificity for NADP+ and NAD+ but under physiological conditions, within the microsomal environment, the native substrates for H6PDH are believed to be G6P and NADP+ (182). Supply of G6P is ensured by the glucose-6-phosphate transporter (G6PT) of the endoplasmic reticulum, which is specific for G6P (182). Currently, little is known about the transcriptional or the translational regulation of H6PDH (183-185).

In a recent study, Lavery *et al.* (179) showed that H6PDH knockout mice have a profound switch in 11βHSD activity from oxo-reductase to dehydrogenase, increasing the corticosterone clearance resulting in a reduction in circulating corticosterone levels. This demonstrated a critical requirement of H6PDH for 11βHSD1 oxo-reductase activity.

1.6.9 P-glycoprotein Expression

The human multidrug-resistance-1 (*MDR-1; ABCB1*) gene encodes P-glycoprotein (P-gp), a 170 kDa integral membrane protein belonging to the adenosine triphosphate-

binding cassette (ABC) superfamily of membrane transporters, the largest gene family known (186, 187). It exports a number of chemically unrelated lipophilic compounds from the inside of cells and from membranes to the extracellular space in an energy-dependent manner (188, 189). P-gp is mainly located in the apical membrane of excretory cells in the liver, kidney and intestine and in the endothelial lining of capillary blood vessels at the blood-brain barrier, and is therefore important for the absorption, distribution, and elimination of xenobiotics (190-192). The degree of expression and the functionality of P-gp can potentially directly affect the (therapeutic) effectiveness of many drugs, including steroid hormones (187, 193).

However, there are still conflicting data on the effect of P-gp on HPA-axis-regulation. *MDR-1ab* knockout mice (the murine P-gp is encoded by *MDR-1a* and *MDR-1b*) show consistently lower plasma ACTH levels and lower evening plasma corticosterone levels (194). It was also demonstrated that specific P-gp inhibitors (e.g. PSC 833) can significantly increase intracellular intestinal epithelial and T-lymphocyte levels of cortisol and cyclosporine in man (195). On the other hand, rabbits treated with PSC 833 show a dose-dependent increase in serum cortisol levels (196), and there are no differences in pituitary uptake of [3H]cortisol between wildtype and *MDR-1a* knockout mice (197).

1.7 Aim and Outline of this Thesis

In clinical medicine, pharmacological doses of GCs are used in therapeutic settings in a vast number of immune diseases (e.g. asthma, rheumatoid arthritis), in hematologic malignancies, as well as in the prevention of rejection after allograft organ transplantation. It is known that a considerable interindividual variation exists in GC-sensitivity in the normal population, resulting in a variable response at the level of both beneficial and side effects. While some individuals develop adverse effects at a low dose of GCs, others can tolerate much higher doses without adverse effects. It is therefore important to identify factors that determine relative GC hyper- or hyposensitivity, including alterations in the affinity or number of the GR, alterations in the ratio of the different splice variants, and alterations in systemic absorption or pharmacokinetic handling of the drugs.

In this thesis, we describe a new method to determine individual GC sensitivity,

the GILZ and IL-2 expression assays, and used this method for the characterization of differences in GC sensitivity (chapter 2). Using this and additional methods, we characterized nine patients with GC sensitivity disorders (chapter 3). Further, we also determined the effects of polymorphisms in the *HSD11B1* and *H6PD* genes (chapter 4), and the *MDR-1* gene (chapter 5) on GC sensitivity. Finally, sideways, we demonstrated the effect of a common polymorphism in the gene coding for the metabolizing enzyme CYP3A7 on circulating serum dehydroepiandrosterone (DHEA) and estrone levels (chapter 6).

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Chapter 2

The Development of the GILZ and IL-2 Expression Assays for the Assessment of 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, Jan W. Koper
   J Clin Endocrinol Metab. 2005 May;90(5):2994-3000

2. "Two Polymorphisms in the Glucocorticoid Receptor Gene Directly affect Glucocorticoid-regulated Gene Expression"
   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
   J Clin Endocrinol Metab. 2005 Oct;90(10):5804-10

3. "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 normal population, there exists considerable variability in the sensitivity to glucocorticoids (GCs), which can be partly explained by polymorphisms in the GC-receptor (GR) gene.

Objective/design: Individual GC-sensitivity was determined by measuring the effects of several clinically used GCs on transactivation of the GC-induced leucine zipper (GILZ) gene and on transrepression of the interleukin-2 (IL-2) gene in peripheral blood mononuclear lymphocytes (PBMLs), using quantitative real-time PCR. To determine whether the *ex vivo* outcomes could predict *in vivo* effects, 15 individuals underwent a 0.25-mg dexamethasone (DEX) suppression test (DST), while determining GILZ and IL-2 mRNA levels in their PBMLs incubated with hydrocortisone, DEX, budesonide, and prednisolone. The GR ER22/23EK and N363S polymorphisms were studied by expressing GR(N363S) and GR(ER22/23EK) in COS-1 cells where we investigated their transactivating and transrepressing capacities, using a GC response element-luciferase reporter and a p65-activated nuclear factor κB-luciferase reporter, respectively. The transactivating and transrepressing capacities of the GR were evaluated in PBMLs of homozygous and heterozygous carriers of these polymorphisms using the GILZ and IL-2 expression assays. Furthermore, we investigated whether it was possible to simplify and validate the GILZ and IL-2 expression assays to make them more applicable for larger populations.

Interventions: Fifteen individuals underwent a 0.25-mg DST.

Results: A clear difference in relative potencies for transactivation and transrepression of the various GCs was observed, suggesting differential effects. No correlations were found between the DST and the GILZ and IL-2 expression assays.However, correlations existed between hydrocortisone and DEX (r = 0.52; *P* = 0.046), hydrocortisone and budesonide (r = 0.48; *P* = 0.069), and hydrocortisone and prednisolone (r = 0.86; *P* = 0.007) regarding GILZ mRNA levels, and between hydrocortisone and DEX (r = 0.62; P = 0.014), hydrocortisone and budesonide (r = 0.71; P = 0.003), and hydrocortisone and prednisolone (r = 0.71; P = 0.047) regarding IL-2 mRNA levels. The ER22/23EK polymorphism resulted in a significant reduction of transactivating capacity, both in transfection experiments (-14 ± 5%, *P* < 0.05) and in PBMLs 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. Further, we demonstrated that the GILZ and IL-2 expression assays can be performed with lower number of cells and less DEX concentration-data points. However, this resulted in larger confidence intervals.

Conclusion: Our results suggest that regulation of the hypothalamic-pituitary-adrenal-axis is complex. However, within an individual person, the GILZ and IL-2 expression assays combined might predict what type and dosage of GC will be preferable for its inhibitory clinical effects, together with relatively less transactivating effects related to adverse effects. The presence of ER22/23EK and N363S polymorphisms and other GC-sensitivity modulating polymorphisms may have consequences for the use of GCs. The downscaled GILZ and IL-2 expression assays increase the convenience for using these assays in larger groups of patients.

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 GC receptor (GR), a member of the nuclear receptor superfamily (4). Prior to ligand binding, the GR primarily resides in the cytoplasm associated with two molecules of heat shock protein 90 and several immunophilins such as FKBP51. Upon ligand binding, the GR undergoes a series of conformational alterations, leading to its dissociation from the cytoplasmic chaperones and translocation to the nucleus. Once inside the nucleus, readily formed GR homodimers recognize and interact with specific sequences called GC-response elements (GREs) in target gene promoters, leading to the enhancement of gene transcription. Alternatively, ligand-bound GR can also bind to negative GREs (nGREs) in promoter regions of target genes to suppress transcription. In addition, transcriptional modulation by the GR can be achieved through its cross talk with other transcription factors such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and signal transduction and activator of transcription (STAT) (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 and osteoporosis) are associated with GC treatment, limiting its therapeutic usefulness (4, 6). Although some patients develop side effects on relatively low doses of topically administered GCs, others appear to be less sensitive to GCs, because they do not show an adequate improvement in response to treatment even on high doses (7). Some patients are even resistant to the anti-inflammatory effects of GCs while at the same time showing side effects known to reflect normal sensitivity to GCs, including suppression of the hypothalamic-pituitary-adrenal axis (7, 8). Cellular GC sensitivity can be measured using several different assays based upon 1) receptor protein characteristics of mononuclear leukocytes (9), 2) inhibition of phytohemagglutinin-induced T-lymphocyte proliferation (10), or 3) changes in gene expression levels (11).

It is generally thought that transactivation is the predominant mechanism by which GCs exert many of their metabolic and cardiovascular side effects (12-14). Conventional GCs do not dissociate transactivation from transrepression. Strategies to develop new GCs aim to maintain transrepression of immune genes in the absence

of significant transactivation of GRE-dependent promoters (15).

One of the genetic factors involved in GC sensitivity is the occurrence of glucocorticoid receptor (GR) gene polymorphisms, generally defined as common variations at the DNA level with a frequency of more than 1% in the normal population. 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 (16, 17). 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) (18). 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 (17, 19, 20). 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-B to GR-A expression ratio leads to an overall decrease in transcriptional activity (21).

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 (18). This polymorphism increases sensitivity to GCs, whereas 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 (22, 23), but others did not (24, 25). 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 (26).

In this study, we aimed to assess individual GC sensitivity by measuring the effects of several clinically used GCs directly on gene expression in peripheral blood mononuclear lymphocytes (PBMLs). For this purpose, the effects of GCs on transactivation of the GC-induced leucine zipper (GILZ) gene and on transrepression of the interleukin-2 (IL-2) gene were determined by means of quantitative real-time PCR. As we were also interested whether these *ex vivo* outcomes could predict *in vivo* potencies of GCs, a group of fifteen healthy volunteers underwent a 0.25 mg dexamethasone suppression test (DST), while determining GILZ and IL-2 expression levels in their PBMLs incubated with hydrocortisone, and the synthetic GCs

dexamethasone (DEX), budesonide, and prednisolone. We also investigated the transactivating and transrepressing capacities of GR(ER22/23EK) and GR(N363S) from a GRE-driven or a p65-activated NF-κB luciferase reporter, respectively, and determined the effects of these polymorphisms on the regulation of GILZ and IL-2. As the GILZ and IL-2 expression assays have their limitations (such as costs, time and the amount of blood needed) we investigated whether is was possible to simplify and validate these assays to make them more applicable for larger populations.

Materials and methods

Materials, plasmids, and subjects

The steroids hydrocortisone, dexamethasone (DEX), prednisolone, triamcinolone acetonide (AC), budesonide, methylprednisolone, beclomethasone dipropionate (DP), deoxycorticosterone, aldosterone, megestrol acetate, progesterone, 6α-methyl-17α-hydroxy-progesterone acetate (MPA), and 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. Oligonucleotide primers for mutagenesis and quantitative real-time 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 pRShGR*α* expression plasmid, the GRE-luciferase (LUC) and NF-κB-LUC reporter plasmid, p65 plasmid, and pTZ plasmid were described previously (27, 28).

For the determination of transactivation and transrepression activities of GCs, peripheral blood from healthy volunteers (all Caucasian) was used. For the first series of experiments in which we tested the whole range of GCs, we obtained cells from a healthy 52-year old male, whereas a study group consisting of 10 males and 5 females (aged 23-37 yr; mean age, 27.7 ± 1.0 yr; not using GCs or oral contraceptives), was used for testing of interindividual variation (study group 1). 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 ten volunteers, all non-carriers of both polymorphisms, was used as control. All subjects were healthy, and none of them was using exogenous GCs. For the downscaling of the GILZ and IL-2 expression assays, we used PBMLs from fifteen healthy volunteers (all Caucasian; 6 males, 9 females; mean age 30 yr; range 22-52 yr; mean BMI 22 kg/m^2) (study group 2). Again, the results of the control group were used as comparison. Informed consent from all subjects and approval from the Medical Ethics Committee of Erasmus MC, The Netherlands, was obtained.

DST

The 0.25 mg-DST was performed as previously described (29). Briefly, venous blood for serum cortisol measurements was obtained between 0800 and 0900 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.

Blood cell preparations and whole cell DEX binding assay

Peripheral blood was collected by venapuncture in heparinized tubes, and PBMLs were obtained after density centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) as previously described (30). The number of GRs per cell (n) were determined as previously described (10).

Cortisol measurements

Blood was drawn by venapuncture and allowed to coagulate for at least 30 min. Subsequently, serum was separated by centrifugation and quickly frozen at –20 ^{0}C. Cortisol concentrations were determined using the Immulite 2000 (Diagnostic Products Corp., Los Angeles, CA, USA). Between-assay variability was less than 10.4%.

Plasmid construction

pcDNA3hGR*α* was generated by digesting pRShGR*α* with *Kpn*I and *Xho*I and cloning the resulting fragment into the *Kpn*I and *Xho*I sites of pcDNA3. The ER22/23EK and N363S polymorphisms were introduced independently into pcDNA3hGR*α* by using a QuickChange site-directed mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands) according the manufacturer's guidelines.

Cells and culture conditions

The acute lymphoblastic T cell leukemia cell line CCRF-CEM (no. CCL-119; American Type Culture Collection, Manassas, VA) was cultured in RPMI 1640 medium containing L-glutamine (Life Technologies, Inc. Europe, Breda, the Netherlands) supplemented with 4.5 g/l glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (Life Technologies, Inc. Europe). Cortisol levels in the culture medium were below detection limits (data not shown). Cells (4x 10^6 per incubation) were incubated at a density of 10 x 10^6 cells/ml for 4 h at 37 ^{0}C with 10^{-7} M steroids (including GR antagonists), after which they were collected.

PBMLs were suspended in RPMI 1640 medium containing L-glutamine (Life Technologies, Inc. Europe) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (Life Technologies, Inc. Europe). Cells were incubated for 30 min at 37 ^{0}C in a shaking water bath in order to remove endogenous cortisol. Afterwards, medium was replaced, and 4 x 10^6 cells per well (2 x 10^6 cells per well for the GR-polymorphism experiments, and 0.25 x 10^6 cells per well for the downscaling experiments) were pre-cultured overnight (5% CO_2, 37 ^{0}C) in 48-well plates at a density of 10 x 10^6 cells/ml (4 x 10^6 cells/ml for the GR-polymorphism experiments). The next day, PBMLs were incubated for 4 h with different concentrations of GCs together with 10 µg/ml phytohemagglutinin (PHA) (Sigma-Aldrich). Afterward, cells were collected.

Monkey kidney (COS-1) cells were maintained in a 5% CO_2 humidified incubator at 37 ^{0}C in DMEM tissue culture medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/l penicillin, 100 mg/l streptomycin, and 1.25 mg/l Fungizone and passaged every 3-4 d.

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 (h)GRα 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 hGRα 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.

Reporter luciferase assay

Five hours after transfection, the indicated concentrations of DEX were added. Twenty hours later, cells were lysed in 100 µl lysisbuffer [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 (Perkin-Elmer, Groningen, 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.

Western Immunoblot Analysis

Cells were lysed in buffer containing 50 mM TrisHCl (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%) (31) 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% non-fatty milk powder for 1 h at room temperature. Blots were incubated overnight at 4 ^{0}C in block buffer, supplemented with the Anti-hGR 57 antibody (1:2500) (10P's, Breda, The Netherlands) or the anti-hGR (P-20) antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA). Thereafter, blots were washed in TBS-T and subsequently incubated with horseradish peroxidase goat anti-rabbit 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).

RNA isolation and RT-PCR

PBMLs were washed with 0.15 M NaCl, and total RNA was isolated from CCRF-CEM cells and PBMLs using a high pure RNA isolation kit (Roche Diagnostics GmbH,

Mannheim, Germany), and directly frozen at –80 ^{0}C. An RT-PCR was performed using 200 ng of total RNA per reaction (400 ng for CCRF-CEM cells and 80 ng for the downscaled assay). 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).

Quantitative real-time PCR

GILZ, IL-2 and GR-α gene expression levels were determined in a quantitative real-time PCR, by using primers and probes that were designed by using the Primer Express software (Applied Biosystems, Foster City, California, USA). Correction for assay variability was performed using the housekeeping gene hypoxanthine phosph oribosyltransferase (HPRT) of which expression levels are stable and not influenced by GCs in this cell-system (not shown). The primer sequences used are presented in Table 1. The reaction mixture to determine GILZ, IL-2 and HPRT mRNA expression levels, with a total volume of 25 µl, contained 2.5 µl cDNA template (obtained from RT-PCR), 12.5 µl Universal Master Mix (Roche, Branchburg, NJ), 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), whereas the reaction mixture to determine GR-α levels contained 2.5 µl cDNA template, 7.5 pmol/µl of each primer, and 5 pmol/µl probe in a qPCR-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 samples from the CCRF-CEM cell line and the 52-yr-old male were analyzed in at least two independent assays with duplicate samples, whereas the samples from the two groups of 15 healthy volunteers were analyzed in one assay with duplicate samples. For the determination of the effects the GR polymorphisms on the regulation of GILZ and IL-2, four assays with quadruplicate measurements were performed per sample. For calculation of the relative amounts of GILZ, IL-2, and HPRT mRNA, the comparative threshold method was used, according to the manufacturer's guidelines.

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'-6FAM-CAA GCT TGC GAC CTT GAC CAT CTT TGG A-TAMRA-3'
GR-α: forward primer	5'-TGT TTT GCT CCT GAT CTG A-3'
GR-α: reverse primer	5'-TCG GGG AAT TCA ATA CTC A-3'
GR-α: probe	5'-FAM-TGA CTC TAC CCT GCA TGT ACG AC-TAMRA-3'

Statistical analysis

Data were analyzed using SPSS for Windows, release 10.1 (SPSS, Chicago, IL) and Instat software version 2.01 (GraphPad Software, Inc., San Diegeo, CA). Spearman rank correlation was used for analyzing relationships between data, and data points were fitted with regression-lines using the least-squares method. EC_{50} values and maximal values were calculated using non-linear regression. 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 non-carriers, were analyzed by the Student's *t* test using the area under the curve. The differences in EC_{50} values of DEX-induced up- and down-regulation of GILZ and IL-2 using less DEX concentration data-points were analyzed using a paired samples *t* test. Data were expressed as mean ± SEM Statistical significance was set at $P < 0.05$

Results

Optimization of GILZ and IL-2 expression assays

We optimized the GILZ and IL-2 expression assays using PBMLs from a healthy 52-yr-old male. The relative increase and decrease of GILZ and IL-2 mRNA levels under the influence of DEX are shown in Figures 1 and 2. Because intra- and interindividual differences were more pronounced at the concentration at which suboptimal effects were achieved (data not shown), we used a DEX concentration of 10^{-7} M in additional experiments. From Figure 1, we concluded that an incubation time of 4 h is the most suitable.

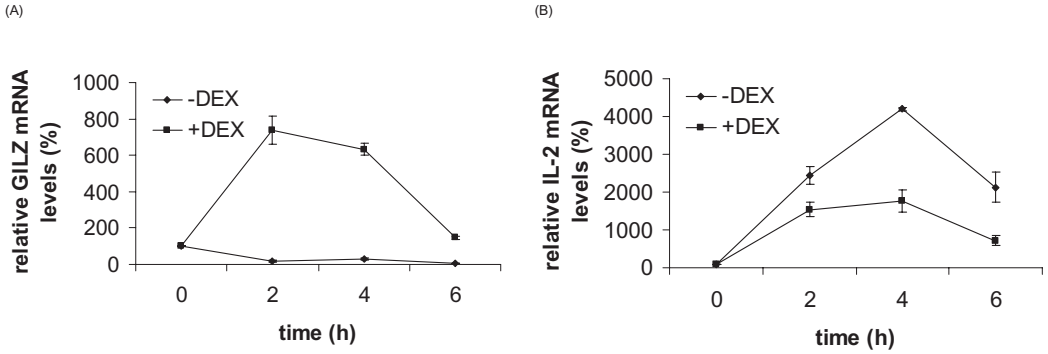

Figure 1. Relative GILZ (A) and IL-2 (B) mRNA levels after a 0-6 h incubation with 10^{-7} M DEX in PBMLs of a healthy volunteer. Data represent means ± SEM of one experiment, each with duplicate measurements.

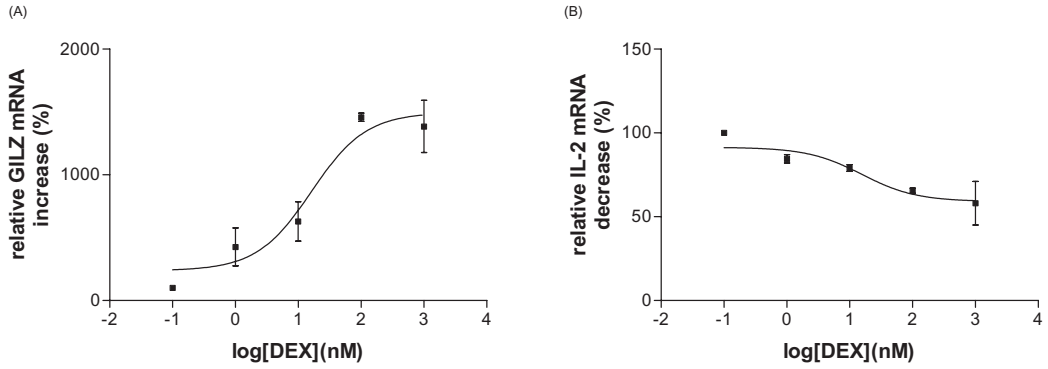

Figure 2. Relative increase in GILZ (A) and relative decrease in IL-2 (B) mRNA levels after a 4 h-incubation at the indicated concentrations of DEX in PBMLs of a healthy volunteer. Data represent means ± SEM of one experiment, each with duplicate measurements.

Specificity of the regulation by GCs and the GR in CCRF-CEM cells

In order to investigate whether up-regulation of GILZ gene expression is specifically regulated by GCs, CCRF-CEM cells were incubated for 4 h at 37^0C with 10^{-7} M of different steroids after which GILZ mRNA levels were determined. The housekeeping gene HPRT was not influenced by GC-treatment (data not shown). The GCs DEX and hydrocortisone were both able to increase GILZ mRNA levels to levels that were respectively 50- and 19-fold higher than in non-stimulated cells (Fig. 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 agonists (deoxycorticosterone and aldosterone), and the estrogen receptor agonist E2 were all unable to increase GILZ mRNA levels. However, incubation of the cells with the PR agonists megestrol acetate and MPA led to a slight induction of GILZ gene expression levels compared to non-stimulated cells.

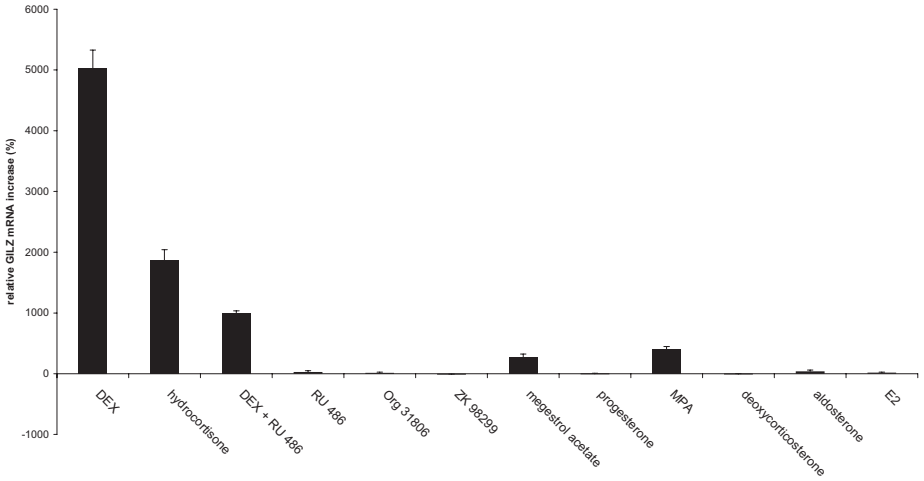

Figure 3. Relative increase in GILZ mRNA levels in CCRF-CEM cells incubated for 4 h with 10^{-7} M of steroids. Data represent means ± SEM of at least 2 experiments, each with duplicate measurements.

Transactivation and transrepression capacities of GCs in a healthy volunteer

When incubating PBMLs from a healthy volunteer for 4 h at 37^0C with 10^{-9} M, 10^{-8} M, and 10^{-7} M 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. Maximal effect and the concentration at which half of the maximal effect is achieved (EC_{50}) were calculated from the mean value of at least two separate assays. The results show that there existed considerable differences between the GCs used (Table 2), both with respect to the EC_{50} and to the maximal effect. Interestingly, high transactivating activity (low EC_{50} / high maximal effect in the GILZ expression assay) did not necessarily correspond to high transrepression activity (low EC_{50} / high maximal effect in the IL-2 expression assay).

Table 2. Maximal relative effect and EC_{50} values calculated from the relative increase in GILZ and relative decrease in IL-2 mRNA levels in PBMLs from a healthy control donor incubated for 4 h with 10^{-9} M, 10^{-8} M, and 10^{-7} M of GCs. Data represent means ± SEM of at least two experiments, each with duplicate measurements.

Glucocorticoid	GILZ EC_{50} (nM)	Max activation (%)	IL-2 EC_{50} (nM)	Max suppression (%)
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

Effects of GCs on GILZ and IL-2 gene expression levels

When incubating PBMLs from 15 healthy volunteers (study group 1) for 4 h at 37^0C with 10^{-7} M of hydrocortisone, DEX, budesonide and prednisolone (n = 8), large intra- and interindividual differences in transactivation and transrepression capacities were found for the different GCs (Table 3). However, studying the whole group, we found correlations in transactivation levels (GILZ) between hydrocortisone and DEX (r = 0.52; *P* = 0.046), hydrocortisone and budesonide (r = 0.48; *P* = 0.069), and hydrocortisone and prednisolone (r = 0.86; *P* = 0.007) (Fig. 4), and in transrepression levels (IL-2) between hydrocortisone and DEX (r = 0.62; *P* = 0.014), hydrocortisone and budesonide (r = 0.71; *P* = 0.003), hydrocortisone and prednisolone (r = 0.71; *P* = 0.047) (Fig. 5). 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 4) of the GCs tested between the GILZ and IL-2

Table 3. Relative increase of GILZ (A) and relative decrease of IL-2 (B) mRNA levels compared in PBMCs from 15 healthy subjects stimulated for 4 h with 10^{-7} M of hydrocortisone, DEX, budesonide, and prednisolone. Data represent means ± SEM.

(A)

	Relative increase in GILZ mRNA levels (%)			
Subjects	**hydrocortisone**	**dexamethasone**	**budesonide**	**prednisolone**
1	1057 ± 412	2248 ± 159	1914 ± 167	1907 ± 892
2	265 ± 81	1619 ± 330	870 ± 72	474 ± 174
3	662 ± 19	1250 ± 117	2223 ± 257	1228 ± 129
4	273 ± 55	776 ± 71	612 ± 72	312 ± 82
5	268 ± 22	1399 ± 525	1038 ± 547	N.D.
6	816 ± 120	1022 ± 135	1554 ± 174	N.D.
7	1099 ± 190	3503 ± 392	1992 ± 562	N.D.
8	209 ± 20	1362 ± 28	2312 ± 238	N.D.
9	1058 ± 146	2765 ± 448	2726 ± 88	N.D.
10	532 ± 78	2050 ± 75	1539 ± 77	1052 ± 157
11	307 ± 52	2355 ± 39	708 ± 77	515 ± 183
12	520 ± 94	2121 ± 370	1386 ± 83	N.D.
13	803 ± 149	2226 ± 659	2215 ± 319	N.D.
14	648 ± 2	2571 ± 144	944 ± 96	548 ± 23
15	537 ± 28	1874 ± 319	2466 ± 120	1357 ± 208

(B)

	Relative decrease in IL-2 mRNA levels (%)			
Subjects	**hydrocortisone**	**dexamethasone**	**budesonide**	**prednisolone**
1	-18 ± 25	32 ± 5	47 ± 11	9 ± 24
2	61 ± 12	61 ± 5	84 ± 4	41 ± 20
3	-2 ± 3	46 ± 13	52 ± 1	22 ± 4
4	24 ± 7	73 ± 2	74 ± 3	62 ± 3
5	63 ± 20	79 ± 3	90 ± 3	N.D.
6	62 ± 11	66 ± 2	72 ± 2	N.D.
7	70 ± 12	76 ± 5	80 ± 1	N.D.
8	43 ± 6	68 ± 7	83 ± 2	N.D.
9	63 ± 5	73 ± 4	86 ± 5	N.D.
10	73 ± 2	95 ± 1	83 ± 1	78 ± 3
11	47 ± 14	30 ± 0	79 ± 7	72 ± 2
12	-17 ± 25	57 ± 9	22 ± 7	N.D.
13	43 ± 5	64 ± 8	72 ± 8	N.D.
14	51 ± 1	33 ± 6	67 ± 2	58 ± 7
15	70 ± 2	60 ± 5	81 ± 1	68 ± 8

N.D.= non determined

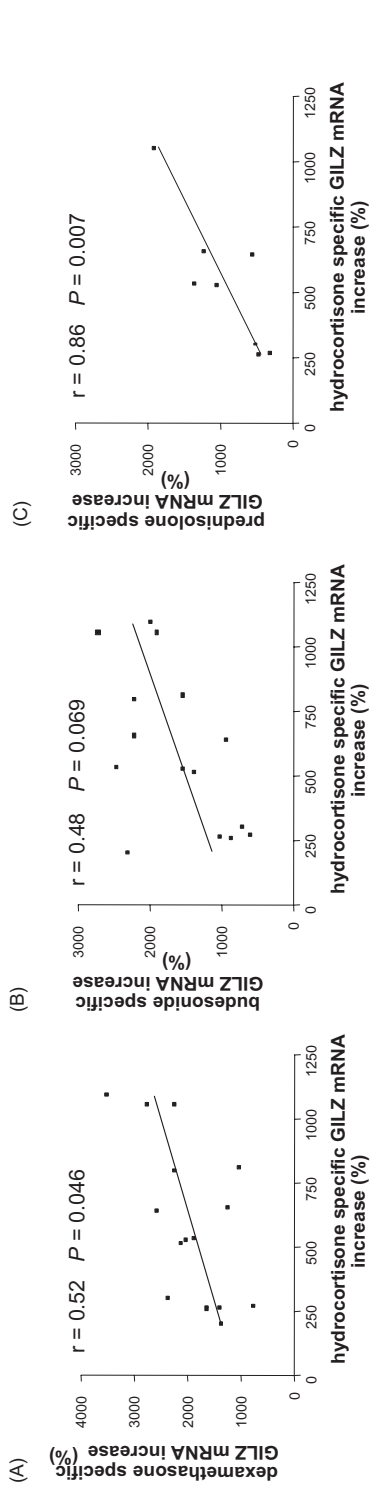

Figure 4. Correlation analysis (Spearman's correlation) between induction of GILZ mRNA levels by 10^{-7} M of hydrocortisone and DEX (A), budesonide (B) and prednisolone (C) in PBMLs of study group 1 (n = 15).

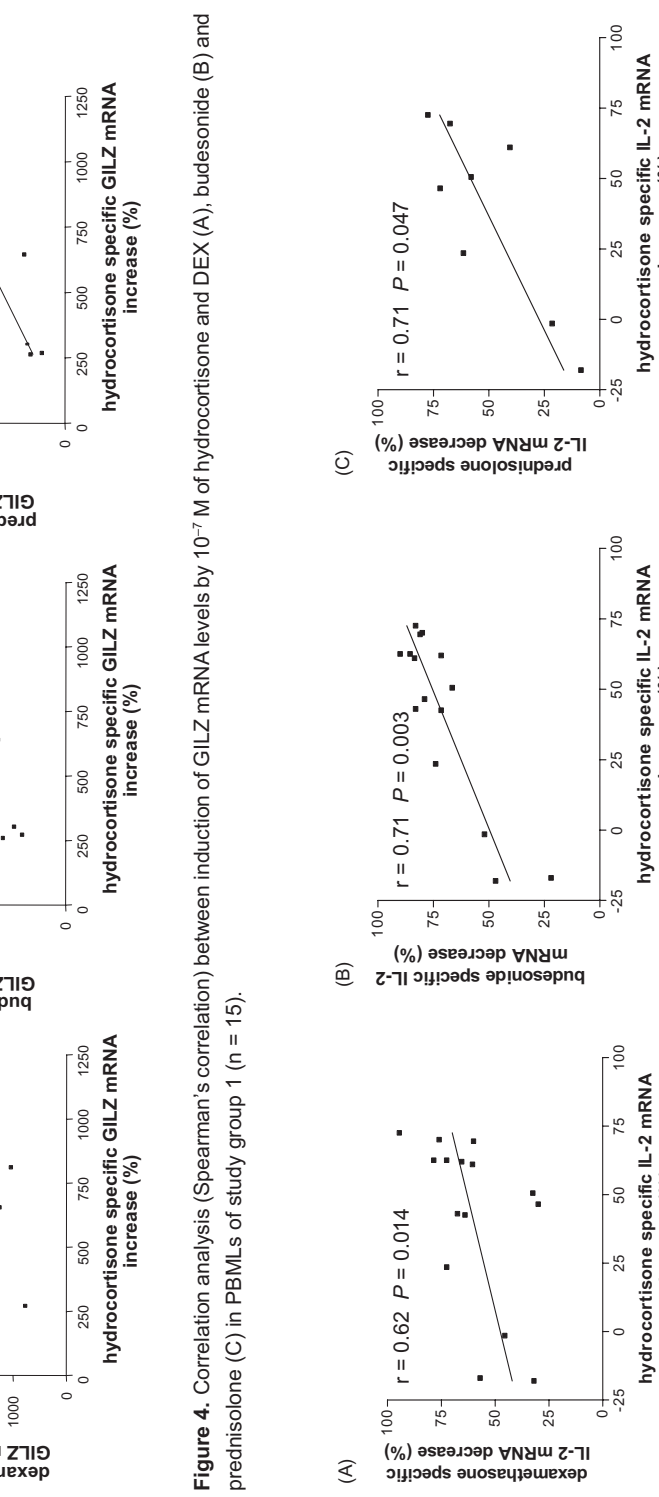

Figure 5. Correlation analysis (Spearman's correlation) between repression of IL-2 mRNA levels by 10^{-7} M hydrocortisone and DEX (A), budesonide (B) and prednisolone (C) in PBMLs of study group 1 (n = 15).

expression assays. In the GILZ expression assay, the order in potencies for the different GCs was, from greatest to least, DEX, budesonide, prednisolone, and hydrocortisone, whereas this was budesonide, DEX, prednisolone, and hydrocortisone in the IL-2 expression assay.

Table 4. Mean rank and mean effect of 10^{-7} M of hydrocortisone, DEX, budesonide and prednisolone in the GILZ expression assay (A) and the IL-2 expression assay (B). Data were calculated from Table 3 and represent means ± SEM of one experiment with duplicate measurements.

(A)

Glucocorticoid	Mean rank	Mean activation (%)
Hydrocortisone	3.7 ± 0.1	603 ± 80
DEX	1.3 ± 0.1	1943 ± 187
Budesonide	1.7 ± 0.1	1633 ± 178
Prednisolone	3.1 ± 0.1	924 ± 196

(B)

Glucocorticoid	Mean rank	Mean repression (%)
Hydrocortisone	3.4 ± 0.2	42 ± 8
DEX	2.3 ± 0.3	61 ± 5
Budesonide	1.1 ± 0.1	71 ± 5
Prednisolone	2.9 ± 0.2	51 ± 9

Effects of 0.25 mg of DEX on serum cortisol concentrations

All 15 subjects from study group 1 underwent a 0.25-mg overnight DST, and serum cortisol levels before and after the administration of DEX were measured (Table 5). No correlations were found between the results of the DST and GILZ and IL-2 expression assays.

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 (27). To examine overall receptor expression, immunoblot analysis was performed with GR-antibody 57, which is raised against amino acid 346-367 (32). Figure 6A 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 (33);

Table 5. Serum cortisol levels of study group 1 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

however, recently additional translation variants have been reported, and the 82-kDa band has been named GR-C (Met-86) (34). 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 semi-quantitative western blotting (21). 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 GR(N363S) was further confirmed by using an antibody (GR antibody P20) directed against a C-terminal epitope (Fig. 6B).

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 7A 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.

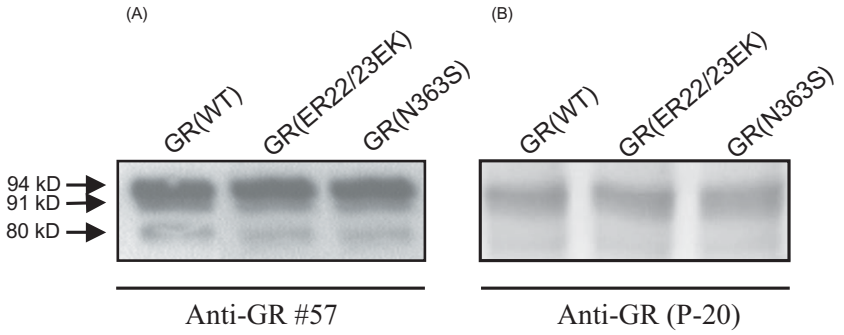

Figure 6. Expression of GR(WT), GR(ER22/23EK), and GR(N363S) variants from recombinant constructs. The pcDNA3.1hGR(WT), pcDNA3.1hGR(ER22/23EK), and pcDNA3.1hGR(N363S) vectors were transfected to COS-1 cells. After 24 h, cells were lysed, and 30 µg of proteins were electrophoresed on an 8% polyacrylamide 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 visualised by enhanced chemoluminescence. The 94-kDa band represents the full-length GR protein, whereas the 91- and 80-kDa bands represent translational variants.

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 co-transfecting a plasmid expressing the p65-subunit, luciferase expression was increased 5-fold, which was maximally repressed to 57 ± 8% by GR(WT) (Fig. 7B). Figure 7B also shows that the ER22/23EK and N363S receptor protein variants seemed to repress the p65-dimer activity to a similar extent, compared to the wildtype. However, the variability in these experiments was higher than in the transactivation experiments, and any effects might be obscured by noise.

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

PBMLs from carriers of the polymorphisms or from non-carrier 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 8. 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

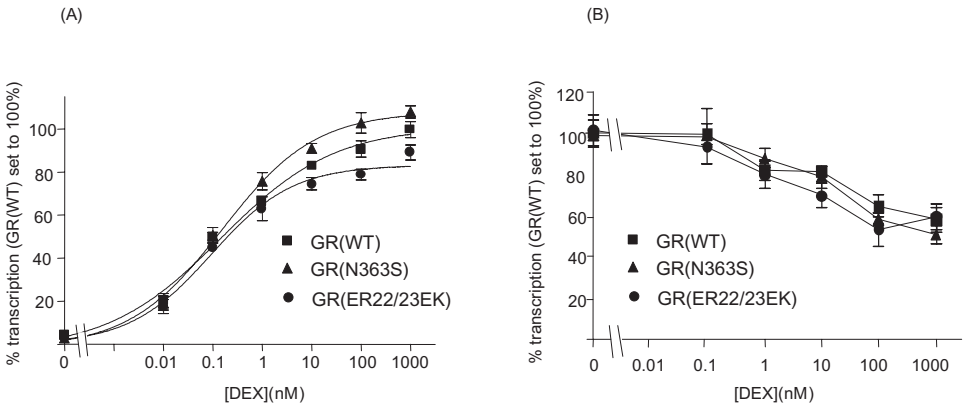

Figure 7. Transcriptional activation and repression capacities of GR(WT) (■), GR(ER22/23EK) (●), and GR(N363S) (▲). COS-1 cells were co-transfected with a GRE-LUC reporter construct (A) or a p65-activated NF-κB reporter (B) and vectors expressing GR(WT), GR(ER22/23EK), or GR(N363S). Five hours after transfection, cells were treated with the indicated amounts of DEX for 20 h, and luciferase activity was measured. Data represent means ± SEM of four experiments, each with quadruplicate measurements.

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 downregulation 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). Upon 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).

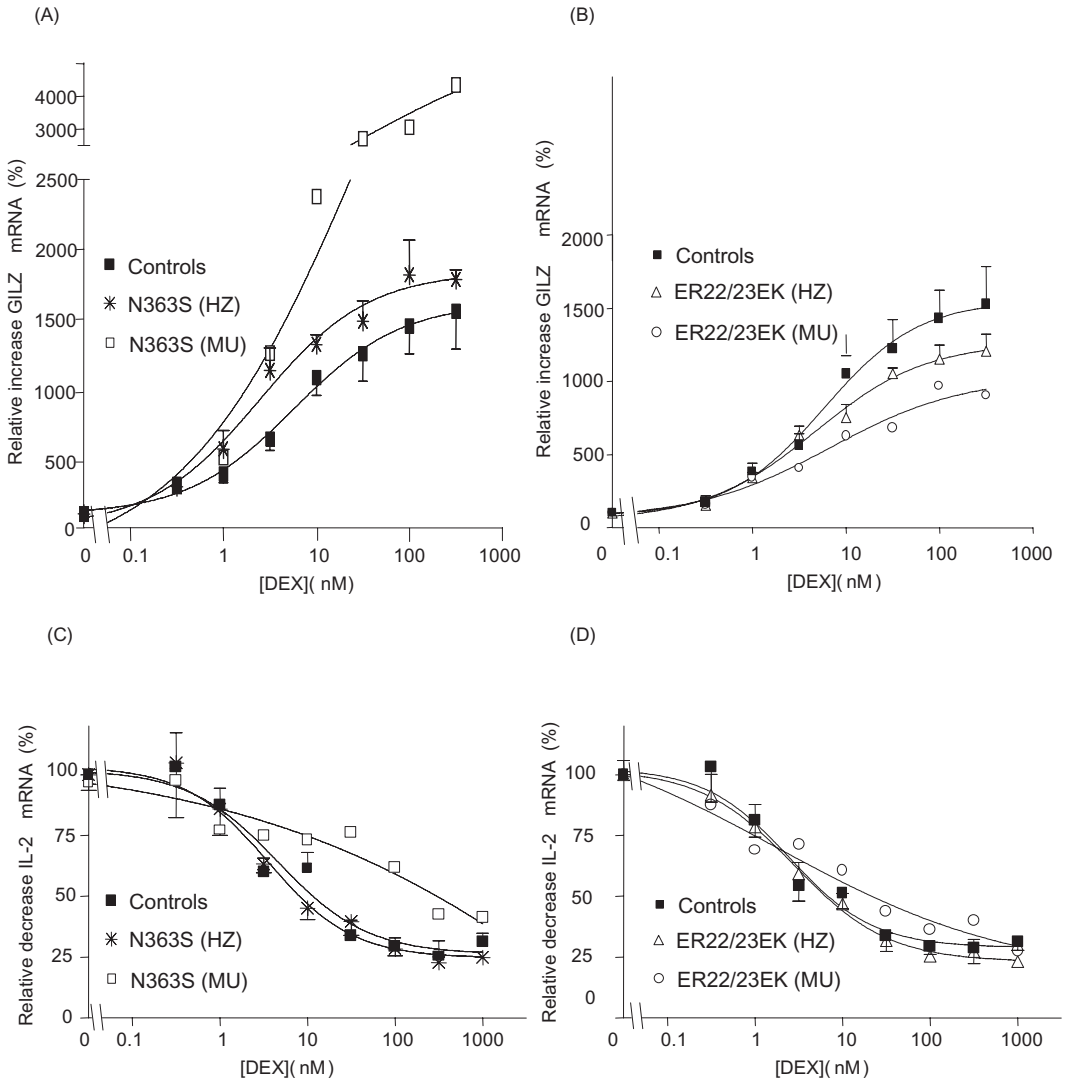

Figure 8. Relative responses of GILZ and IL-2 mRNA expression to DEX in PBMLs of non-carriers and carriers of either the ER22/23EK or N363S polymorphism in the GR gene. PBMLs of homozygous (MU, n = 1) and heterozygous (HZ, n = 3) carriers of either the ER22/23EK or N363S polymorphisms in the GR gene 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 non-carriers 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 ± SEM of the average response in PBMLs of the indicated number of subjects. No systemetic 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).

Reduction of cell number for the GILZ and IL-2 expression assays

Our first objective was to reduce the amount of PBMLs necessary for the GILZ and IL-2 expression assays. We compared two assays with PBMLs from a 25-yr-old healthy female, using 0.25 x 10^6 cells per well and 2 x 10^6 cells per well. The relative increase and decrease of GILZ and IL-2 mRNA levels under the influence of DEX for the two assays are presented in Figure 9.

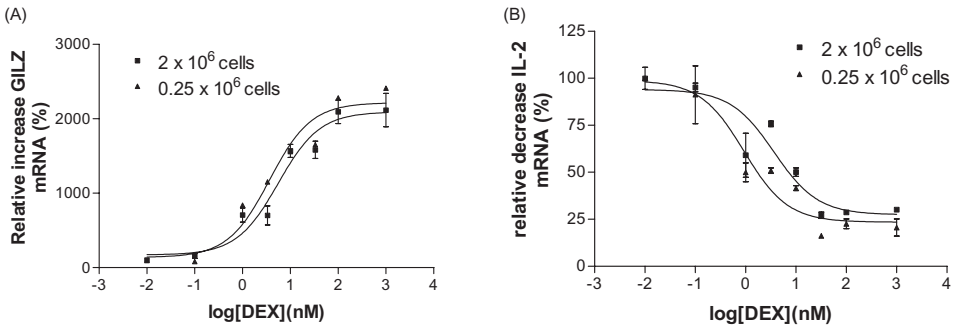

Figure 9. Relative increase in GILZ (A) and relative decrease in IL-2 (B) mRNA levels of 2 x 10^6 cells compared with 0.25 x 10^6 cells with PBMLs of the same person after 4h incubation at the indicated concentrations of DEX. Data represent means ± SEM of one experiment, each with duplicate measurements.

We measured the transactivation of GILZ and transrepression of IL-2 by determining the effect of DEX on the mRNA expression level using PBMLs from 15 healthy volunteers (study group 2) (data not shown). The assay was performed with eight different DEX concentrations ranging from 10^{-11} M to 10^{-6} M, and EC_{50} was calculated. The results were compared with the results previously obtained from the control group (Fig. 10).

Reduction of data-point number for the GILZ and IL-2 expression assays

We also calculated the EC_{50} values with six DEX concentrations from the collected data from study group 2, ignoring the results of the DEX concentrations of 10^{-10} and 10^{-7} M. No significant difference was shown between the EC_{50} calculated on the basis of six and eight DEX concentrations for GILZ (P = 0.44) and for IL-2 (P = 0.20) expression. The EC_{50} values calculated with 8 and 6 DEX concentration data-points are shown in Figure 11.

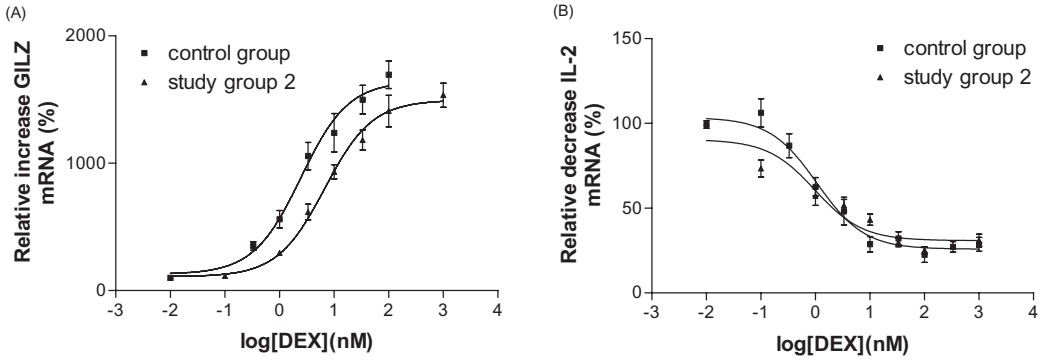

Figure 10. Relative increase in GILZ (A) and relative decrease in IL-2 (B) mRNA levels of the control group performed with 2 x 10^6 cells compared with study group 2 (n = 15) performed with 0.25 x 10^6 cells after 4h incubation at the indicated concentrations of DEX. Data represent means ± SEM. Individual experiments were carried out at least once with duplicate measurements.

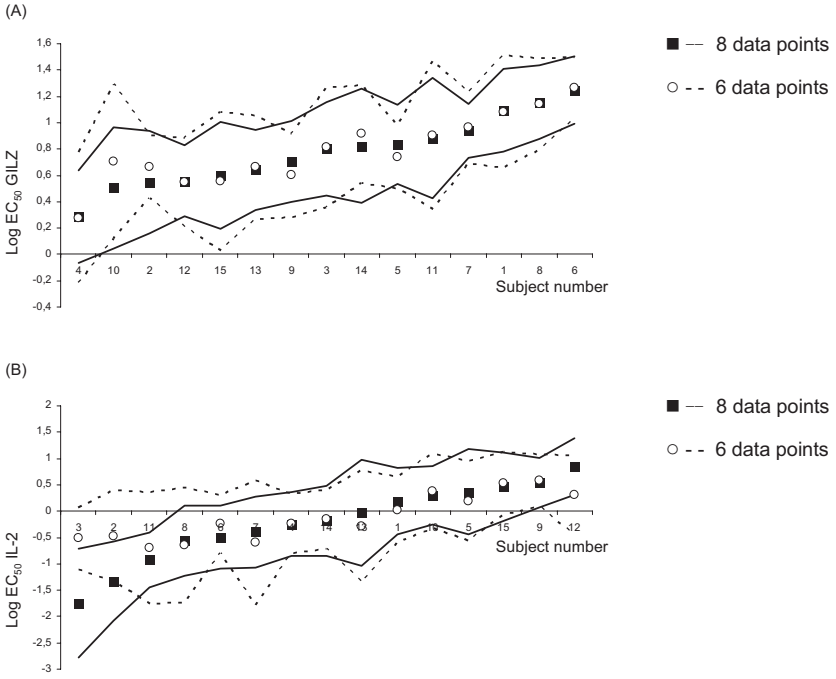

Figure 11. Logarithms of EC_{50} concentrations for GILZ (A) and IL-2 (B) expression from study group 2 (n = 15) calculated with 8 and with 6 DEX concentration data points.

Discussion

Differential regulation of GCs on GILZ and IL-2 expression

In this study, we investigated individual sensitivity towards several clinically used GCs by determining the effects on gene expression levels in PBMLs. For this purpose, we developed two new assays for the determination of the potencies of these GCs in transactivation and transrepression using quantitative real-time PCR analysis for expression of GILZ and IL-2. We demonstrated that GILZ gene expression is 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 comparing the regulation of GILZ and IL-2 gene expression levels by several GCs in one person, large differences in both EC_{50} and maximal values were shown. Because it is thought that most known side effects of GCs are driven by transactivation rather than transrepression (12-14), 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 GCs showing a high EC_{50} and a low maximal value in the GILZ expression assay in combination with a low EC_{50} and a high maximal value in the IL-2 expression assay might therefore be an indication for a beneficial outcome with minor adverse effects in an individual patient.

We were also interested whether there is a relationship between the *ex vivo* outcomes from our expression assays and the *in vivo* effects of GCs. For this purpose, we selected a group of 15 healthy volunteers (study group 1; not using GCs or oral contraceptives) and subjected them to a 0.25-mg DST. In parallel, we measured *ex vivo* the induction of the expression of GILZ and the suppression of the expression of IL-2 by several GCs 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. This lack of correlation may reflect the mechanistic differences between stimulation and repression of gene expression *ex vivo* and the process of HPA-axis regulation *in vivo*.

Although we found large intra- and interindividual variation in the GILZ and IL-2 assays, 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. With the GILZ expression assay, the order was, from greatest to least, DEX, budesonide, prednisolone, hydrocortisone, whereas it was budesonide, DEX, prednisolone, hydrocortisone in the IL-2 expression assay. Whelan *et al.* (35) compared in their study the potency of budesonide, DEX and hydrocortisone (and beclomethasone DP) in inhibition of IL-5 and IFN-γ and found a ranking of, from greatest to least, budesonide, DEX, hydrocortisone, which is similar to our results in the IL-2 expression assay.

Effects of ER22/23EK and N363S polymorphisms on GILZ and IL-2 expression

The ER22/23EK polymorphism influences the transactivating capacity of the GR: in transfected cells a reduced capacity for GRE-driven LUC activation (Fig. 7A) was found, 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 (Fig. 8A). 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 (Fig. 7), 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 (Fig. 8D).

It was 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 (21), of which the latter had a stronger transactivating effect in transient transfection experiments (33). 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 (33), explaining the unchanged transrepression.

With respect to the N363S polymorphism, we found that it increased transactivating capacity of the GR. In transfection experiments, the N363S polymorphism increased transcription from the GRE-LUC reporter (Fig. 7A), whereas in PBMLs, expression of GILZ mRNA was increased in cells both from heterozygous and homozygous carriers

of this polymorphism (Fig. 8A). 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 (Fig. 7B) 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 (Fig. 8C), 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 (36-38). 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 (39, 40) 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 9 heterozygous carriers whose PBMLs were tested in a mitogen-stimulated proliferation assay (16). There a tendency was found 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 (39, 40), and it has been suggested that the N363S variant introduces a new phosphorylation site, possibly altering interactions with other transcription factors (26). However, this serine residue is not in one of the currently known consensus phosphorylation sites (40).

Simplification of GILZ and IL-2 expression assays

Downscaling the GILZ and IL-2 expression assays by using 0.25 x 10^6 cells instead of 2 x 10^6 per well within the same person, resulted in comparable response in transactivation of GILZ, but different results for transrepression of IL-2 (Fig. 9). When

we compared the results of 15 healthy persons (study group 2) with the control group in which 2 x 10^6 cells per well were used (Fig. 10), the results were comparable. This makes the GILZ and IL-2 expression assays more applicable to larger study populations, and even small infants.

Using less data points (e.g. DEX concentrations), the estimated EC_{50} values with 6 DEX concentrations were overall not different from using 8 DEX concentrations. However, we had to accept a greater uncertainty as indicated by a wider confidence interval (Fig. 11). This might limit the usefulness of this assay to detect small differences in GC sensitivity.

In conclusion, in this study we describe the usage of the GILZ and IL-2 expression assays using quantitative real-time PCR. We showed that whereas there exists considerable interindividual variation in GC sensitivity within the healthy population, GC sensitivity is rather stable within an individual person. We also demonstrated that the ER22/23EK and N363S polymorphisms in the GR gene can partly explain interindividual variation in sensitivity to GCs. Finally, we showed that it is possible to downscale the GILZ and IL-2 expression assays, making these assays more applicable for larger study populations and even small infants. These GILZ and IL-2 expression assays may therefore be useful in determining the optimal type and dosage of GC in individual patients.

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Chapter 3

Strategies for the Characterization of Disorders in Cortisol Sensitivity

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
* both authors contributed equally to this work

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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/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 interleukin-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.

Introduction

Glucocorticoids (GCs) are key-hormones in metabolic and immunologic 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 in order 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, oligomenorrhoea, 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 (6-8), 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 co-activators (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 (17-19) 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.

Methods

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 (Δ_4) 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/l; normal range (N), <145nmol/l]. Urinary free cortisol [250–340 nmol/24 h (N, 40-200 nmol/24 h)], as well as early morning cortisol [1280 nmol/l (N, <850 nmol/l)], 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/l (N, 1-3 nmol/l); dehydroepiandrosterone sulfate was 37-43 µmol/l (N, 3-13

μmol/l). 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 years after transplantation were clinically largely uneventful. He has no other specific diseases. Blood pressure is normal.

Because of this extra-ordinary 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 (Timmermans, M. A., F. H. de Jong, unpublished results). He was treated with GCs and MCs (final height, 167cm; -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 in order to overcome adrenal insufficiency, indicating GC resistance.

Currently, 20 mg of hydrocortisone three times per day (N, 8-15 mg/m^2•d) or 0.5mg 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) in order 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 (budenoside 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/l was too low (N, 200-600 nmol/l) 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.

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 venapuncture. 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 phytohaemagglutinine (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 quantitative real-time PCR, as described previously (21, 22).

EBV 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.

Sequence analysis

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

Results

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 single-nucleotide 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). Codon 750 and 588 in, respectively, patient 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 (Fig. 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 Fig. 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 quantitative 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.

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, 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

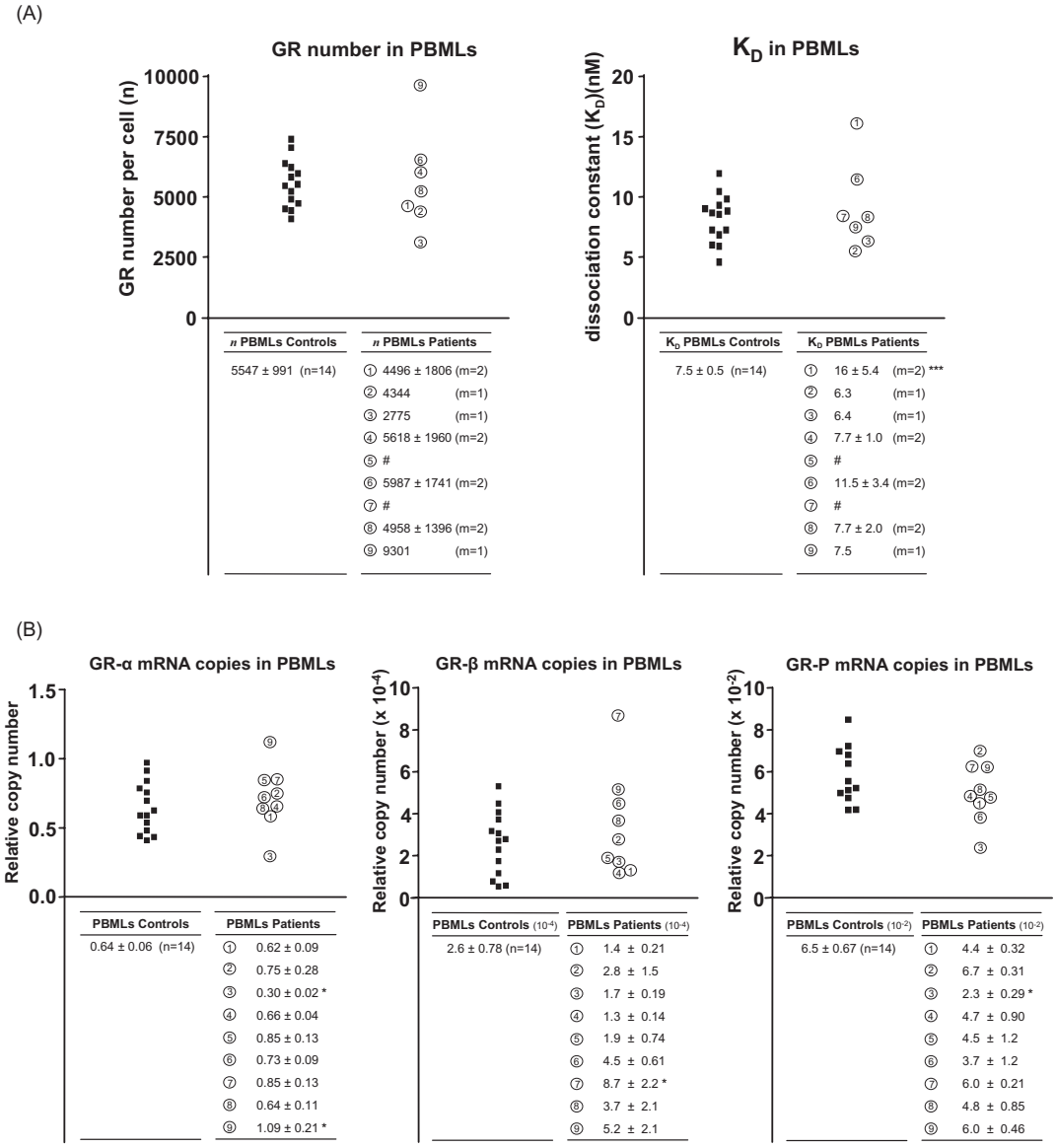

Figure 1. GR characteristics and GR mRNA copy numbers in PBMLs of patients and healthy controls. GR number per cell (n) and dissociation constant (K_D) (A) and relative copy numbers of GR-α, GR-β, and GR-P splice variants (B) in PBMLs of patients with affected GC sensitivity (*circled numbers*) and controls (■). 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 phosphoribosyl transferase (HPRT) by applying the formula $2^{[CT\ (HPRT)\ -\ CT\ (GR)]}$. For further details, see Livak and Schmittgen (32). Data represent means ± SEM, 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.

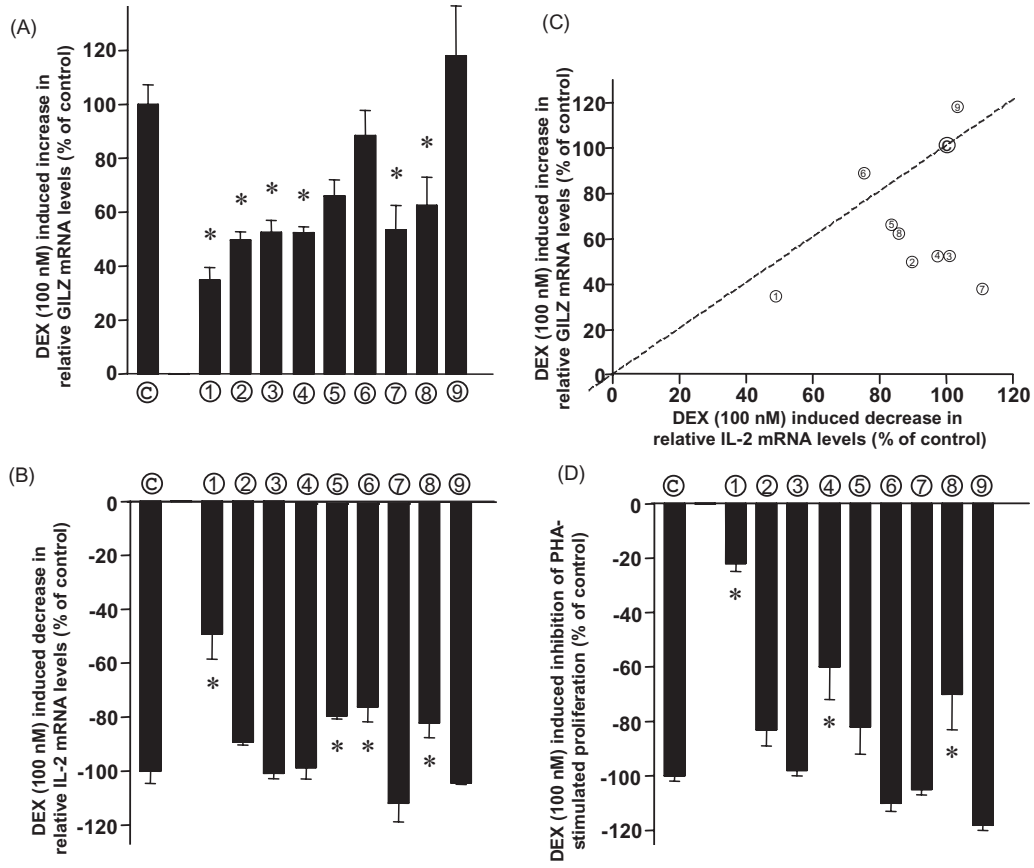

Figure 2. GILZ and IL-2 mRNA expression levels and repression of PHA-stimulated proliferation in PBMLs. Relative increase of GILZ (A) and decrease of IL-2 (B) mRNA levels induced by 100 nM DEX in PBMLs of patients (*circled numbers*) and controls (©). Cells were incubated for 4 h with PHA and with or 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 ± SEM, 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.

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 upregulation 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 Fig. 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 patient 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.

EBV transformed B lymphocytes

To obtain permanent cell lines, native B lymphocytes of patients and controls were transformed with EBV in order to obtain immortalized lymphoblast cell lines, and then GR number and ligand K_D were measured (Fig. 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

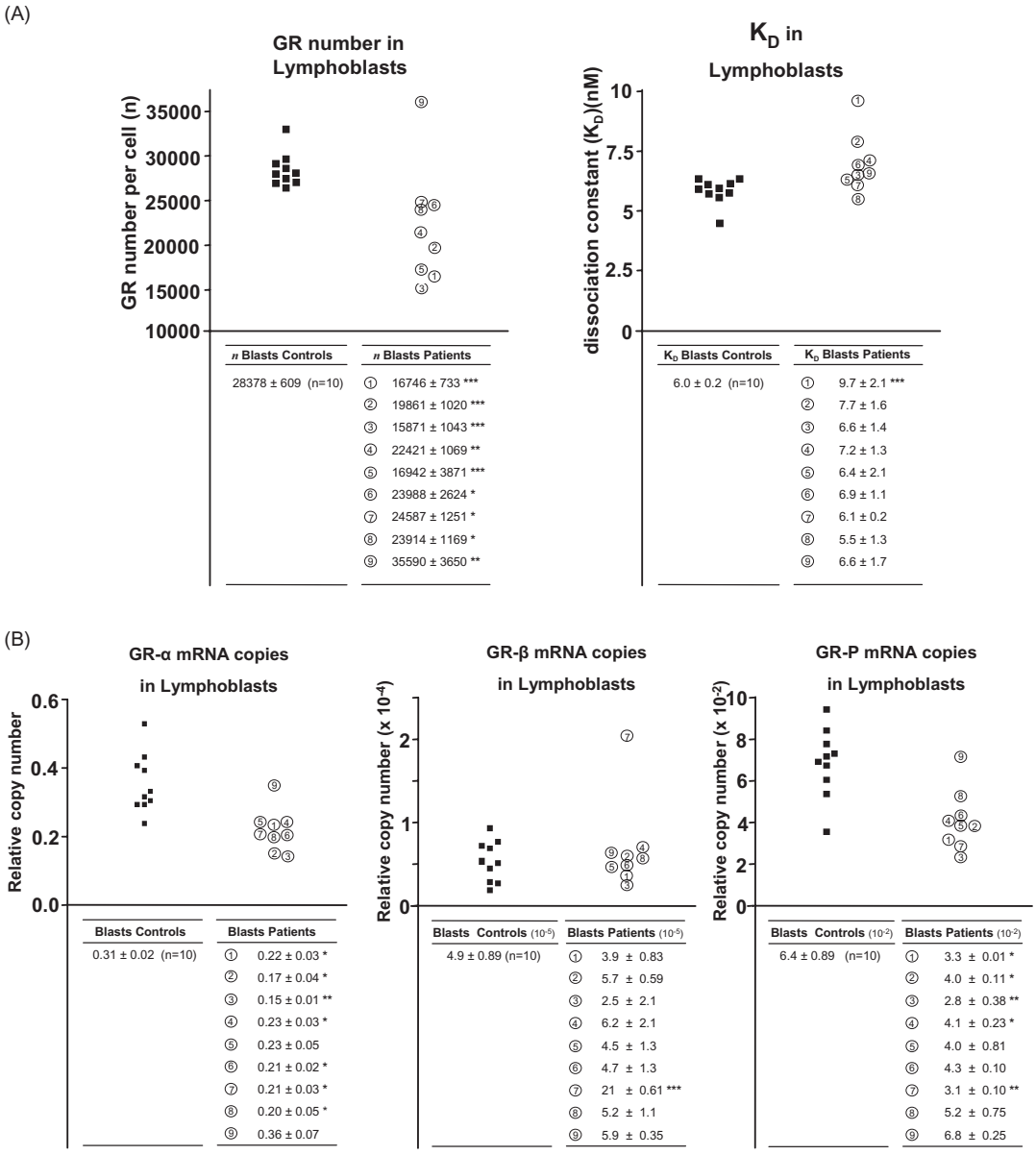

Figure 3. GR characteristics and GR mRNA copy numbers in lymphoblast cell lines of patients and healthy controls. GR number per cell (n) and K_D (A) and relative copy numbers of GR-α, GR-β, and GR-P splice variants (B) in lymphoblasts of patients with affected GC sensitivity (*circled numbers*) and controls (■). 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 ± SEM, and assays were performed at least three times in duplicate, exactly 3 months after transfection. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ by Student's *t*-test.

volume (data not shown). The increase in receptor number after EBV transformation 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 (Fig. 3A). Quantifying GR-α and GR-P mRNA expression levels also showed that GR expression is affected after viral transformation (Fig. 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 (Fig. 1B).

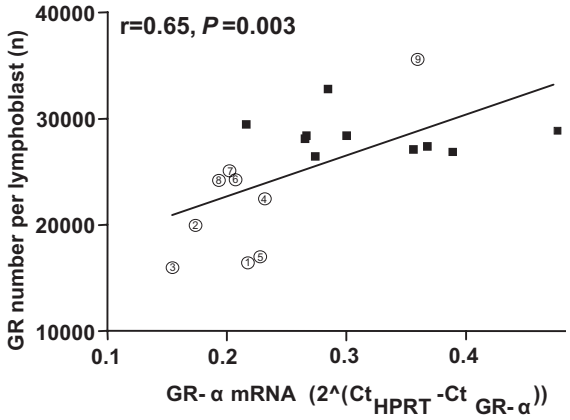

Figure 4. Correlation analysis (Spearman's correlation) between GR number and relative GR-α mRNA copy numbers in lymphoblast cell-lines of patients and healthy controls.

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. A table describing patients, genotype, clinical presentation, biochemical phenotype, and a summary of the data obtained from this study is shown in the supplementary table.

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 characterised 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 GR, might demonstrate the strong relationship between GR number and GC sensitivity. Transactivation is 50% reduced (Fig. 2A), whereas maximal transrepression was unaffected (Fig. 2, B and C). 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 patient 4 and 5 (Fig. 2) and on transrepression of IL-2 also in patients 5 and 6 (Fig. 2). GR expression

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

levels and characteristics were normal (Figs. 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 normalised 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 resuling in skipping of exon 9α in favour 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 real-time PCR did show that the GR-β expression was three to four times higher than in controls, both in PBMLs and in lymphoblasts (Figs. 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 (Fig. 2), whereas GR characteristics were normal (Fig. 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 (Figs. 1 and 3) and slightly increasing cellular sensitivity (Fig. 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 (Figs. 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 (Fig. 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 signalling 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 (Fig. 4) grouped all patients diagnosed as

CR in 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 4

No Association of the 11ß-Hydroxysteroid Dehydrogenase Type 1 Gene 83,557insA and Hexose-6-Phosphate Dehydrogenase Gene R453Q Polymorphisms with Body Composition, Adrenal Androgen Production, Blood Pressure, Glucose Metabolism and Dementia

Pauline Smit*, Marieke J.H.J. Dekker*, Frank Jan de Jong, Annewieke W. van den Beld, Jan W. Koper, Huibert A.P. Pols, Albert O. Brinkmann, Frank H. de Jong, Monique M.B. Breteler, Steven W.J. Lamberts
* both authors contributed equally to this work

Submitted

Abstract

Context: Cortisone reductase deficiency (CRD) is a rarely diagnosed disorder resulting in an ACTH-mediated androgen excess. Recently, it was proposed that a combination of the 83,557insA polymorphism in the 11ß-hydroxysteroid dehydrogenase type 1 gene (*HSD11B1*) and the R453Q polymorphism in the hexose-6-phosphate dehydrogenase (*H6PD*) gene interacts to cause CRD when at least three alleles are affected.

Objective: To study the separate and combined effects of these polymorphisms on body composition, adrenal androgen production, blood pressure, glucose metabolism, and the incidence of dementia in the healthy elderly population.

Design/Setting/Participants: The Rotterdam Study and the Frail Old Men Study, two population-based cohort studies in the elderly.

Main outcome measures: Genotype distributions and influences of (combined) genotypes on body mass index, adrenal androgen production, waist to hip ratio, systolic and diastolic blood pressure, fasting glucose levels, glucose tolerance test, and incidence of dementia.

Results: No influence of the *HSD11B1* 83,557insA (allele frequencies 22.0% and 21.5%) and *H6PD* R453Q (allele frequencies 22.9% and 20.2%) variants was found for the different outcome measures that were investigated, neither separately, nor when at least three alleles were affected.

Conclusions: Two population-based studies among Caucasian elderly showed no evidence for (combined) effects of two polymorphisms in the *HSD11B1* and *H6PD* genes on body composition, adrenal androgen production, blood pressure, glucose metabolism, and incidence of dementia. Moreover, the high frequencies observed for these two polymorphisms do not correspond to the low incidence of CRD observed in the general population. Altogether, it is unlikely that these polymorphisms cause CRD.

Introduction

Glucocorticoids (GCs) are ubiquitous, highly pervasive nuclear hormones, which exert their actions in almost all tissues. Binding of GCs to the glucocorticoid receptor is followed by a multistep process, ultimately leading to changes in the transcription rate of target genes (1). The two isoenzymes of 11β-hydroxysteroid dehydrogenase (11β-HSD) play a pivotal role in the prereceptor regulation of GC hormone action by catalyzing the interconversion of cortisol and cortisone: *in vivo*, 11β-HSD type 1 (11β-HSD1) converts cortisone to cortisol whereas 11β-HSD type 2 converts cortisol to cortisone.

11β-HSD1 is widely expressed. *HSD11B1*, located on chromosome 1q32.2, encodes a 34-kDa protein that resides within the lumen of the endoplasmatic reticulum (2-4). The enzymatic activity of 11β-HSD1 is bi-directional, processing both dehydrogenase (cortisol to cortisone) and oxo-reductase (cortisone to cortisol) components (3, 4). In intact cells or organs *in vivo*, it acts predominantly as an oxo-reductase (5).

The oxo-reductase activity of 11β-HSD1 requires NADPH. There is evidence that hexose-6-phosphate dehydrogenase (H6PDH) is the only source of NADPH within the lumen of the endoplasmatic reticulum and therefore it is crucial for the oxo-reductase activity of 11β-HSD1 (6-8). H6PDH, encoded by *H6PD,* which is localized on 1p36.2, is present in most tissues, but the highest expression levels are found in liver and adipose tissue, major sites of 11β-HSD1 oxo-reductase activity.

Individuals with cortisone reductase deficiency (CRD) excrete virtually all GCs as cortisone metabolites (tetrahydrocortisone) with low or absent cortisol metabolites (tetrahydrocortisols). This, together with low circulating cortisol levels after treatment with oral cortisone, suggests a defect in 11β-HSD1. Recently, Draper *et al.* (7) concluded from a study in kindreds with CRD that a combination of mutations in *HSD11B1* and *H6PD* interacts to cause CRD supposedly caused by a combination of reduction in 11β-HSD1 expression and impaired provision of NADPH. They proposed a digenic triallelic mode of inheritance, in which three alleles, from two (or more) loci (*HSD11B1* and *H6PD*) are necessary for trait manifestation. In this study, they described one polymorphism in *HSD11B1* (83,557insA) and two polymorphisms in *H6PD* (620ins29bp621 and R453Q). Subsequent analysis showed that the *H6PD* 620ins29bp621is absent in the normal population.

The aim of our study was to determine the role of two known single nucleotide polymorphisms (SNPs), *HSD11B1* 83,557insA (in complete linkage disequilibrium with T83,597G, rs12086634) and *H6PD* R453Q (rs6688832), either separate or combined, on body composition, adrenal androgen levels (androstenedione, dehydroepiandrosterone (DHEA), and DHEA sulfate (DHEAS)), blood pressure, glucose levels and incidence of dementia in the elderly. For this, we studied the Rotterdam Study, a population-based cohort study in the elderly and the Frail Old Men Study, a cross-sectional study in independently living elderly men.

Materials & Methods

The Rotterdam Study

We genotyped a total of 6105 subjects for *HSD11B1* 83,557insA and *H6PD* R453Q from the Rotterdam Study. This is a population-based, prospective cohort study of men and women aged 55 years and over (9). All residents aged 55 or older of Ommoord, a district of Rotterdam, The Netherlands, were invited to participate. A total of 7983 men and women (78% of those eligible) entered the study. Baseline examinations, including a home interview and an extensive physical examination at the research center, took place between 1990 and 1993. The Rotterdam Study was approved by the medical ethics committee of the Erasmus MC and written informed consent was obtained from all participants.

Frail Old Men Study

We genotyped a total of 347 Caucasian individuals for *HSD11B1* 83,557insA and *H6PD* R453Q from the Frail Old Men Study (10). This is a population-based cohort study in 403 independently living men, 70 yr of age and higher. Participants were recruited by letters of invitation, which were sent to all male inhabitants of Zoetermeer aged over 70, a medium-sized town in the Netherlands. All subjects provided informed consent to participate in the study, which was approved by the Medical Ethics Committee of the Erasmus MC. Subjects were judged sufficiently healthy to participate in the study if they were physically and mentally able to visit the study center independently. No additional health-related criteria were used.

DNA analysis

The appropriate Assay-by-Design mixes were designed, synthesized and supplied by Applied Biosystems (Foster City, CA, USA) (sequences shown in Table 1). Five-µl PCR reactions containing ~10 ng of DNA, 0.0625 µl of 80x Assay-by-Design mix, 2.4375 µl water, and 2.5 µl Universal Master Mix (Applied Biosystems) were performed in 384-well plates. The reaction conditions were: 2 min 50 ^{0}C followed by 40 cycles of 10 min 95 ^{0}C- 15 sec 92 ^{0}C- 60 sec 60 ^{0}C. A number of water controls were run in parallel with the DNA samples. Plates were analyzed using the Applied Biosystems 7900HT Sequence Detection System and SDS version 2.0 software (Applied Biosystems).

Table 1. TaqMan primer and probe sequences used for *HSD11B1* 83,557insA and *H6PD* R453Q genotyping

Gene	Primer/ probe name	Sequence 5'-3'
HSD11B1	Forward primer	CTT-ACC-TCC-TCC-TCT-GAA-CTT-TGC
	Reverse primer	TCC-TCC-TGC-AAG-AGA-TGG-CTA-TAT-T
	Wildtype-specific probe (FAM)	CAC-CAA-GAG-CTT-TT
	InsA-specific probe (VIC)	CAC-CAA-AGA-GCT-TTT
H6PD	Forward primer	TCT-GTC-CGA-TTA-CTA-CGC-CTA-CA
	Reverse primer	GGC-CAT-GGA-AGA-TAT-GGG-ATA-AGA-G
	453R-specific probe (FAM)	CTG-TGC-GGG-AGC-G
	453Q-specific probe (VIC)	CCT-GTG-CAG-GAG-CG

Anthropometric measurements

Body weight and height of the subjects were measured, and body mass index (BMI) was defined as weight divided by the square of height (kg/m^2). The waist circumference was measured at the level of the umbilicus and the hip circumference was measured at the level of the greater trochanter. Waist-to-hip ratio (WHR), which represents a measure of upper adiposity, was calculated from these measurements. In the Frail Old Men Study, total lean body mass and fat mass were measured by dual energy x-ray absorptiometry (DEXA) as previously described (11, 12).

Hormonal measurements

In a limited number of subjects of the Rotterdam Study, plasma levels of androstenedione (n = 1608) and DHEAS (n = 1654) were determined. Plasma levels of androstenedione

and DHEAS were estimated in 12 separate batches of samples using coated-tube RIAs purchased from Diagnostic Systems Laboratories, Inc. (Webster, TX, USA). Due to the relatively small volumes of plasma available, all values reported are single-sample estimations. Intraassay coefficients of variation, determined on the basis of duplicate results of internal quality control (QC) plasma pools with three different levels of each analyte, were below 12 and 7% for androstenedione and DHEAS, respectively. Since interassay variations were relatively large (23% for androstenedione and 24% for DHEAS) results of all batches were normalized by multiplying all concentrations within a batch with a factor, which made results for the internal QC pools comparable. This reduced interassay variations to 9% for androstenedione and 10% for DHEAS and was considered justified because the patterns of the results of these pools and the mean results for male and female sera in one assay batch were very similar.

In the Frail Old Men Study, serum DHEA and DHEAS levels were determined by radioimmunoassay (Diagnostics Products Corporation, Los Angeles, USA) in nearly all subjects (n = 346). The intraassay coefficients of variation (CV) for these assays were: 3.8% and 2.1%, respectively. The interassay CVs were 8.6% and 5.1%, respectively.

Blood pressure measurement

Blood pressure was measured in sitting position at the right upper arm with a random-zero sphygmomanometer (the Rotterdam Study, n = 5919; Frail Old Men Study, n = 346). Persons using blood pressure lowering drugs and persons without data on blood pressure lowering drugs were excluded from the statistical analysis with regard to blood pressure. 1903 persons of the Rotterdam Study were excluded versus 138 of the Frail Old Men Study.

Assessment of glucose metabolism

In the Rotterdam Study, participants who did not use anti-diabetic medication received a glucose drink of 75 g in 200 ml water (n = 4844). Two hours later a venous blood sample was obtained. Glucose levels were determined using the glucose hexokinase method. In the Frail Old Men Study, fasting glucose levels were measured (n = 346).

Subjects with diabetes mellitus (DM) and subjects without data on DM were excluded from the statistical analysis with regard to glucose metabolism. In the Rotterdam Study, DM was defined as the use of blood glucose-lowering medication

or random serum glucose concentration of at least 11.1 mmol/l, or both. In the Frail Old Men Study, DM was defined as the use of blood glucose-lowering medication only. 322 persons of the Rotterdam Study were excluded versus 31 of the Frail Old Men Study.

Diagnosis of dementia

Dementia screening and diagnosis in the Rotterdam Study followed a three-step protocol (13). Briefly, all subjects were screened with the Mini-Mental State examination (MMSE) and the Geriatric Mental State schedule, organic level (14, 15). Subjects with positive screening results (MMSE score <26 or GMS organic level >0 underwent the Cambridge examination for mental disorders of the elderly (16), and an informant interview was obtained. Subjects who were suspected of having dementia were examined by a neuropsychologist if additional neuropsychological testing was required for diagnosis. When available, imaging data were used. In addition, the total cohort was continuously monitored for incident dementia through computerized linkage between the study database and digitalized medical records from general practitioners and the Regional Institute for Outpatient Mental Health Care. The diagnosis of dementia and subtype of dementia was made in accordance with internationally accepted criteria for dementia (17) and Alzheimer's disease (AD) (NINCDS_ADRDA) (18) by a panel of a neurologist, neuropsychologist and research physician. Of all participants of the Rotterdam Study, 597 subjects met the definition of dementia of which 466 subjects were diagnosed with AD. In the Frail Old Men Study, no data on dementia were available.

Combined genotype analysis

As Draper *et al.* (7) proposed a digenic triallelic mode of inheritance, in which three distinct alleles, from two loci (*HSD11B1* and *H6PD*) are necessary for the manifestation of CRD, we compared carriers of at least three affected alleles with the rest of the study group. The combination of three or four affected alleles is called the 'CRD genotype'.

Statistical analysis

Data were analyzed using SPSS for Windows, release 10.1 (SPSS, Chicago, IL, USA). All data are presented as means ± SEM. Statistical analyses on body composition,

DHEA(S),androstenedione, blood pressure, and glucose metabolism were carried out by using the General Linear Model (GLM) procedure and linear regression (P_{trend}), and were corrected for age and sex. The analyses on DHEA(S) were also corrected for smoking. Analyses on body composition and DHEA(S) were stratified for sex. Statistical analysis on the incidence of dementia was performed using Cox' proportional hazards models adjusted for age and sex.

Results

The baseline characteristics of the Rotterdam Study and the Frail Old Men Study are shown in Table 2.

HSD11B1 83,557insA

In the Rotterdam Study, the wildtype genotype was observed in 3704 (60.7%) subjects, whereas 2110 (34.6%) subjects were heterozygous, and 291 (4.8%) subjects were homozygous for the *HSD11B1* 83,557insA SNP. Allele frequencies were 78.0% reference allele and 22.0% variant allele, respectively. In the Frail Old Men Study we found 217 (62.5%) homozygous wildtype carriers, 111 (32.0%) heterozygous carriers, and 19 (5.5%) homozygous carriers for the *HSD11B1* 83,557insA SNP. Allele frequencies were 78.5% reference allele and 21.5% variant allele, respectively. Both populations were found to be in Hardy-Weinberg equilibrium.

In the Rotterdam Study, no statistically significant associations of this SNP with the anthropometric parameters weight, BMI, waist circumference, hip circumference, and WHR were found. In female carriers, a significant trend towards higher length was found (wildtypes, 161.0 m ± 0.14; heterozygous carriers, 161.5 m ± 0.19; homozygous carriers 161.8 m ± 0.51; P_{ANOVA} = 0.06; P_{trend} = 0.02). There were no differences in adrenal androgen levels, blood pressure, glucose levels after a glucose drink or incidence of dementia between the different genotypes of the *HSD11B1* 83,557insA SNP. In the Frail Old Men Study, no differences in weight, BMI, WHR, trunk fatmass, trunk leanmass, total leanmass, and total fatmass were found for the different genotypes of *HSD11B1* 83,557insA. However, there was an association with lower height in carriers of the *HSD11B1* 83,557insA SNP (wildtypes, 173.4 m ± 0.43; heterozygous carriers, 172.2 m ± 0.59; homozygous carriers 170.4 m ± 1.44;

P_{ANOVA} = 0.049; P_{trend} = 0.02). There were no differences in adrenal androgen levels, blood pressure and glucose levels (no data shown).

Table 2. Baseline characteristics of the Rotterdam Study (n = 6105) and the Frail Old Men Study (n = 347). Data are presented as means (SD) unless stated otherwise.

Characteristic	Rotterdam Study	Frail Old Men Study
Sex, No. (%)		
male	2472 (40.5)	347 (100)
female	3633 (59.5)	0 (0)
Age (yrs)	69.5 (9.1)	77.6 (3.4)
Current Smoking, No. (%)		
yes	1347 (22.1)	60 (17.3)
no	4585 (75.1)	286 (82.4)
missing	173 (2.8)	1 (0.3)
Diabetes Mellitus, No. (%)		
yes	614 (10.1)	-
no	5089 (83.4)	-
missing	402 (6.6)	-
Use of Glucose Lowering Medication, No. (%)		
yes	261 (4.3)	30 (8.6)
no	4137 (67.8)	316 (91.1)
missing	1707 (27.9)	1 (0.3)
Glucose Tolerance Test (mmol/l)	6.9 (3.1)	-
Fasting Glucose (mmol/l)	-	5.8 (2.7)
Use of Blood Pressure lowering medication, No.(%)		
yes	1979 (32.4)	138 (39.8)
no	4124 (67.6)	208 (59.9)
missing	2 (0.0)	1 (0.3)
Systolic Blood Pressure (mmHg)	139.3 (22.1)	155.8 (23.9)
Diastolic Blood Pressure (mmHg)	73.7 (11.4)	83.5 (11.0)
Weight (kg)		
Male	78.4 (10.7)	76.3 (10.2)
Female	69.4 (11.3)	-
Height (cm)		
Male	174.7 (6.8)	172.9 (6.3)
Female	161.2 (6.6)	-
BMI (kg/m^2)		
Male	25.7 (3.0)	25.5 (2.9)
Female	26.7 (4.0)	-
WHR		
Male	0.96 (0.07)	0.98 (0.05)
Female	0.87 (0.09)	-
Trunk fatmass (kg)	-	10.7 (2.5)
Trunk leanmass (kg)	-	25.6 (29.7)
Total leanmass (kg)	-	52.1 (5.4)
Total fatmass (kg)	-	21.1 (5.5)
DHEA (nmol/l)	-	7.39 (3.91)
DHEAS (µmol/l)		
Male	4.38 (2.95)	1.98 (1.39)
Female	2.65 (2.03)	-
Androstenedione (nmol/l)		
Male	4.37 (1.87)	-
Female	3.58 (1.89)	-

H6PD R453Q

In the Rotterdam Study, the wildtype genotype was observed in 3655 (59.9%) subjects, whereas 2105 (34.5%) subjects were heterozygous, and 345 (5.7%) subjects were homozygous for the *H6PD* R453Q SNP. Allele frequencies were 77.1% reference allele and 22.9% variant allele. In the Frail Old Men Study we found 224 (64.6%) homozygous wildtype carriers, 106 (30.5%) heterozygous carriers, and 17 (4.9%) homozygous carriers for the *H6PD* R453Q SNP. Allele frequencies were 79.8% reference allele and 20.2% variant allele. Both populations were in Hardy-Weinberg equilibrium.

For the Rotterdam Study, we found no statistically significant associations of this SNP with the anthropometric parameters weight, height, BMI, waist circumference. Male carriers of the *H6PD* R453Q SNP had a smaller hip circumference compared to wildtypes (wildtypes, 98.7 cm ± 0.17; heterozygous carriers, 98.2 cm ± 0.23; homozygous carriers, 97.2 cm ± 0.60; P_{ANOVA} = 0.02; P_{trend} = 0.005). In female carriers, a significant trend towards higher WHR was found (wildtypes, 0.87 ± 0.002; heterozygous carriers, 0.87 ± 0.003; homozygous carriers, 0.88 ± 0.006; P_{ANOVA} = 0.10; P_{trend} = 0.035). No differences in adrenal androgen levels, blood pressure, glucose levels or incidence of dementia were found between the different genotypes of the *H6PD* R453Q SNP. In the Frail Old Men Study, no differences were found between the different genotypes of *H6PD* R453Q for the anthropometric parameters weight, height, BMI, WHR, trunk fatmass, trunk leanmass, total leanmass, and total fatmass. There were also no differences in adrenal androgen levels, blood pressure or glucose levels (no data shown).

Combined genotype groups

In the Rotterdam Study, 233 persons (3.8%) presented with the CRD genotype versus 14 persons (4.0%) in the Frail Old Men Study. In the Rotterdam Study, we found no statistical significant differences for the anthropometric parameters weight, height, BMI, waist circumference, hip circumference, and WHR in carriers of the CRD genotype (Table 3). There were also no differences in adrenal androgen levels (Table 3), blood pressure, glucose levels (data not shown), or incidence of dementia (Table 4). In the Frail Old Men Study, no differences were found for weight, height, BMI, WHR, trunk fatmass, trunk leanmass, total leanmass, total fatmass, adrenal androgen levels (Table 5), blood pressure or glucose levels (data not shown).

Table 3. Effect of CRD genotype on anthropometric parameters and hormonal measurements in the Rotterdam Study. Data are presented as means ± SEM. *P* values were adjusted for age. *P* values of DHEAS were also adjusted for smoking. No CRD genotype, carriers of two or less affected alleles; CRD genotype, carriers of at least three affected alleles.

	Women			Men		
	No CRD genotype	**CRD genotype**	***P***	**No CRD genotype**	**CRD genotype**	***P***
Weight (kg)	69.4 ± 0.20 (n = 3320)	69.4 ± 0.95 (n = 141)	0.80	78.4 ± 0.22 (n = 2336)	78.4 ± 1.20 (n = 80)	0.91
Heigth (cm)	161.2 ± 0.12 (n = 3313)	160.9 ± 0.56 (n = 140)	0.95	174.7 ± 0.14 (n = 2333)	174.2 ± 0.76 (n = 80)	0.42
BMI (kg/m^2)	26.7 ± 0.07 (n = 3310)	26.7 ± 0.34 (n = 140)	0.97	25.7 ± 0.06 (n = 2333)	25.8 ± 0.34 (n = 80)	0.68
Waist (cm)	87.7 ± 0.21 (n = 3139)	89.4 ± 1.00 (n = 132)	0.16	94.2 ± 0.20 (n = 2221)	95.2 ± 1.07 (n = 78)	0.38
Hip (cm)	101.1 ± 0.15 (n = 3138)	100.9 ± 0.75 (n = 130)	0.79	98.5 ± 0.14 (n = 2222)	98.2 ± 0.73 (n = 78)	0.66
WHR	0.87 ± 0.00 (n = 3137)	0.88 ± 0.01 (n = 130)	0.05	0.96 ± 0.00 (n = 2221)	0.97 ± 0.01 (n = 78)	0.11
DHEAS (µmol/l)	2.64 ± 0.07 (n = 837)	3.05 ± 0.31 (n = 42)	0.07	4.37 ± 0.11 (n = 709)	4.10 ± 0.59 (n = 25)	0.60
Androstenedione (nmol/l)	3.58 ± 0.07 (n = 838)	3.63 ± 0.30 (n = 39)	0.82	4.39 ± 0.07 (n = 703)	4.10 ± 0.35 (n = 28)	0.39

Table 4. Hazard ratios of dementia for carriers of the CRD genotype**.** *P* values were adjusted for age and sex. Reference, carriers of two or less affected alleles; CRD genotype, carriers of at least three affected alleles. All, all forms of dementia; AD, Alzheimer's disease; CI, confidence interval; HR, hazard ratio.

		HR	95% CI for HR	*P*
All (597/5758)	Reference	1.00		
	CRD genotype	1.09	0.74-1.59	0.67
AD (466/5758)	Reference	1.00		
	CRD genotype	1.05	0.54-1.40	0.87

Table 5. Effect of CRD genotype on anthropometric parameters and hormonal measurements in the Frail Old Men Study. Data are presented as means ± SEM. *P* values were adjusted for age. *P* values of DHEA and DHEAS were also adjusted for smoking. No CRD genotype, carriers of two or less affected alleles; CRD genotype, carriers of at least three affected alleles.

	No CRD genotype		CRD genotype		*P*
Weight (kg)	76.3 ± 0.56	(n = 14)	76.1 ± 2.72	(n = 333)	0.67
Height (cm)	172.9 ± 0.35	(n = 14)	171.9 ± 1.68	(n = 333)	0.44
BMI (kg/m^2)	25.5 ± 0.16	(n = 14)	25.7 ± 0.79	(n = 333)	0.98
WHR	0.98 ± 0.00	(n = 14)	0.98 ± 0.01	(n = 330)	0.88
Trunk fatmass (kg)	10.7 ± 1.39	(n = 14)	10.5 ± 6.77	(n = 332)	0.60
Trunk leanmass (kg)	25.6 ± 1.63	(n = 14)	25.5 ± 7.96	(n = 332)	0.74
Total leanmass (kg)	52.1 ± 0.30	(n = 14)	51.9 ± 1.44	(n = 332)	0.62
Total fatmass (kg)	21.1 ± 0.30	(n = 14)	20.7 ± 1.47	(n = 333)	0.63
DHEA (nmol/l)	7.33 ± 0.22	(n = 14)	8.92 ± 1.04	(n = 331)	0.20
DHEAS (µmol/l)	1.96 ± 0.08	(n = 14)	2.32 ± 0.37	(n = 331)	0.63

Discussion

Recently, Draper *et al.* (7) reported the relationship of the *HSD11B1* 83,557insA and *H6PD* R453Q SNPs and their relationship towards CRD in kindreds with CRD and unaffected controls. They concluded from their study that a combination of mutations in *HSD11B1* and *H6PD* interacts to cause CRD, because of a reduction of 11β-HSD1 expression and impaired provision of NADPH. They proposed a triallelic mode of inheritance, in which at least three distinct alleles, from two (or more) loci are necessary for trait manifestation. However, in a recent study by San Millan *et al*., (19) it was shown that triallelic genotypes of *HSD11B1* 83,557insA and *H6PD* R453Q SNPs

do not always cause CRD. Four out of 76 non-hyperandrogenic controls and 5 out of 116 patients with polycystic ovary syndrome (PCOS), all without CRD, presented three or four mutant alleles. Moreover, the *HSD11B1* 83,557insA and *H6PD* R453Q SNPs, either separately or in combination, did not influence 11βHSD oxo-reductase activity. However, they found that PCOS patients had higher allele frequencies of *H6PD* R453Q compared to controls, and that PCOS patients homozygous for *H6PD* R453Q had increased cortisol and 17-hydroxyprogesterone levels (19).

White (20) showed that the *HSD11B1* 83,557insA and *H6PD* R453Q SNPs occur more frequently than previously reported. They found no differences between genotypes for BMI, WHR, visceral adiposity, insulin sensitivity, testosterone, FSH or LH (females), and the risk of PCOS. There was also no effect on urinary free cortisol/cortisone ratio or the corticosteroid metabolite ratios (20). However, Gambineri *et al*. (21) found that the *HSD11B1* 83,557insA was significantly related to PCOS status (mainly attributable to lean PCOS patients, rather than obese patients), lower 0800-0830 h plasma cortisol and higher cortisol response to $ACTH_{1-24}$ in all women with PCOS, and with higher DHEAS levels, greater suppression of DHEAS by dexamethasone, and lower fasting plasma LDL-cholesterol levels in lean PCOS women.

In our study, we were interested in the genotype distributions and influence of the separate and combined genotypes of the *HSD11B1* 83,557insA and *H6PD* R453Q SNPs in the elderly. It was shown that these SNPs are relatively common in the elderly population. For *HSD11B1* 83,557insA, the allele frequencies were higher in our study groups than in the Scottish and Indo-Asian populations described by Draper *et al.* (7). This might indicate a survival effect of the *HSD11B1* 83,557insA SNP. Indeed, in the Rotterdam Study, homozygous carriers of this SNP had a higher survival (P = 0.046) compared to non-carriers or heterozygous carriers (data not shown). However, the allele frequencies found by White *et al.* (20) in a younger population were in accordance with our results. The allele frequencies of *H6PD* R453Q in our study groups were in between the frequencies reported by Draper *et al.* (7) and White *et al.* (20). As a considerable part in both our elderly study groups (3.8% in the Rotterdam Study *vs.* 4.0% the Frail Old Men Study) is carrier of at least three affected alleles, it is very unlikely that *HSD11B1* 83,557insA and *H6PD* R453Q interact to cause CRD. Moreover, we showed that carriers of at least three affected alleles do not have higher androgen production, which is the key factor causing the symptoms observed in patients with CRD.

The conversion of cortisone to cortisol might be involved in obesity and its complications (3). In rodents, Masuzaki *et al.* (22) showed that alterations in the activity of 11β-HSD1 have functional significance for body composition. Targeted overexpression of 11β-HSD1 in adipocytes caused a threefold increase of visceral adipose tissue. In humans, however, there is much controversy about the role of 11β-HSD1 on body composition (2, 23, 24). In both our study groups, no effect of *HSD11B1* 83,557insA on body composition was found. However, we found contradictory results for height in both study groups and for both sexes. In the Rotterdam Study, we found a trend towards greater length in females, and in the Frail Old Men Study we found the opposite.

Central obesity is strongly associated with insulin resistance which is considered to play an important pathogenetic role in the development of type 2 DM (25). The inhibition of 11β-HSD1 has been proposed for the treatment of DM and central obesity (26-28). Indeed, 11β-HSD1-null mice have enhanced hepatic and adipose insulin sensitivity and are protected from weight gain and hyperglycemia on a high-fat diet (29, 30). In cross-sectional studies in humans, 11β-HSD1 activity and variability in *HSD11B1* have been associated with obesity, diabetes, and glucose tolerance (2, 31, 32). However, Kerstens *et al*.(33) found no relationship between 11β-HSD setpoint and insulin sensitivity in healthy subjects and patients with type 2 DM. In our 2 study populations, all Caucasian, there were no differences in blood pressure and glucose metabolism between carriers and non-carriers of *HSD11B1* 83,557insA.

GCs have numerous biological effects on the brain, affecting neurotransmission, metabolism, neuronal structure and maturation and survival. Chronically high levels of GCs have been associated with neuro-psychiatric disorders, cognitive impairment, structural deterioration and neuroendocrine dysfunction (34). Especially the hippocampus, a key locus for memory, cognition and neuroendocrine control and known to be among the first tissues to be degenerated in AD, is sensitive to GCs (35, 36). 11β-HSD1 is highly expressed in the cerebellum, hippocampus, and cortex (37, 38). In two small, randomized, double-blind, placebo-controlled cross-over studies, administration of the 11β-HSD inhibitor carbenoxolone (3 times a day 100 mg) improved verbal fluency after 4 weeks in 10 healthy elderly men (aged 55-75 yr) and improved verbal memory after 6 weeks in 12 patients with type 2 DM (39). Furthermore, de Quervain *et al.* (40) showed that a specific haplotype of *HSD11B1* was associated with a 6-fold increased risk of AD. In our study, there was no difference in incidence of

dementia between carriers and non-carriers of *HSD11B1* 83,557insA.

In a recent study, Lavery *et al.* (8) showed that H6PDH knockout mice have a profound switch in 11βHSD activity from oxo-reductase to dehydrogenase, increasing the corticosterone clearance resulting in a reduction in circulating corticosterone levels. This demonstrated a critical requirement of H6PDH for 11βHSD1 oxo-reductase activity. However, in our study, no effect of the *H6PD* R453Q SNP was found. The only associations we found were that male carriers of *H6PD* R453Q in the Rotterdam Study had a smaller hip circumference, and that female carriers had a trend towards higher WHR. However, this was not confirmed in the Frail Old Men Study. Furthermore, the trend towards higher WHR in female carriers was not in line with our hypothesis.

Persons with three or more affected alleles showed no differences in body composition (including weight, height, BMI, waist and hip circumference, WHR, total lean and fat mass), the adrenal production of androgens, blood pressure, glucose levels or incidence of dementia. No additional effect of *H6PD* R453Q on *HSD11B1* 83,557insA was shown. For this reason, we think that the *H6PD* R453Q SNP does not have an (additional) effect, neither separately nor in combination with *HSD11B1* 83,557insA, on cortisol metabolism.

We conclude from our study, in two independent elderly populations, that we have not been able to detect any influence of the *HSD11B1* 83,557insA and *H6PD* R453Q SNPs, neither separately nor when using three or more affected alleles on body composition, adrenal androgen production, blood pressure, glucose levels, or incidence of dementia in the elderly. We also demonstrated that the presence of at least three affected alleles is relatively common in those two populations. Taking this together, it is unlikely that these SNPs cause CRD. However, as Lavery *et al.* (8) demonstrated the critical role H6PDH for 11βHSD1 oxo-reductase activity, it is important to search for other possible functional SNPs in the *HSD11B1* and *H6PD* genes.

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Chapter 5

The C3435T Polymorphism in the Multidrug Resistance-1 *(MDR-1)* Gene is Associated with Increased Activity of the Hypothalamic-Pituitary-Adrenal-axis

Pauline Smit, Ron H. N. van Schaik, Ilse P. van der Heiden, Marieke J.H.J. Dekker, Henning Tiemeier, Jan W. Koper, Jan Lindemans, Huibert A.P. Pols, Albert O. Brinkmann, Frank H. de Jong, Steven W.J. Lamberts

Submitted

Abstract

Context: Variations in expression and functionality of P-glycoprotein (P-gp), encoded by *MDR-1*, might contribute to differences in hypothalamic-pituitary-adrenal (HPA)-axis activity and glucocorticoid (GC) sensitivity.

Objective: To investigate the effects of *MDR-1* C3435T and G2677T on HPA-axis activity and GC sensitivity.

Design/Setting/Participants: 209 subjects randomly selected from the Rotterdam Study, a population-based cohort study in the elderly.

Intervention: A 1-mg overnight dexamethasone (DEX) suppression test.

Main Outcome Measures: Serum cortisol and insulin levels (both pre- and post-DEX), salivary cortisol levels, and serum androstenedione levels.

Results: Allele frequencies of C3435T and G2677T were 53.1% and 42.8%, respectively. Both polymorphisms were not associated with differences in post-DEX early morning cortisol levels, or with serum DEX concentrations. C3435T was associated with higher serum total cortisol concentrations (P_{trend} = 0.01), and an elevated insulin response to DEX (P_{trend} = 0.02). However, neither calculated 'free' cortisol nor salivary cortisol levels were increased. C3435T-carriers showed higher androstenedione levels, in the whole study group (P = 0.01; 3435CC vs. 3435CT, P < 0.01; 3435CC *vs.* 3435TT, P < 0.01, respectively), and in females (P = 0.06; 3435CC *vs.* 3435CT, P = 0.04 and 3435CC *vs.* 3435TT, P = 0.03, respectively). No associations with G2677T were found using haplotype analysis.

Conclusions: C3435T was associated with increased HPA-axis activity, illustrated by higher early morning serum total cortisol and androstenedione levels, without affecting the response to DEX. Unexpectedly, 'free' and salivary cortisol levels were not increased in parallel. These results indicate that P-gp is not involved in cortisol feedback regulation of the HPA-axis. The increased insulin response to DEX in C3435T carriers might not be related to P-gp.

Introduction

Glucocorticoids (GCs) are important regulators of most physiological systems, including the immune and cardiovascular systems (1-3). The anti-inflammatory effects of GCs are used in the treatment of patients with chronic inflammatory or autoimmune diseases. However, whereas some patients develop side-effects at relatively low doses of topically administered GCs, others appear to be less sensitive to GCs as they do not show an adequate improvement in response to treatment even at high doses (4). This suggests an inter-individual variation in GC sensitivity. These differences in sensitivity to GCs may be caused by polymorphisms in the GC receptor gene (5, 6). Alternatively, the effectiveness of mechanisms determining intracellular GC concentrations (such as metabolism and active transport) might also be involved.

The human multidrug-resistance-1 (*MDR-1; ABCB1*) gene encodes P-glycoprotein (P-gp), a 170 kDa integral membrane protein belonging to the adenosine triphosphate-binding cassette (ABC) superfamily of membrane transporters, the largest gene family known (7, 8). It exports a number of chemically unrelated lipophilic compounds from the inside of cells and from membranes to the extracellular space in an energy-dependent manner (9, 10). P-gp is mainly located in the apical membrane of excretory cells in the liver, kidney and intestine and in the endothelial lining of capillary blood vessels at the blood-brain barrier, and is therefore important for the absorption, distribution, and elimination of xenobiotics (11-13). The degree of expression and the functionality of P-gp can potentially directly affect the (therapeutic) effectiveness of many drugs, including steroid hormones (8, 14).

Recently, a number of single nucleotide polymorphisms (SNPs) in the *MDR-1* gene have been described which affect expression levels and the activity of P-gp (15, 16). These SNPs may contribute to the variability in hypothalamic-pituitary-adrenal (HPA)-axis activity and GC sensitivity in the normal population. In our study, we aimed to determine the functional relevance of two known SNPs in the *MDR-1* gene, C3435T and G2677T, in relation to HPA-axis activity and GC sensitivity. C3435T is a silent SNP located in exon 26, and is associated with decreased P-gp expression (15). Also for G2677T, located in exon 21 and leading to an amino acid change (Ala893Ser), an association with decreased P-gp function has been described (16-18). We studied a group of 209 persons in whom a 1-mg dexamethasone suppression test (DST) was performed. Serum hormone levels were determined, as well as salivary cortisol

concentrations collected in the evening (n = 86). All persons were analyzed for *MDR-1* C3435T and G2677T variant alleles.

Subjects and methods

Subjects

A total of 213 subjects were randomly selected from the Rotterdam Study, a population-based cohort study (comprising 7,983 subjects) in a suburb of Rotterdam, the Netherlands, in whom the determinants of chronic disabling diseases in the elderly are studied (19). Subjects with acute psychiatric or endocrine diseases, including diabetes treated with medication, were not invited. Compared with all participants of the Rotterdam Study, there were no differences in age and sex distribution and cardiovascular risk factors. The subjects gave their written consent to participate in the study, which received the approval of the Medical Ethics Committee of the Erasmus University Medical Center.

Four subjects were excluded from the analysis; two female subjects taking estrogen-containing medication (because of the significant effect of estrogens on corticosteroid binding globulin (CBG) concentration and therefore on cortisol), one male subject with a pathologically low baseline cortisol level (41 nmol/l), and one male subject with a very high estradiol level (1778 pmol/l). The age in the resulting study group (n = 209) varied between 53 and 82 years (99 men and 110 women with mean ages of 67.7 ± 0.6 and 66.1 ± 0.6, respectively). Mean age and body mass index (BMI) are shown in Table 1. All female subjects were postmenopausal.

Dexamethasone suppression test

The 1-mg DST was performed as previously described (20). Briefly, venous blood was obtained in SSTII tubes (BD Vacutainer Systems, Plymouth, UK) between 08.00 and 09.00 h after an overnight fast. Subjects were asked to ingest a tablet of 1 mg of dexamethasone (DEX) at 23.00 h. Fasting blood was drawn by venapuncture the next morning at the same time as the previous day. In two persons, serum DEX levels were near zero together with no suppression of cortisol levels, suggesting non-compliance. These subjects were excluded from the analysis regarding the DST.

Table 1. Description of study population (n = 209). Data represent means ± SEM. BMI, body mass index.

	Mean	*SEM*
Age (years)	66.9	0.4
Males/Females	99/110	
BMI (kg/m^2)	26.4	0.3
Allele frequencies C3435T		
C-allele	46.9%	
T-allele	53.1%	
Allele frequencies G2677T		
G-allele	57.2%	
T-allele	42.8%	

Hormonal measurements

Fasting blood was drawn by venapuncture and allowed to coagulate for 30 min. Subsequently serum was separated by centrifugation and quickly frozen in liquid nitrogen. Serum cortisol concentrations were determined using RIA kits obtained from Diagnostics Products Corporation (DPC) (Los Angeles, CA). Intra- and interassay coefficients of variation (CVs) were below 8.0% and 9.5%, respectively. To check for compliance and possible abnormalities in the metabolism, DEX concentrations were measured by a radioimmunoassay (RIA) using an anti serum supplied by IgG Corporation (Nashville, TN) and [^{3}H]-DEX from Amersham (Little Chalfont, UK). Intra- and interassay CVs were below 8.5% and 14.2%, respectively. Circulating insulin and cortisol-binding globulin (CBG) concentrations were determined using commercially available RIAs (Medgenix Diagnostics, Brussels, Belgium). Intraassay CVs were 12.0% and 8.0%, respectively. Interassay CVs were 13.7% and 4.4%, respectively. 'Free' cortisol concentrations were calculated using a formula that included cortisol, CBG, and albumin concentrations according to the method of de Ronde *et al.* (21), using $Ka_{albumin} = 0.3 \times 10^4\ M^{-1}$ and $Ka_{CBG} = 0.5 \times 10^8\ M^{-1}$ (22, 23). Androstenedione concentrations were determined using RIA kits obtained from DPC. Intra- and interassay CVs were 8.3 and 9.2%, respectively.

From 86 subjects, saliva was collected before sleeping time by using Salivette collection devices (Sarstedt, Rommelsdorf, Germany). We chose to study evening cortisol levels as they are, in principle, less sensitive to influences of daily stress. The devices were stored at –20 ^{0}C until biochemical analysis. Before the saliva samples

were assayed for cortisol, they were thawed and spun at 3000 rpm for 10 min, which results in low-viscosity saliva. Cortisol concentrations were determined by a time-resolved immunoassay with fluorometric detection as described in detail elsewhere (24). Intra- and interassay CVs were below 6% and 9%, respectively.

Anthropometric measurements

Body weight and height of the subjects were measured, and BMI was defined as weight divided by the square of height (kg/m^2).

Genotyping

Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. Polymerase chain reaction (PCR)-restriction fragment length polymorphisms (RFLP) for C3435T was performed as previously described (25). Briefly, approximately 5 ng of DNA was used in a PCR volume of 25 µl containing 1x buffer (10 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$; 50 mM KCl, and 0.001% [wt/vol] gelatin [Perkin-Elmer, Inc, Wellesley, Mass]), 0.2 M each of the deoxynucleotide triphosphates (Roche), 1.25 U of Ampli*Taq* Gold (Perkin-Elmer), and 40 pmol of each forward and reverse primer. Forward primer 5'-CATGCTCCCAGGCTGTTTAT-3' and reverse primer 5'-GTAACTTGGCAGTTTCAGTG-3' were used. PCR conditions were: 7 min at 94 ^{0}C; 35 cycles of 1 min at 94 ^{0}C, 1 min at 55 ^{0}C, and 1 min at 72 ^{0}C; finally 7 min at 72 ^{0}C. PCR products were digested with DpnII, and subsequently analyzed on agarose/Tris-borate-ethylenediaminetetraacetic acid (EDTA) gel with ethidium bromide staining.

For the G2677T SNP, PCR-RFLP was performed in a volume of 25 µl, using ~5 ng of genomic DNA. The PCR mixture contained 1x buffer [10 mM Tris-HCL, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mg/L gelatin (Perkin-Elmer)], 0.2 mM each of the deoxynucleotide triphosphates (Roche), 1.25 U of Amplitaq Gold (Perkin-Elmer), and 40 pmol each of the forward primer (5'-TAG TTT GAC TCA CCT TCC C<u>G</u>G-3') and reverse primer (5'-GGC TAT AGG TTC CAG GCT TG-3'). The underlined nucleotide is a mismatch with the *MDR-1* sequence, creating a restriction site in the PCR product. PCR conditions were: 7 min at 94 ^{0}C, 35 cycles of 1 min at 94 ^{0}C, 1 min at 57 ^{0}C, 1 min at 72 ^{0}C; and finally 7 min at 72 ^{0}C. PCR products were digested with Ban1 (New England Biolabs) and subsequently analyzed on agarose/Trisborate-EDTA gel with ethidium bromide staining.

Haplotype analysis

Haplotype analysis for C3435T and G2677T was performed according to the nomenclature of Johne *et al.* (26). Each genotype was assigned to haplotype pairs. For those individuals who were homozygous at both variants or who were heterozygous at only one variant, haplotypes could be assigned unambiguously. For genotype 11, haplotype pairs 12/21 and 11/22 are possible, but the latter is more likely according to Johne *et al.* (26).

Statistical analysis

All results are presented as means ± SEM. Data were analyzed using SPSS for Windows, release 10.1 (SPSS, Chicago, IL, USA). The variables serum CBG levels, calculated 'free' cortisol levels, salivary evening cortisol levels, serum androstenedione levels, serum cortisol levels after 1 mg of DEX, and serum DEX levels were logarithmic transformed to normalize these variables and to minimize the influence of outliers. Associations of the *MDR-1* C3435T and G2677T SNPs and the different haplotypes with continuous variables were tested by using linear regression, the General Linear Model (GLM), and Least-significant test (LSD), adjusting for age, sex and BMI. A *P* value of less than 0.05 was considered to indicate a significant difference.

Results

MDR-1 C3435T

The wild-type genotype (3435CC) was observed in 46 (22%) subjects, whereas 104 (49.8%) subjects were heterozygous (3435CT), and 59 (28.2%) subjects were homozygous (3435TT) for the C3435T SNP. The population was found to be in Hardy-Weinberg equilibrium.

In the whole group, early morning serum total cortisol concentrations were elevated in an allele-dosage dependent way in heterozygous and homozygous T-allele carriers (P_{trend} = 0.01, 3435CC *vs.* 3435TT, *P* = 0.01) (Fig. 1A). CBG concentrations did not differ between the genotypes. 'Free' cortisol concentrations were calculated on the basis of cortisol, CBG, and albumin concentrations and turned out to be not different between the groups. In order to investigate this result, we carried out additional studies by measuring salivary evening cortisol concentrations. A total of

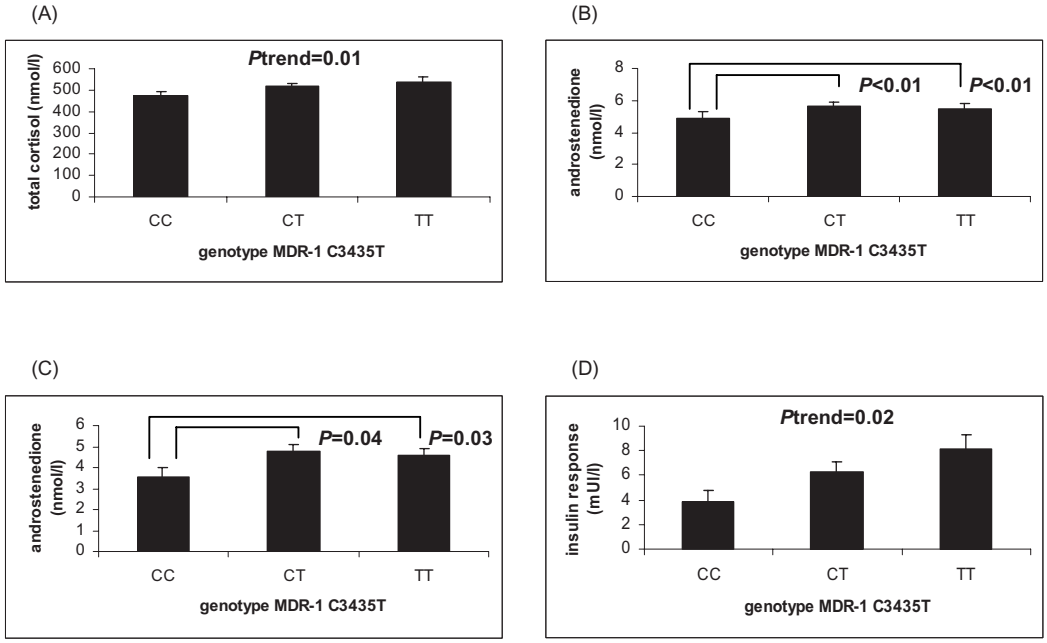

Figure 1. (A) Baseline total cortisol concentrations (n = 209), androstenedione concentrations (B) in the whole study group (n = 208) and (C) in females (n = 109), and (D) insulin response to 1 mg of DEX (n = 201) for the different variant alleles of *MDR-1* C3435T. Data are presented as means ± SEM. *P* value was adjusted for age, sex, and BMI.

86 subjects participated in this additional study, which was carried out 9 years later. These concentrations did not differ in carriers of all three genotypes. Finally, in order to investigate the dose-allele dependent increase in total cortisol levels further, we measured serum androstenedione levels. Serum androstenedione concentrations were increased in C3435T-carriers in the whole study group (P = 0.01; 3435CC *vs.* 3435CT, P < 0.01; 3435CC *vs.* 3435TT, P < 0.01) (Fig. 1B), as well as in the female participants (P = 0.06; 3435CC *vs.* 3435CT, P = 0.04; 3435CC *vs.* 3435TT, P = 0.03) (Fig. 1C). The C3435T SNP did not influence the feedback effect of 1 mg of DEX at night on serum total cortisol concentrations the next morning; neither serum total cortisol levels, nor serum DEX concentrations measured in the early morning differed between the genotypes.

Furthermore, we observed that 1 mg of DEX significantly increased serum fasting insulin levels in an allele-dose dependent manner (P_{trend} = 0.02; 3435CC *vs.* 3435TT, P = 0.02) (Fig. 1D).

MDR-1 G2677T

The wild-type genotype (2677GG) was observed in 69 (33.0%) subjects, whereas 101 (48.3%) subjects were heterozygous (2677GT), and 39 (18.7%) subjects were homozygous (2677TT) for the G2677T SNP. The population was found to be in Hardy-Weinberg equilibrium.

With this SNP, we found no differences in early morning serum total cortisol levels, CBG levels, calculated 'free' cortisol levels, salivary evening cortisol levels or androstenedione concentrations. Also serum total cortisol and DEX concentrations after 1 mg of DEX were not statistically different between the three genotypes.

Serum fasting insulin levels after 1 mg of DEX, however, were significantly increased in an allele-dosage dependent way in T-allele carriers (P_{trend} = 0.02; 2677GG *vs.* 2677TT, P = 0.049). This increase was not different from that observed in the C3435T-carriers. (data not shown)

Haplotypes

Comparisons were made between carriers of a particular haplotype and all non-carriers of that haplotype. In total, we found 148 carriers (70.8%) of haplotype 11 (2677G/3435C, representing the wild type), 46 (22.0%) carriers of haplotype 12 (2677G/3435T), 5 (2.4%) carriers of haplotype 21 (2677T/3435C) and 137 carriers (65.6%) of haplotype 22 (2677T/3435T).

There were significant differences in early morning serum total cortisol levels between carriers and non-carriers of haplotypes 11 and 12. Wildtypes (carriers of haplotype 11) had lower serum total cortisol levels (P = 0.03) compared to persons with at least one of the SNPs (non-carriers of haplotype 11), while persons with 2677G/3435T (haplotype 12) had higher total cortisol levels compared to non-carriers (P = 0.049). No differences in CBG, calculated 'free' cortisol or salivary evening cortisol levels were found between carriers and non-carriers of haplotype 11 and 12. For haplotype 11, there were no differences in androstenedione concentrations in the whole study group, or in females. However, haplotype 12 was associated with higher baseline androstenedione levels, which was only significant in females (P = 0.02). No differences in serum total cortisol and DEX concentrations after 1 mg of DEX were found between the different haplotypes.

In carriers of haplotype 22 (2677T/3435T) serum fasting insulin levels after 1 mg of DEX at 23.00u were significantly increased (P = 0.01) compared to non-carriers. This

increase of insulin was not higher than that elicited by both polymorphisms seperately. Moreover, the insulin increase after DEX observed in G2677T-carriers was dependent on the simultaneous presence of the C3435T variant, as no differences in the insulin response were found between carriers and non-carriers of haplotype 21 (data not shown).

Discussion

In this study, we determined the functional relevance of two recently discovered SNPs in the *MDR-1* gene, C3435T and G2677T, with regard to HPA-axis activity and GC sensitivity. We found that early morning serum total cortisol levels in the whole group, as well as androstenedione levels in (female) carriers were higher in an allele-dosage dependent way in carriers of C3435T but not G2677T. No differences in calculated circulating 'free' cortisol levels or salivary evening cortisol concentrations were observed in carriers of C3435T and G2677T. Salivary cortisol can be used as index of plasma free cortisol levels (27). These results indicate that activation of the HPA-axis in carriers of C3435T resulted in elevated total cortisol and androstenedione levels without affecting the calculated 'free' 'bioactive' and salivary 'free' cortisol concentrations.

According to the suggested effects in the literature (25), we expected carriers of the *MDR-1* C3435T SNP to have higher intracellular cortisol concentration in the pituitary which would lead (via suppression of the HPA-axis) to lower serum ACTH and cortisol levels. However, we observed the opposite. *MDR-1ab* knockout mice (the murine P-gp is encoded by *MDR-1a* and *MDR-1b*) show consistently lower plasma ACTH levels and lower evening plasma corticosterone levels (28). It was also demonstrated that specific P-gp inhibitors (e.g. PSC 833) can significantly increase intracellular human intestinal epithelial and T-lymphocyte levels of cortisol and cyclosporin (29). On the other hand, rabbits treated with PSC 833 show a dose-dependent increase in serum cortisol levels (30). Karssen *et al.* demonstrated that the pituitary uptake of [^{3}H]cortisol was not different between wildtype and *MDR-1a* knockout mice (31), which indicates that P-gp is apparently not involved in the feedback regulation of the HPA-axis at the level of the pituitary. This is in accordance with our results showing no difference between carriers and non-carriers on suppression of the HPA-axis by

means of the 1-mg DST.

P-gp is known to be present in the liver, where it is expressed on the biliary canalicular front of hepatocytes and on the apical surface of epithelial cells in small biliary ductules (11-13). As the C3435T SNP is associated with lower levels of P-gp protein and a reduced function (15), one could indeed expect that carriers of this SNP have higher GC levels due to the reduced elimination into bile, leading to suppression of the HPA-axis. The observed activation of the HPA-axis, resulting in increased adrenal gland activity with higher total cortisol and androstenedione levels, is therefore unexpected. That the increase in serum androstenedione levels is only significant in post-menopausal women can be explained by the adrenal glands being the only source of androstenedione. In men, serum androstenedione levels are partially dependent on direct testicular secretion and peripheral conversion from testosterone as well. However, it remains unexplained why the elevated total cortisol concentrations are not accompanied by a parallel increase in 'free' cortisol levels, calculated on the basis of cortisol, CBG, and albumin concentrations, and measured by salivary evening cortisol levels. Also, the increased insulin response to DEX cannot be attributed to HPA-axis regulation as the effect of C3435T is opposite compared to cortisol and androstenedione.

As C3435T is a silent SNP, it has been proposed that its observed functional effects might be due to linkage to G2677T that encodes an amino acid change (Ala893Ser) (16, 17). However, in our study, no associations with this SNP were found. We cannot exclude the possibility of linkage of the C3435T SNP to other, yet unknown, functionally important SNPs. Further, there is also a possibility that the C3435T SNP causes mRNA instability. These questions will hopefully be addressed in future studies.

In summary: we investigated the *MDR-1* C3435T and G2677T SNPs in an elderly population. No associations with the G2677T SNP were found. However, C3435T was associated with an increased activity of the HPA-axis, instead of the hypothesized lower activity, as we found higher total cortisol levels as well as higher androstenedione levels in (female) carriers of C3435T.

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Chapter 6

A Common Polymorphism in the *CYP3A7* Gene is Associated With a Nearly 50% Reduction in Serum DHEAS levels

Pauline Smit*, Ron H.N. van Schaik*, Marloes van der Werf, Annewieke W. van den Beld, Jan W. Koper, Jan Lindemans, Huibert A.P. Pols, Albert O. Brinkmann, Frank H. de Jong, Steven W.J. Lamberts
* both authors contributed equally to this work

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Abstract

Context: CYP3A7, expressed in the human fetal liver and normally silenced after birth, plays a major role in the 16α-hydroxylation of dehydroepiandrosterone (DHEA), DHEAS, and estrone. Due to a replacement of part of the *CYP3A7* promoter with a sequence identical to the same region in the *CYP3A4* promoter (referred to as *CYP3A7*1C*), some individuals still express a variant of the *CYP3A7* gene later in life.

Objective: The objective of this study was to examine the effect of the *CYP3A7*1C* polymorphism on serum steroid hormone levels.

Design/Setting/Participants: Two population-based cohort studies were performed. Study group 1 consisted of 208 subjects randomly selected from the Rotterdam Study, and study group 2 consisted of 345 elderly independently living men.

Main Outcome Measures: Serum DHEA(S), androstenedione, estradiol, estrone, and testosterone levels were the main outcome levels.

Results: In study groups 1 and 2, heterozygous *CYP3A7*1C* carriers had almost 50% lower DHEAS levels compared with homozygous carriers of the reference allele [study group 1: 1.74 ± 0.25 *vs.* 3.33 ± 0.15 μmol/l ($P = 0.02$); study group 2, 2.09 ± 0.08 *vs.* 1.08 ± 0.12 μmol/l ($P < 0.001$)]. No differences in circulating DHEA, androstenedione, estradiol or testosterone levels were found. However, in study group 2, serum estrone levels were lower in heterozygous *CYP3A7*1C* carriers compared with homozygous carriers of the reference allele (0.11 ± 0.002 *vs.* 0.08 ± 0.006 nmol/l; $P < 0.001$).

Conclusion: The *CYP3A7*1C* polymorphism causes the persistence of the enzymatic activity of CYP3A7 during adult life, resulting in lower circulating DHEAS and estrone levels.

Introduction

Cytochrome P450 enzymes are important for the metabolism of many endogenous compounds, procarcinogens and drugs. The CYP3A subfamily is one of the major subfamilies of the CYP superfamily expressed in the human liver and comprises CYP3A4, CYP3A5, and CYP3A7. The *CYP3A* genes are located adjacent to each other on chromosome band 7q21, but are differentially regulated (1). CYP3A4 is the most abundant form of CYP3A (~30% of total CYP), whereas CYP3A5 accounts for approximately 20% of the CYP3A content in adult liver and is known to be polymorphically expressed (2, 3). Of the oxidative enzymes, it has been shown that CYP3A7 accounts for up to 50% of the total fetal hepatic CYP content and up to 87-100% of total fetal hepatic CYP3A content (4, 5). Among the endogenous substrates and exogenous chemicals, the fetal/neonatal CYP3A7 has a high catalytic activity for the 16α-hydroxylation of estrone (E1) and dehydroepiandrosterone (DHEA) (6, 7). Presumably, these latter effects of CYP3A7 are necessary during development in order to protect the fetus against placental production of estradiol (E2) from DHEA (8, 9). DHEA has a short half-life, with a high metabolic clearance rate (2000ls/day), whereas DHEAS circulates in relatively large quantities and undergoes delayed metabolism (10). During adult life, DHEA is known to be a precursor steroid in the (peripheral) production of androgens and estrogens (11). CYP3A7 expression sharply decreases or stops shortly after birth, although some individuals still express CYP3A7 into adulthood due to replacement of an approximately 60-bp stretch [nucleotides (nt) –129 to –188] of the *CYP3A7* promoter with a sequence identical with the same region in the *CYP3A4* promoter. This genotype is referred to as *CYP3A7*1C* (12).

The aim of our study was to clarify the role of the *CYP3A7*1C* polymorphism in the regulation of serum DHEAS levels and the effects of DHEAS levels on the serum levels of other steroid hormones in the elderly. For this, we genotyped a group of 208 elderly subjects and a group of 345 elderly men (all Caucasian) for this polymorphism and determined serum DHEA(S) concentrations as well as serum androstenedione, E1, E2, and testosterone levels.

Material and methods

Study group 1

We genotyped a total of 212 subjects who were randomly selected from the Rotterdam Study, a longitudinal population-based cohort study (comprising 7983 subjects) in a suburb of Rotterdam, The Netherlands, in which the determinants of chronic disabling diseases in the elderly are studied (13). The medical ethics committee of the Erasmus Medical Center approved this study, and all participants gave written informed consent. Serum DHEAS, androstenedione, E2, and testosterone levels were measured.

Four subjects were excluded from the statistical analysis: two females taking estrogen-containing medication (because of the effects of oral contraceptives on serum DHEAS levels), one male with a very high estrogen level, and one male with a pathologically low baseline cortisol level. The age in the resulting study group (n = 208) varied between 53 and 82 yr (98 men and 110 women with mean ages of 67.7 ± 0.6 and 66.1 ± 0.6 yr, respectively).

Study group 2

A group of 345 independently living men, all Caucasian, aged 73 yr or older (mean age 77.6 ± 0.2 yr), were genotyped, and serum DHEAS, DHEA, E1, E2, and testosterone levels were measured. Participants were recruited by letters of invitation, which were sent to the oldest male inhabitants of Zoetermeer, a medium-sized town in The Netherlands. All subjects provided informed consent to participate in the study which was approved by the medical ethics committee of the Erasmus Medical Center. Subjects were judged sufficiently healthy to participate in the study if they were physically and mentally able to visit the study independently. No additional health-related criteria were used.

*Genotyping of CYP3A7*1C*

Within the replacement of a 60-bp stretch (nt –129 to –188) of the *CYP3A7* promoter sequence, 7 bp changed compared with the reference *CYP3A7* allele. Genotyping of the *CYP3A7*1C* was performed by PCR-restriction fragment length polymorphism based upon the T-167G variant. A PCR amplification was performed in a 50-µl reaction volume, using approximately 10 ng genomic DNA, 1x PCR Buffer II (PerkinElmer, Wellesley, MA), 1.25 mM $MgCl_2$, 0.2 mM each of the deoxynucleotide triphosphates

(Roche, Indianapolis, IN), 1.25 U AmpliTaq Gold (PerkinElmer) and 40 pmol each of forward primer 5'- CCATAGAGACAAGAGGAGAG – 3' and reverse primer 5'- CTGAGTCTTTTTTTCAGCAGC – 3'. Amplification consisted of an initial denaturation step (7 min at 94 ^{0}C), followed by 35 cycles (each consisting of 1 min at 94 ^{0}C, 1 min at 58 ^{0}C and 1 min at 72 ^{0}C) and ending with an extension cycle (7 min at 72 ^{0}C). For restriction analysis, 10 µl from the PCR amplification was digested for 2h at 37 ^{0}C in a final volume of 15 µl containing 1x restriction buffer and 5 U *SspI* (New England Biolabs, Beverly, MA). The digested fragments were separated by electrophoresis on a 3% agarose gel with ethidium bromide staining. The fragments produced were 244 and 126 bp for the wildtype sequence; 370, 244 and 126 bp for heterozygous sequences; and 370 bp for homozygous variant sequences.

Hormonal measurements

Serum DHEAS, DHEA, E1, E2, and androstenedione levels were determined by RIA (Diagnostics Products Corp., Los Angeles, CA). The intraassay coefficients of variation (CV) for these assays were 5.3% or less, 3.8%, 5.6%, 7.0% or less, and 8.3%, respectively. The interassay CVs were 7.0% or less, 8.6%, 10.2%, 8.1%, and 9.2% respectively. Testosterone was measured with a noncommercial RIA (intra- and interassay CVs, 5.6% and 9.0%) (14) in study group 1 and with an RIA in study group 2 (Diagnostics Products Corp.; intra- and interassay CVs, 8.1% and 10.5%).

Anthropometric measurements

Body weight and height of the subjects were measured, and body mass index was defined as weight (kilograms) divided by the square of height (meters).

Statistical analysis

Data were analyzed using SPSS for Windows, release 10.1 (SPSS, Inc., Chicago, IL). Data are expressed as the mean ± SEM. Statistical analysis was carried out using the general linear model procedure, and results were adjusted for age and, if necessary, sex, body mass index, alcohol use and smoking habits. $P < 0.05$ was considered significant.

Results

We studied the C*YP3A7*1C* polymorphism in two independent populations consisting of 208 healthy elderly subjects (study group 1) and 345 independently living males (study group 2), and found 6.7% (n = 14) and 8.4% (n = 29) heterozygous carriers, respectively. Allele frequencies for study group 1 and 2 were 96.6% reference allele and 3.4% variant allele, and 95.8% reference allele and 4.2% variant allele, respectively. Both populations were found to be in Hardy-Weinberg equilibrium.

In study group 1, the *CYP3A7*1C* polymorphism was associated with lower serum DHEAS levels (P = 0.02) when corrected for age, sex, alcohol use, and smoking habits. Heterozygous carriers of the *CYP3A7*1C* polymorphism had 47.7% lower mean serum DHEAS levels compared with the wildtypes (1.74 ± 0.25 *vs.* 3.33 ± 0.2 µmol/l, respectively; Fig. 1). Because all female subjects in our study group were postmenopausal, males and females were analyzed together. Separate analysis for males and females followed the same trend, although no significance was reached, presumably due to the small numbers of subjects in both groups [males: wildtype, 4.08 ± 0.22 µmol/l; heterozygous mutant, 2.34 ± 0.71 µmol/l (P = 0.18); females: wildtype, 2.63 ± 0.16 µmol/l; heterozygous mutant, 1.51 ± 0.19 µmol/l (P = 0.07)]. Serum androstenedione, E2, as well as testosterone levels did not differ between homozygous carriers of the reference allele and heterozygous carriers of the *CYP3A7*1C* polymorphism (data not shown).

In study group 2, consisting of 345 males, we found the *CYP3A7*1C* polymorphism to be significantly associated with lower serum DHEAS levels (2.09 ± 0.08 *vs.* 1.08 ± 0.12 µmol/l, $P < 0.001$) when corrected for age and smoking habits (data about alcohol use were not available). We also found that heterozygous carriers of the *CYP3A7*1C* polymorphism had significantly lower E1 levels compared with wildtype subjects when corrected for age (0.11 ± 0.002 *vs.* 0.08 ± 0.006 nmol/l; $P < 0.001$; Fig. 2). No differences were found in serum E2, testosterone, or DHEA levels (data not shown).

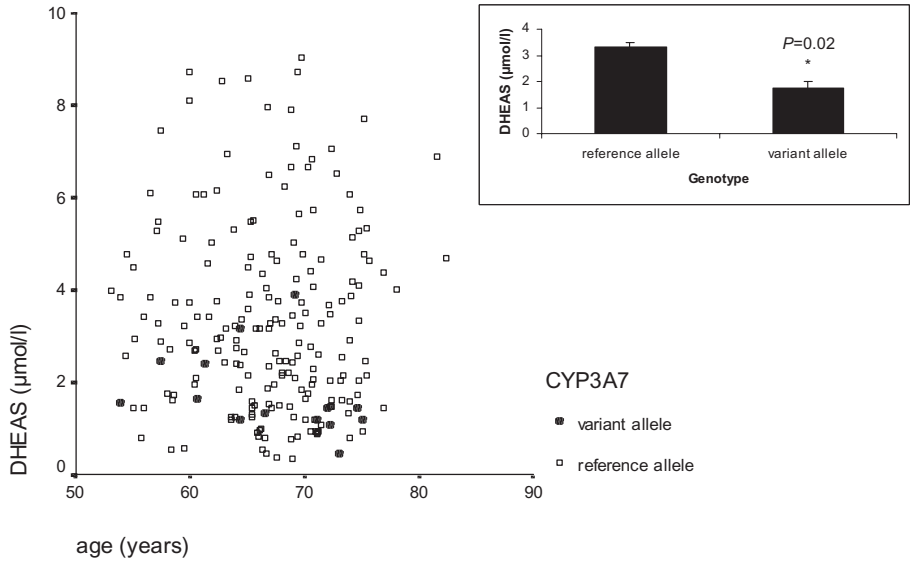

Figure 1. Serum DHEAS concentrations in wild-type and heterozygous mutant carriers of the *CYP3A7*1C* polymorphism in a group of 208 healthy elderly individuals. The *P* value was adjusted for age, sex, alcohol use, and smoking habits. *, *P* = 0.02 *vs.* reference allele. Values are the mean ± SEM.

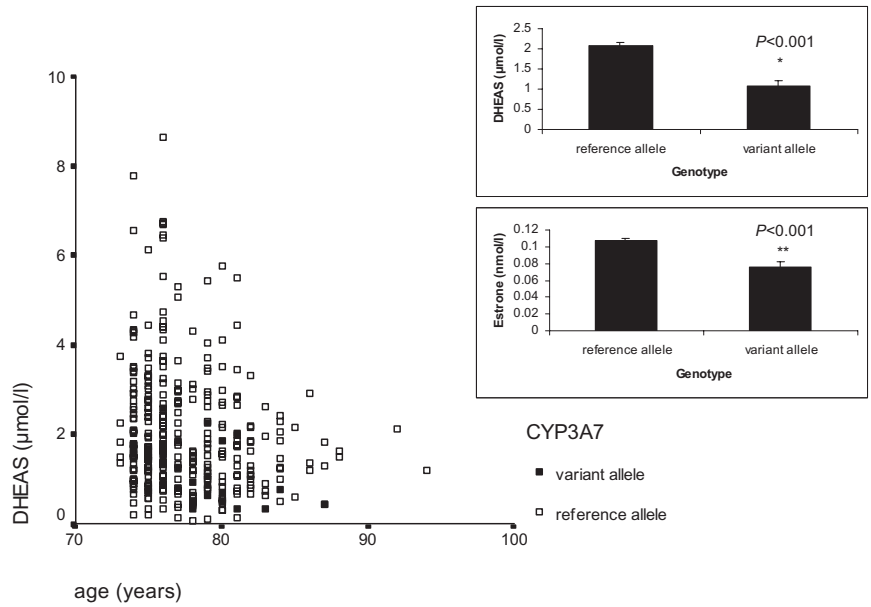

Figure 2. Serum DHEAS and E1 concentrations in wildtype and heterozygous mutant carriers of the *CYP3A7*1C* polymorphism in a group of 345 independently living elderly men. The *P* value for DHEAS was adjusted for age and smoking habits; the *P* value for E1 was adjusted for age. *, *P* < 0.001; **, *P* < 0.001 (*vs.* reference allele). Values are the mean ± SEM.

Discussion

In this study it was demonstrated that a substantial part of two healthy elderly Caucasian populations carries the *CYP3A7*1C* polymorphism heterozygously. It is suggested that this polymorphism is functional, because circulating DHEAS levels in these carriers were nearly 50% lower than those in the reference population, in whom CYP3A7 is presumably not functionally active in adult life. The fact that we did not observe differences in DHEA levels in study group 2 can be explained by the short half-life of DHEA together with a high metabolic clearance rate (10).

Much controversy exists about the potential significance of (lowered) DHEA(S) levels in the aging process (15). In both groups, no changes in circulating androgen and E2 levels were observed in the individuals who had possibly lived a life with decreased DHEAS levels due to the presence of this polymorphism. However, according to de Ronde e*t al.* (16), strong correlations exist between DHEAS levels and E2, E1, androstenedione, and testosterone levels in both men and women. The significantly lower serum E1 levels observed in heterozygous *CYP3A7*1C* carriers in study group 2 can be explained by the high catalytic activity of CYP3A7 for this estrogen (7).

Previous studies suggested that lower(ed) DHEAS levels might be associated with a higher mortality (17). Therefore, it is possible that selective survival bias already occurred in our study populations. We found allele frequencies of 3.4% (study group 1) and 4.2% (study group 2) for the variant *CYP3A7* allele. In a separate study, a frequency of 3.2% was found in 500 healthy adult Caucasian blood donors (van Schaik, R. H. N., M. van der Werf, J. Lindemans., unpublished observations). This is in agreement with the frequencies observed in previous studies performed by Kuehl *et al.* (18) and Burk *et al.* (19) (3.0% and 3.5%, respectively). However, no description of these study populations in terms of age was given.

In conclusion, we found that a common heterozygous variant allele of the *CYP3A7 g*ene results in a nearly 50% reduction in DHEAS levels in two populations of healthy elderly individuals. However, no indications were found that such lowered levels are associated with an acceleration of the aging process, suggesting that even these lower(ed) DHEAS levels are sufficiently high to enable the steroid to act as an adequate precursor for peripheral estrogen and androgen formation. However, these intriguing data are still preliminary and deserve additional studies.

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Chapter 7

General Discussion

The secretion of glucocorticoids (GCs) is under the control of the hypothalamic-pituitary-adrenal (HPA)-axis. In humans, cortisol is the main GC produced by the adrenal cortex. It exerts a strong negative feedback both at the level of the hypothalamus and the pituitary, completing a negative feedback-loop in which a balance is maintained between production and need (1).

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 and osteoporosis) are associated with GC-treatment, limiting its therapeutic usefulness (2, 3). Whereas some patients develop side effects on relatively low doses of topically administered GCs, others appear to be less sensitive to GCs, as they do not show an adequate improvement in response to treatment even on high doses (4). Some patients are even resistant to the anti-inflammatory effects of GCs while at the same time showing side effects known to reflect normal sensitivity to GCs, including suppression of the hypothalamic-pituitary-adrenal (HPA)- axis (3, 4). Therefore, it is important to determine individual GC sensitivity in order to apply the optimal treatment.

Although the GC receptor (GR) is always the prime suspect, when changes in GC sensitivity are observed, it is not the only protein involved in GC action. The GC signalling pathway is very complex and many proteins play a role, each of them a possible cause for abnormal GC sensitivity.

In this thesis, we describe the investigation of abnormal GC sensitivity in patients, and we introduce two new bioassays to determine cellular GC sensitivity *ex vivo*. Furthermore, we have investigated the effects of genetic variations in a number of other genes involved in cellular bioavailability of cortisol on GC signaling.

7.1 The GILZ and IL-2 Expression Assays

7.1.1 Optimization of the GILZ and IL-2 Expression Assays

So far, cellular GC sensitivity was measured using several different assays based upon 1) receptor protein characteristics of mononuclear leukocytes (5), 2) inhibition of phytohemagglutinin (PHA)-induced T-lymphocyte proliferation (6), or 3) changes in gene expression levels (7). Also the skin blanching test was used to study

vasoconstriction in the skin in response to GCs which should correlate with GC sensitivity (8).

It is generally thought that transactivation is the predominant mechanism by which GCs exert many of their metabolic and cardiovascular side effects (9-11). The anti-inflammatory effects of GCs are thought to be mainly the result of transrepression of many genes encoding inflammatory mediators (12). For this reason, we developed two new bioassays to measure individual GC sensitivity using quantitative real-time PCR. As a prototypical gene that is up-regulated under the influence of GCs, we chose the GC-induced leucine zipper (GILZ) gene. Transactivation of this gene occurs via direct interaction of the GC receptor (GR) with different GC response elements (GREs) in the promoter region (13). The interleukin-2 (IL-2) gene was chosen as an example of a gene that is strongly downregulated by GCs. The expression of this cytokine is, like other genes involved in the inflammatory response, mediated by protein-protein interactions of the GR with the activating protein-1 (AP-1) and nuclear factor-κB (NFκB) transcription factors (14).

With these new bioassays we showed that, whereas there exists a large interindividual variation in GC sensitivity, intraindividual GC sensitivity is rather stable. This has also been demonstrated in other studies, and is independent of age and sex (15, 16). We were also able to discriminate between suppressive and stimulatory effects of GCs. However, conventional clinically used GCs do not dissociate transactivation from transrepression. Strategies to develop new GCs should focus on maintaining transrepression of immune genes in the absence of significant transactivation of GRE-dependent promoters (12).

The usefulness of the GILZ and IL-2 expression assays was demonstrated by showing the differences in GC sensitivity in carriers and non-carriers of the GR ER22/23EK and N363S polymorphisms (chapter 2). We showed that the ER22/23EK polymorphism influences the transactivating capacity of the GR: in peripheral blood mononuclear lymphocytes (PBMLs) from hetero- and homozygous carriers a significant reduction in the activation of GILZ transcription compared to controls was observed, whereas no differences were detected in transrepression of IL-2. This effect is probably due to a shift in translation in favour of the translation variant GR-A over GR-B in ER22/23EK carriers. As GR-A and GR-B are equally potent in inhibiting NF-κB activity, no changes in transrepression were found (17).

Concerning the N363S polymorphism, PBMLs from hetero- and homozygous

carriers showed an increased expression of GILZ compared to controls. The response of PBMLs from heterozygous carriers with respect to IL-2 transcription did not differ from that of controls. However, PBMLs from homozygous N363S carriers showed a reduced response to DEX, suggesting a decreased sensitivity. As it was shown that the polymorphism does not interfere with the NF-κB-driven transcription of the LUC reporter gene, it is thought that it causes a GR-induced upregulation of inhibitory-κBα. It is also possible that GILZ or other aspects of the signalling cascade are affected. However, the exact molecular mechanism underlying this polymorphism is so far unknown.

An important step was the downscaling of these assays by using a lower number of PBMLs (0.25×10^6 instead of 2×10^6) per assay and fewer DEX concentrations. This makes them more applicable for larger study populations and even small infants as only 5 mL rather than 40 ml of blood is needed. Although it was possible to use a lower number of cells for these assays with comparable results, using fewer DEX concentrations resulted in a wider confidence interval compared to the original method. However, in the future, (downscaled) GILZ and IL-2 expression assays may be useful in a clinical setting to determine the optimal type and dosage of GC in individual patients.

7.1.2 Determining Cortisol Resistance and Hypersensitivity Syndromes

In the normal healthy population, interindividual variations do not lead to clinical manifestations. However, within a small number of patients, large differences in GC sensitivity give rise to clinical signs and symptoms.

As complete GC resistance is not compatible with life, only the syndrome of partial cortisol resistance (CR) has been described. In this syndrome, the relative insensitivity of all target tissues to GCs leads to hypercortisolism without Cushingoid features, together with clinical signs and symptoms due to hyperandrogenism and/or hypermineralocorticoidism (6, 18, 19). On the other hand, clinical signs and symptoms of Cushing's syndrome characterize the syndrome of cortisol hypersensitivity in the absence of high endogenous cortisol levels (6).

In chapter 3, 9 patients with GC sensitivity disorders are described. In these patients, different approaches were used 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* using the GILZ and IL-2 expression assays and the inhibition of mitogen stimulated proliferation; and 3) obtaining permanent cell lines by transfection of B lymphocytes with Epstein-Barr virus.

Transfection of B-lymphocytes with Epstein-Barr virus led to increased GR levels. It was shown that this increase was less in patients diagnosed as GC resistant compared to controls. The molecular mechanism explaining this phenomenon is still unknown. However, it can be used as an additional indicator of altered GC sensitivity.

In four patients (numbers 1, 2, 3, 7), mutations in the hormone-binding domain of the GR gene have been identified (supplementary table, chapter 3). The results from our assays were in accordance with the clinical signs and symptoms of these patients. Patients numbers 1, 2, and 3 have been described earlier (20-22). Patient number 7, who was treated with low dose GCs to prevent kidney transplant rejection, was suspected of being GC hypersensitive. However, the GILZ and IL-2 assays showed a significantly reduced transactivation capacity together with normal transrepression activity. Furthermore, elevated levels of the GR-β splice variant were found which might be caused by the alterations in the GR gene sequence that were identified in this patient and that could induce changes in the splicing of exon 9α and 9β. This might lead to decreased GC sensitivity, although elevated GR-β levels have been associated with acquired GC resistance rather than with generalized GC resistance (23-30). It is thought 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 while the immunosuppressive function is not affected.

The other 5 patients described in chapter 3 also have clinical signs and symptoms of altered GC sensitivity. In these patients, however, no mutations in the GR gene, nor changes in mRNA or protein expression levels were identified that could explain their clinical manifestations. Alterations in co-regulators of GR activity could be responsible, but many of them are involved in the functioning of more than one nuclear receptor (31) and no differences in response to other steroid hormones were identified in these patients. Further research is needed to investigate whether the altered GC sensitivity in our patients can be attributed to differences concerning these co-factors and their complex formation.

7.2 Factors Involved in GC Sensitivity

7.2.1 11β-Hydroxysteroid Dehydrogenase Type 1 and Hexose-6-phosphate Dehydrogenase Activity

11β-Hydroxysteroid Dehydrogenase type 1 (11β-HSD1) is widely expressed and is encoded by *HSD11B1* (32-34). Its enzymatic activity is bi-directional, having both dehydrogenase (cortisol to cortisone) and oxo-reductase (cortisone to cortisol) activities (33, 34). In intact cells or organs *in vivo*, however, it acts predominantly as an oxo-reductase (35). As hexose-6-phosphate dehydrogenase (H6PDH) is the only source of NADPH within the lumen of the endoplasmatic reticulum, it is crucial for the oxo-reductase activity of 11β-HSD1 (36-38). It is encoded by *H6PD*, and is expressed in most tissues.

In chapter 4, we investigated the effect of the *HSD11B1* 83,557insA polymorphism and the *H6PD* R453Q polymorphism on body composition, adrenal androgen production, blood pressure, glucose metabolism, and the incidence of dementia in two population-based cohort studies in the elderly. A previous report had indicated that a combination of these two polymorphisms interacts to cause cortisone reductase deficiency (CRD) when at least three alleles are affected (37). Therefore, we analysed the effects of these polymorphisms not only separately, but also in combination and especially the presence of at least three affected alleles.

Within our two study populations, both representing the healthy elderly population, the presence of at least three affected alleles was relatively common (4.0 and 4.2 %), as also observed by others. However, we found no evidence that these polymorphisms have an effect on body composition, adrenal androgen production, blood pressure, glucose metabolism, and the incidence of dementia. All together, it was concluded that it is very unlikely that the *HSD11B1* 83,557insA and the *H6PD* R453Q polymorphisms can cause CRD. This conclusion was supported by work from San Millan *et a.l* (39) and White (40). However, in view of the important role of these two genes in cortisol bioavailability, it is important to search for other possible functional polymorphisms in the *HSD11B1* and *H6PD* genes. So far, no other mutations in the coding regions and intron/exon borders of *HSD11B1* have been found in patients with CRD. Maybe, these must be searched within the promoter region or 3' UTR.

7.2.2 P-glycoprotein Expression

P-glycoprotein (P-gp), encoded by *MDR-1*, exports a number of chemically unrelated lipophilic compounds from the inside of cells and from membranes to the extracellular space in an energy-dependent manner (41, 42). It is mainly located in the apical membrane of excretory cells in the liver, kidney and intestine and in the endothelial lining of capillary blood vessels at the blood-brain barrier (43-45). However, the role of P-gp on HPA-axis-regulation is still not clear.

In chapter 5, we studied the effect of the *MDR-1* C3435T and G2677T polymorphism, separately and by using haplotype analysis, on HPA-axis activity and GC sensitivity in a subset of the Rotterdam Study. We found that the C3435T polymorphism was associated with increased HPA-axis activity, illustrated by higher early morning serum total cortisol and androstenedione levels, without affecting the response to DEX. However, 'free' and salivary cortisol levels were not increased in parallel. This indicates that P-gp is not involved in cortisol feedback regulation of the HPA-axis. In the literature, however, there are still conflicting data about the role of P-gp in HPA-axis regulation (46-49). The increased insulin response to DEX cannot be attributed to HPA-axis regulation as the effect of C3435T is opposite compared to cortisol and androstenedione.

There are still discrepancies in the role of C3435T. As C3435T is a silent polymorphism, it has been proposed that its observed functional effects might be due to linkage to the G2677T polymorphism that encodes an amino acid change (Ala893Ser) (50, 51). The effects found might be explained by linkage of the C3435T polymorphism to other, yet unknown, functionally important polymorphisms. Further, there is also a possibility that the C3435T SNP causes mRNA instability.

7.2.3 CYP3A7 Activity

The fetal/neonatal CYP3A7, a member of the family of P450 cytochrome enzymes, has a high catalytic activity for the 16α-hydroxylation of estrone (E1) and dehydroepiandrosterone (DHEA) (52, 53). Its expression sharply decreases or stops shortly after birth, although some individuals still express CYP3A7 into adulthood due to a replacement of an approximately 60-bp stretch (nt –129 to –188) of the *CYP3A7*

promoter with a sequence identical to the same region in the *CYP3A4* promoter. This confers the CYP3A4 expression pattern on CYP3A7. This genotype is referred to as *CYP3A7*1C* (54).

As we were interested whether CYP3A7 also plays a role in the metabolism of steroid hormones, we studied the effect of the *CYP3A7*1C* polymorphism on several steroid hormone levels in 2 elderly study populations (chapter 6). Interestingly, carriers of the *CYP3A7*1C* polymorphism showed nearly 50% reduction in serum DHEAS levels and a significant reduction in serum E1 levels, indicating that CYP3A7 is indeed still active in these persons. As there is much controversy about the potential significance of (lowered) DHEA(S) levels in the aging process (55), further research on the *CYP3A7*1C* polymorphism might be useful to clarify the role of DHEA(S).

7.3 Concluding remarks

More and more, it becomes evident that many factors contribute to interindividual variation in GC sensitivity. For a long time, it is known that variation within the GR is important. However, other factors are also involved.

This thesis focussed on the influences of several genes involved in the (local) metabolism and absorption of GCs. The different effects of these factors on the availability of GCs to the individual cell are shown in Figure 1. In this figure, the process of cortisol secretion and the separate actions of the GR, 11β-HSD1, H6PDH, and P-gp on the bioavailability of cortisol are illustrated in the different organs and eventually in the individual cell. So far, the role of P-gp on HPA-axis regulations is still unknown.

However, many other (so far undiscovered) factors might also play an important role, and need to be considered as well. Proteins that should be considered in this respect include cortisol binding globulin, heatshock protein 90 and other GR-associated proteins, and certainly also proteins involved in GC signalling such as co-activators or co-repressors.

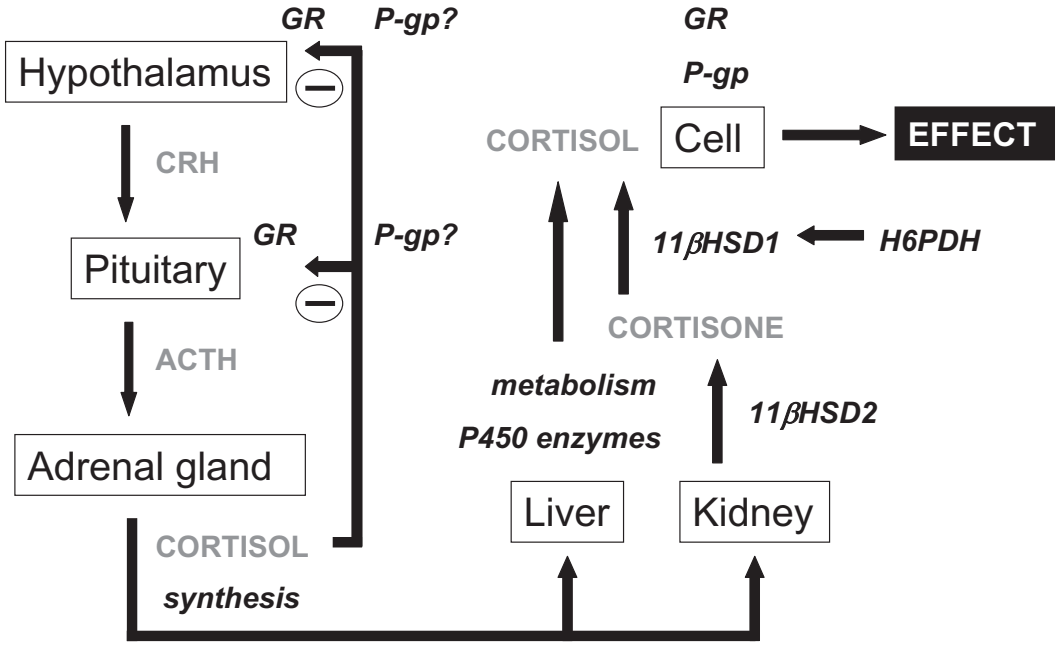

Figure 1. Effects of different factors involved in the bioavailability of cortisol to the individual cell. Cortisol is secreted under the control of the HPA-axis and has a negative feedback-action at the level of the hypothalamus and pituitary. Both the hypothalamus and pituitary contain GR. The role of P-gp on HPA-axis regulation is still unknown. In the liver, metabolizing enzymes such as P450 enzymes metabolize cortisol. In the kidney, 11β-HSD2 converts cortisol to the inactive cortisone. At cellulair level (mainly liver and adipose tissue), 11β-HSD1 converts cortisone back to cortisol. The activity of 11β-HSD1 is influenced by H6PDH. Finally, P-gp and GR determine the intracellulair cortisol concentration. HPA-axis, hypothalamic-pituitary-adrenal-axis; GR, glucocorticoid receptor; P-gp, P-glycoprotein; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; H6PDH, hexose-6-phosphate dehydrogenase

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Chapter 8

Summary
Samenvatting
List of Abbreviations
Dankwoord
Curriculum Vitae

Summary

As described in **chapter 1**, cortisol is the main glucocorticoid (GC) produced by the adrenal cortex in humans in a process called steroidogenesis. The secretion of GCs is the under control of the hypothalamic-pituitary-adrenal (HPA)-axis, the activity of which is under the influence of a circadian rhythm. Physical and psychological stress factors lead to the activation of the HPA-axis.

GCs exert a wide variety of functions throughout the human body, including mediation of the stress response, regulation of lipid and glucose metabolism, immunosuppressive and anti-inflammatory actions, vascular effects, increase of bone resorption, as well as effects on the brain and development and function of numerous organs. The effects of GCs are exerted through the GC receptor (GR), which modulates target gene transcription and protein expression via direct interaction with GC-response elements (GREs) and negative GREs (nGREs), or indirectly via cross talk with other transcription factors.

The immunosuppressive effects of GCs are routinely used in the treatment of chronic inflammatory or immune diseases. However, as severe side effects are associated with GC treatment, its therapeutic usefulness is limited. Furthermore, large inter-individual variation in GC sensitivity exists within the normal population. Some of the factors determining this variation have been studied in this thesis.

In order to apply the optimal dosage of GC for an individual patient, it is important to be able to measure GC sensitivity *ex vivo*. For this reason, we introduced the GILZ and IL-2 expression assays of which the development is decribed in chapter 2. Using these assays, we determine the effects of GCs on transactivation of the GC-induced leucine zipper (GILZ) gene and on transrepression of the interleukin-2 (IL-2) gene using quantitative real-time PCR. We showed that in the healthy population a wide variation exists in interindividual sensitivity to GCs, whilst intraindividual variation is rather stable. We also demonstrated that it is possible to differentiate in GC sensitivity with these assays between carriers and non-carriers of two known polymorphisms within the GC receptor (GR) gene, N363S and ER22/23EK. Downscaling of the GILZ and IL-2 expression assays increases the convenience for using these assays in larger groups of patients.

Chapter 3 describes the characterization of a group of 9 patients with affected GC sensitivity. Differences in GR number per cell, GR affinity, GR splice variants and

effects on transactivation of GILZ or transrepression of IL-2 were observed between these patients and healthy controls. Epstein-Barr virus transformation of lymphoblasts in order to obtain permanent cell-lines had no influence on the affinity of the GR but increased the GR number 5-fold in healthy controls, however significantly less in patients with glucocorticoid resistance. This effect might be used to discriminate between affected and non-affected GC sensitivity in patients.

11β-HSD type 1 (11β-HSD1) plays a pivotal role in the prereceptor regulation of GC hormone action by catalyzing the conversion of cortisone to cortisol. The NADPH required for this reaction is provided by hexose-6-phosphate dehydrogenase (H6PDH). Recently, it was proposed that a combination of *HSD11B1* 83,557insA and *H6PD* R453Q polymorphisms interacts to cause cortisone reductase deficiency when at least three alleles are affected. However, in **chapter 4**, we demonstrated that this is unlikely, as we found high frequencies of both polymorphisms in two study groups in the elderly, together with no effects of these polymorphisms on body composition, adrenal androgen levels, blood pressure, glucose levels, and incidence of dementia in the elderly.

In **chapter 5** we describe the effects of the C3435T and G2677T polymorphisms within the human multidrug-resistance-1 (*MDR-1*) gene, encoding for P-glycoprotein (P-gp), on HPA-axis activity and GC sensitivity. The C3435T polymorphism was associated with increased HPA-axis activity, illustrated by higher early morning serum total cortisol and androstenedione levels (without affecting the response to dexamethasone), but with no parallel increase in free and salivary cortisol levels. We concluded that P-gp is not involved in cortisol feedback regulation of the HPA-axis and that the observed increased insulin response to dexamethasone in C3435T carriers might not be related to P-gp. Using haplotype analysis we found no associations with the G2677T polymorphism.

As the fetal/neonatal enzyme CYP3A7 has a high catalytic activity for the 16α-hydroxylation of estrone (E1) and dehydroepiandrosterone (DHEA), we describe in **chapter 6** our research into the effects of this enzyme on steroid hormone metabolism in two study groups in the elderly. Due to a replacement of part of the *CYP3A7* promoter with a sequence identical to the same region in the *CYP3A4* promoter (referred to as *CYP3A7*1C*), some individuals still express a variant of the *CYP3A7* gene later in life**.** Within carriers of this polymorphism, we observed significantly lower DHEA sulfate and E1 levels, indicating that CYP3A7 is indeed still active.

Chapter 7 contains a general discussion about the research described in this thesis, concluding that more insight has been gained into factors that determine the availability of GCs to the individual cell, with an important role for the conversion/ metabolism and transcellulair transport of GCs.

Samenvatting

In **hoofdstuk 1** is beschreven dat cortisol het voornaamste glucocorticoïd bij mensen is en dat het wordt geproduceerd in een proces dat steroïdgenese wordt genoemd. De secretie van glucocorticoïden wordt gereguleerd door de hypothalamus-hypofyse-bijnier-as, waarvan de activiteit onder invloed staat van een circadiaan ritme. Fysieke en psychische stressfactoren leiden tot activatie van de hypothalamus-hypofyse-bijnier-as.

Glucocorticoïden hebben veel verschillende functies in het menselijk lichaam, waaronder het mediëren van de stress respons, regulatie van vet- en glucose metabolisme, immunosuppressieve en anti-inflammatoire werking, vasculaire effecten, toename van bot resorptie, alsmede effecten op hersenen en ontwikkeling en functie van vele organen. De effecten van glucocorticoïden worden bewerkstelligd door de glucocorticoïd receptor (GR), die doel gen transcriptie en eiwitexpressie regelt via directe interactie met glucocorticoïd-respons elementen en negatieve glucocorticoïd-respons elementen, of indirect via interactie met andere transcriptie factoren.

De immunosuppressieve effecten van glucocorticoïden worden vaak gebruikt bij de behandeling van chronische inflammatoire- of ontstekingsziekten. Er zijn echter veel bijwerkingen geassocieerd met de behandeling met glucocorticoïden hetgeen de therapeutische bruikbaarheid beperkt. Daarnaast bestaat er binnen de normale populatie grote interindividuele variatie in glucocorticoïd gevoeligheid. Enkele factoren die deze variatie bepalen zijn bestudeerd in dit proefschrift.

Om de optimale dosis glucocorticoïden voor een individuele patiënt toe te passen is het belangrijk om in staat te zijn glucocorticoïd gevoeligheid *ex vivo* te meten. Om deze reden introduceerden wij de GILZ en IL-2 expressie assays waarvan de ontwikkeling is beschreven in **hoofdstuk 2**. Met deze assays bepalen we de effecten van glucocorticoïden op de transactivatie van het glucocorticoïd-geïnduceerde leucine zipper (GILZ) gen en op de transrepressie van het interleukine-2 (IL-2) gen met behulp van quantitatieve real-time PCR. We hebben laten zien dat er in de gezonde populatie een grote variatie bestaat in interindividuele gevoeligheid voor glucocorticoïden, maar dat de variatie binnen individuen stabieler is. We hebben ook gedemonstreerd dat het mogelijk is om met behulp van assays onderscheid te maken tussen dragers en niet-dragers van twee bekende polymorfismen in het GR gen, N363S en ER22/23EK. Het omlaag schalen van de GILZ en IL-2 expressie assays vergroot de mogelijkheid om

deze assays te gebruiken in grote groepen patiënten.

Hoofdstuk 3 beschrijft de karakterisatie van een groep van 9 patiënten met aangedane glucocorticoïd gevoeligheid. Er werden verschillen gevonden in het aantal GR per cel, GR affiniteit, GR splice varianten en effecten op transactivatie van GILZ en transrepressie van IL-2 tussen deze patiënten en gezonde controles. Eppstein-Barr virus transformatie van lymfoblasten om permanente cellijnen te verkrijgen had geen invloed op de affiniteit van de GR maar verhoogde het aantal GR 5-voudig in gezonde controles, echter significant minder in patiënten met glucocorticoïd resistentie.

11β-HSD type 1 (11β-HSD1) speelt een centrale rol in de prereceptor regulatie van glucocorticoïd hormoon werking door het katalyseren van de omzetting van cortison naar cortisol. Het NADPH dat nodig is voor deze reactie wordt geleverd door hexose-6-fosfaat dehydrogenase (H6PDH). Recent werd voorgesteld dat een combinatie van *HSD11B1* 83,557insA en *H6PD* R453Q polymorfismen op elkaar inwerken om cortison reductase deficiëntie te veroorzaken wanneer ten minste drie allelen aangetast zijn. Wij demonstreerden echter in **hoofdstuk 4** dat dit onwaarschijnlijk is aangezien we hoge frequenties van beide polymorfismen vonden in twee studiegroepen binnen ouderen, zonder effecten van deze polymorfismen op lichaamssamenstelling, bijnier androgenen, bloeddruk, glucose waarden en incidentie van dementie bij ouderen.

In **hoofdstuk 5** beschrijven we de effecten van de C3435T en G2677T polymorfismen binnen het humane multidrug-resistentie-1 (*MDR-1*) gen, dat codeert voor P-glycoproteïne (P-gp), op hypothalamus-hypofyse-bijnier-as activiteit en glucocorticoïd gevoeligheid. Het C3435T polymorfisme was geassocieerd met verhoogde hypothalamus-hypofyse-bijnier-as activiteit, geïllustreerd door hogere vroege ochtend serum totaal cortisol en androsteendion waarden (zonder effect te hebben op de respons op dexamethason), maar zonder een parallelle toename in vrij cortisol en speeksel cortisol waarden. We concludeerden dat P-gp niet betrokken is bij de cortisol terugkoppeling-regulatie op de hypothalamus-hypofyse-bijnier-as en dat de geobserveerde toegenomen insuline respons op dexamethason in C3435T dragers mogelijk niet gerelateerd is aan P-gp. Gebruik makend van haplotype analyse vonden we geen associaties met het G2677T polymorfisme.

Omdat het foetale/neonatale enzym CYP3A7 een hoge affiniteit heeft voor de 16α-hydroxylatie van estron (E1) en dehydroepiandrosteron (DHEA), beschrijven we in **hoofdstuk 6** ons onderzoek naar de effecten van dit enzym op steroïd hormoon metabolisme in twee studiegroepen binnen ouderen. Door vervanging van een deel

van de *CYP3A7* promoter door een sequentie die indentiek is aan dezelfde regio in de *CYP3A4* promoter (waarnaar verwezen wordt met *CYP3A7*1C*), brengen sommige individuen later in het leven nog steeds een variant van het *CYP3A7* gen tot expressie**.** We observeerden binnen dragers van dit polymorfisme significant lagere DHEA sulfaat en E1 waarden, wat indiceert dat CYP3A7 inderdaad nog steeds actief is.

Hoofdstuk 7 bevat een algemene discussie over het onderzoek dat beschreven staat in dit proefschrift, waarbij wordt geconcludeerd dat meer inzicht is verkregen in factoren die de beschikbaarheid van glucocorticoïden aan de individuele cel bepalen, met een belangrijke rol voor omzetting/metabolisme en transcellulair transport van glucocorticoïden.

List of Abbreviations

11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
AC	acetonide
AD	Alzheimer's disease
AP-1	activator protein-1
AVP	arginine vasopressin
BMI	body mass index
CH	cortisol hypersensitivity
CMV	cytomegalovirus
CR	cortisol resistance
CRD	cortison reductase deficiency
CRH	corticotropin releasing hormone
CV	coefficient of variation
DBP	diastolic blood pressure
DEX	dexamethasone
DHEA	dehydroepiandrosterone
DHEAS	DHEA sulfate
DM	diabetes mellitus
DP	dipropionate
DST	DEX suppression test
E1	estrone
E2	estradiol
EBV	Epstein-Barr virus
GC	glucocorticoid
GILZ	GC-induced leucine zipper
GMS	Geriatric Mental State
GR	GC receptor
GRE	GC responsive element
nGRE	negative GC responsive element
h	human
H6PDH	hexose-6-phosphate dehydrogenase
HPA	hypothalamo-pituitary-adrenal
HPRT	hypoxanthine phosphoribosyltransferase

hsp	heat shock protein
HZ	heterozygous
IL-2	interleukin-2
K_D	dissociation constant
LBD	ligand-binding domain
LUC	luciferase
MC	mineralocorticoids
MDR-1	multidrug-resistance-1
MMSE	Mini-Mental State examination
MPA	6α-methyl-17α-hydroxy-progesterone acetate
MR	MC receptor
MU	mutant
NF-κB	nuclear factor- κB
PBML	peripheral blood mononuclear lymphocytes
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
P-gp	P-glycoprotein
PHA	phytohemaglutinin
POMC	pro-opiomelanocortin
PR	progesteron receptor
SBP	systolic blood pressure
SEM	standard error of the mean
SNP	single nucleotide polymorphism
STAT	signal transducer and activator of transcription
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
WHR	waist-to-hip ratio
WT	wildtype

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Curriculum Vitae

De auteur van dit proefschrift werd op 30 juli 1979 geboren te Laren. In 1997 behaalde zij het Atheneum diploma aan het Mgr. Frencken College te Oosterhout, waarna ze in september van datzelfde jaar begon aan de studie Gezondheidswetenschappen aan de Universiteit Maastricht (afstudeerrichting Biologische Gezondheidkunde). In september 2001 werd het doctoraal diploma behaald, na o.a. een afstudeeronderzoek op de afdeling Hematologie aan de Universiteit van Cambridge (UK) onder leiding van Dr. N.A. Watkins, getiteld: “Identification of a novel polymorphism in the human αIIbβ3 integrin”. In november van datzelfde jaar is zij begonnen als assistent in opleiding (AIO) bij de afdeling Inwendige Geneeskunde aan de Erasmus Universiteit Rotterdam, in het kader van het NWO-project: “Factors determining glucocorticoid sensitivity in man”, onder leiding van Prof.dr. S.W.J. Lamberts en Dr. J.W. Koper. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Per 1 september 2005 is zij begonnen aan de 4-jarige studie geneeskunde School for Utrecht Medical Masters (SUMMA) aan de Universiteit Utrecht.