VARIABILITY IN GLUCOCORTICOID SENSITIVITY;

The role of the glucocorticoid receptor

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VARIABILITY IN GLUCOCORTICOID SENSITIVITY; THE ROLE OF THE GLUCOCORTICOID RECEPTOR

VERSCHILLEN IN GEVOELIGHEID VOOR GLUCOCORTICOIDEN; DE ROL VAN DE GLUCOCORTICOID RECEPTOR

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Nannette Adriana Tobina Maria Huizenga,

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Promotores:	Prof. dr S.W.J. Lamberts
	Prof. dr F.H. de Jong
Overige leden:	Prof. dr H.A.P. Pols
	Prof. dr D.E. Grobbee
	Prof. dr H.C. Hoogsteden

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Aan mijn ouders

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List of Abbreviations

AIDS	acquired immuno deficiency syndrome
ACTH	adrenocorticotropin
AP-1	activating protein 1
AVP	arginine vasopressin
BMD	bone mineral density
BMI	body mass index
CR	cortisol resistance
CRH	corticotropin releasing hormone
DEX	dexamethasone
DOC	deoxycorticosterone
DST	dexamethasone suppression test
GCs	glucocorticoids
GGR	generalized glucocorticoid resistance
GR	glucocorticoid receptor
GRE	glucocorticoid responsive element
nGRE	negative glucocorticoid responsive element
HPA-axis	hypothalamo-pituitary-adrenal-axis
IL	interleukin
K_d	dissociation constant
LOH	loss of heterozygosity
LUC	luciferase
MMTV	mouse mammary tumor virus
α-MSH	α-melanocyte stimulating hormone
NF-κB	nuclear factor κB
PBML	peripheral bood mononuclear leukocytes
POMC	pro-opiomelanocortin
TNF-α	tumor necrosis factor-α

CHAPTER 1

INTRODUCTION

1. GLUCOCORTICOIDS

1.1 The adrenal glands and steroid hormone biosynthesis.

The adrenal glands are paired organs localized superomedially to the kidneys. Each gland is composed of a cortex and a medulla which are embryologically and functionally distinct. The cells in the medulla are the principal site of adrenaline production in the body. In the adrenal cortex, the adrenal steroids are synthesized. The cortex is composed of three histologically different zones, the zona glomerulosa, which is the outermost portion, the zona fasciculata and the zona reticularis, which is the innermost part. The adrenal cortex produces aldosterone, which is the principal mineralocorticoid, the glucocorticoid cortisol and the adrenal androgens. All steroid hormones produced by the adrenal cortex, are derived from cholesterol. About 80% of the cholesterol used for steroid synthesis is provided by circulating plasma lipoproteins (1-3). The cells of steroidogenic tissues can also synthesize cholesterol de novo from acetate or mobilize intracellular cholesteryl ester pools (1-3). A series of enzymatic steps convert cholesterol into steroids with glucocorticoid, mineralocorticoid or androgen activity. The biosynthetic pathways for adrenal steroidogenesis are shown in Figure 1.

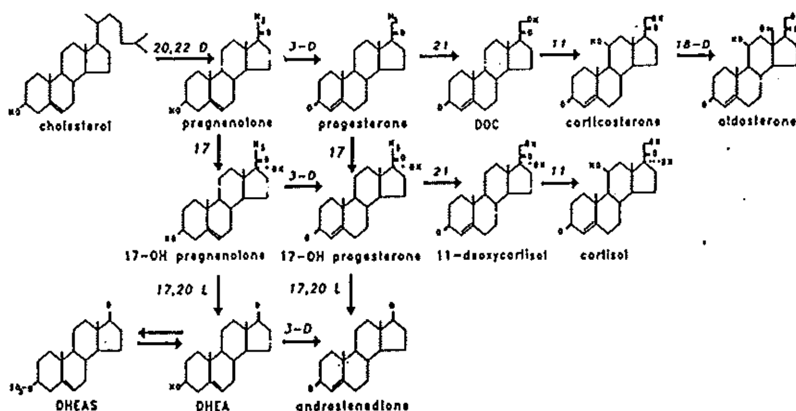

Figure 1. Diagram of the biosynthetic pathways and enzymes involved in the synthesis of glucocorticoids, mineralocorticoids and adrenal androgens from cholesterol.

1.2. Regulation of glucocorticoid secretion; The Hypothalamo-Pituitary-Adrenal-axis.

Glucocorticoid (GC) secretion is regulated by hormonal interactions between the hypothalamus, the pituitary, and the adrenal glands by neural and other stimuli (4-6). In response to neural stimuli from the brain (e.g.stress), corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) are released from the paraventricular nuclei of the hypothalamus into the hypophysial portal system (6). They are carried to the pituitary where the corticotropic cell is stimulated to synthesize and secrete corticotropin (ACTH) into the systemic blood (5). CRH is probably the most potent ACTH secretagogue (7). AVP is a weak stimulator of ACTH secretion, but it potentiates the action of CRH (8). ACTH is synthesized as part of a large precursor, the pro-opiomelanocortin (POMC) molecule (9), which is posttranslationally processed to form different biologically active peptides, among which are ACTH and α- melanocyte stimulating hormone (α-MSH) (9). ACTH in its turn stimulates the adrenal cortex to systhesize cortisol. To increase the cortisol secretion, ACTH primary acts by increasing cortisol synthesis because intra-adrenal cortisol storage is minimal (10). Apart from stimulation of cortisol secretion, ACTH stimulates the synthesis and secretion of adrenal androgens and mineralocarticoids as well (11). Adrenal growth stimulation is another effect of ACTH. Longterm supraphysiological plasma ACTH concentrations result in adrenocortical hypertrophy and hyperplasia (12).

On the other hand, GCs have an inhibitory effect on hormone synthesis and secretion at both the hypothalamic and the pituitary level, which completes a negative feedback loop. In the corticotropic cell, GCs inhibit both POMC gene transcription and ACTH secretion (9). To a lesser extent, GCs also decrease CRH and AVP mRNA levels in the hypothalamic paraventricular nuclei (13-15). Finally, GCs block the stimulatory effect of CRH on POMC gene transcription (9). A simplified model of the HPA axis is shown in Figure 2.

The activity of the HPA axis is under the influence of an endogenous pacemaker that generates a circadian rhythm. The timing of the circadian rhythm is synchronized with the solar day by dark-light shifts, which normally are a reflection of the habitual sleep-wake pattern (16-18). Plasma ACTH and cortisol concentrations are the highest at about the time of waking in the morning, are low in the late afternoon and in the evening and reach a nadir about an hour or two after sleep begins.

In addition, severe illness, infections, trauma, anesthesia, and surgery but also psychological stress are accompanied by activation of the HPA axis, as demonstrated by increased serum ACTH and cortisol concentrations (4, 19-26). This activation is an essential component of the general adaptation to stress and contributes to the maintenance of homeostasis (4, 26).

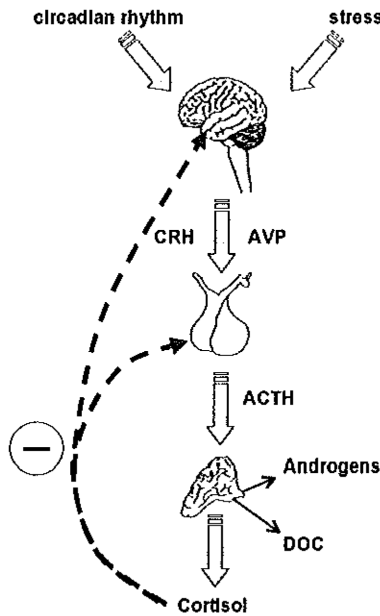

Figure 2. A simplified model of the HPA-axis: The activity of the axis is under the influence of a circadian rhythm and different forms of stress. CRH and AVP are secreted into the hypophysial portal system thus increasing the ACTH concentration. In reaction, the adrenal glands increase the secretion of cortisol along with the other adrenal steroids with androgen and mineralocorticoid activity. Cortisol has a negative feedback action at both the hyothalamic and pituitary level to complete a negative feedback loop. In this way, a perfect balance between cortisol requirement and secretion can be achieved.

1.3. Effects of glucocorticoids

GCs serve a variety of important functions in cells and tissues including mediation of the stress response, induction of gluconeogenesis in the liver, regulation of fat metabolism, and effects on the immune- and central nervous systems as well as on the development and function of numerous organs (20). Furthermore, cortisol has a vital supportive role in the maintenance of vascular tone, endothelial integrity, vascular permeability and the distribution of total body water within the vascular compartment in cases of severe illness or trauma (26). In the next section, several functions of GCs will be discussed in more detail.

Effects on glucose and lipid metabolism

The overall effect of GCs on glucose metabolism is to reduce glucose utilisation and to increase glucose production (27-29) (Figure 3). GCs inhibit glycogenolysis in order to maintain hepatic glycogen stores by activating the enzyme glycogen synthetase and inactivating the glycogen mobilising enzyme glycogen phosphorylase (27,28). Furthermore, GCs increase hepatic glucose production (gluconeogenesis) by increasing substrate availability (lactate and pyruvate) and stimulating the release of glucogenic amino acids from peripheral tissues such as skeletal muscle (29). GCs also directly activate hepatic gluconeogenetic enzymes like glucose-6-phosphatase and phosphoenol pyruvate carboxy kinase (29).Other gluconeogenic hormones such as glucagon and adrenaline are ineffective without the permissive effect of GCs (29,30). Finally, GCs inhibit glucose uptake and utilisation by peripheral tissues, partly by a direct inhibition of glucose transport into the cell, causing insulin resistance (31,32).

GCs acutely activate lipolysis in adipose tissue, thus providing free fatty acids as energy supply for gluconeogenesis (33,34). GCs also have chronic effects on lipid metabolism. After longterm GC excess, there is a striking redistribution of fat, with marked depositions of adipose tissue on the abdomen and the trunck (35) (see further section 3.2.).

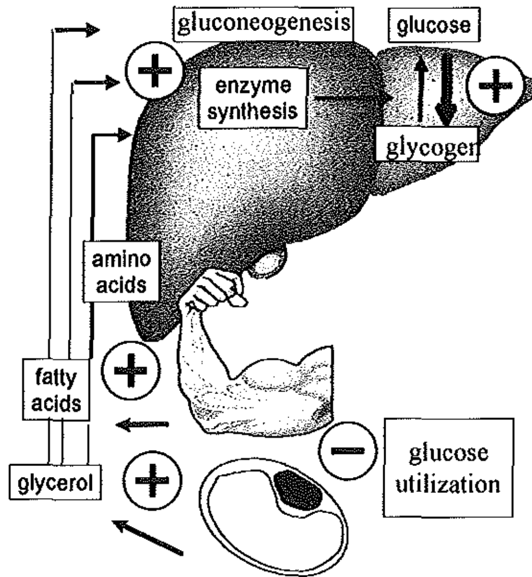

Figure 3. GC effect on hepatic glucose metabolism and peripheral tissue metabolism of protein and fat. Stimulation by GCs is indicated by a plus sign, inhibition by a minus sign.

Effects on inflammatory processes and immunological function.

Although GCs are well known for their anti-inflammatory and immunosuppressive activities, many of these probably reflect pharmacological effects (4, 25). Indeed, many of the effects of GCs have been evaluated under supraphysiological concentrations of these steroids. Despite the importance of GC therapy in modern medicine, the physiological effect of endogenous GCs on the immune system is poorly understood. This might be a consequence of the fact that GC therapy was extensively used long before the mechanisms that regulate glucocorticoid receptor activation and gene regulation (see section 2.1) were determined (36).

Inhibition of chemotaxis and bactericidal activity in neutrophils and monocytes, lymphopenia, decreased macrophage function and disturbed complement function are well known effects of GC administration (25). On the other hand, several circulating mediators of inflammation have

a major role in activating the HPA-axis (4).Three cytokines, tumor necrosis factor α (TNF-α), interleukin -1 (IL-1) and interleukin-6 (IL-6), account for most of the HPA-axis stimulating activity in plasma during inflammation. TNF-α appears first, followed by secretion of IL-1 and IL-6 (37-39). TNF-α, IL-1 and IL-6 stimulate the HPA-axis independently, but in combination, their effects are synergistical. All three cytokines stimulate their own secretion (4). Most of the GC effects on the immune system are thought to be mediated via interference with transcription of the genes encoding for these and other cytokines. The inhibition of IL-1 and IL-6 synthesis in monocytes and macrophages (40) but also the inhibition of IL-2 synthesis in T-lymphocytes (41,42) are major sites of GC action. These paradoxial effect of GCs on the immune system (the system is challenged, the defence is suppressed) have been suggested to be necessary to protect the organism against overreaction of the immune system (4,26).

Effects on bone and mineral metabolism.

One of the most important side effects in patients treated with GCs is the appearance of osteoporosis. GCs enhance bone resorption and decrease bone formation. Consequently they decrease bone mass and increase the risk of fractures (43).

The increased bone resorption is the result of direct effects of GCs on the skeleton, probably through increased osteoclast function, in combination with a decrease of intestinal calcium resorption and an increased urinary calcium excretion (43). Parathyroidectomy prevents the excessive bone resorption which is associated with GC, suggesting that *in vivo* PTH might enhance GC-induced bone resorption. Although increased PTH serum levels have been observed during GC therapy, other investigators found them to be in the normal range (45), suggesting a transient serum PTH increase or an increment of PTH activity as other possible mechanisms for the enhancement of bone resorption during steroid therapy (43).

Finally, the decreased bone formation is mostly the result of direct inhibitory effects of GCs on cells of the osteoblastic lineage (43).

Developmental effects

Supraphysiological concentrations of exogenous or endogenous GCs inhibit linear skeletal growth in children (46, 47). The mechanism of this effect of GCs is unknown: growth hormone

levels are usually within the normal range and IGF-1 concentrations are not decreased (48). Growth arrest is probably due to indirect effects of GCs on bone and connective tissue (49-51).

GCs stimulate the differentiation of many cell types. In the lung, GCs induce the production of surfactant by pneumocytes type II. This normal developmental process is accelerated by pharmacological amounts of exogenous GCs (50).

2. SYSTEMIC GLUCOCORTICOID SENSITIVITY

There is a considerable variability in the sensitivity to GCs within the normal population. As is true for the anti-inflammatory actions of GCs, the majority of our knowledge about variability in GC sensitivity in the normal population has been collected by observations in patients, who received pharmacological amounts of GCs in order to treat non-endocrine diseases. Exogenous GCs play a major role in the acute and chronic therapy of a number of immune diseases such as asthma and rheumatoid arthritis, and contribute considerably to the prevention of allograft rejection after organ transplantation (53). Nevertheless, some disadvantages of GC therapy have reduced the enthousiasm to use these agents. The first disadvantage is the unpredictable occurrance of side effects during (chronic) therapy (54-56). Although plasma concentrations can be measured, it is currently impossible to define a safe GC dose for a given patient which provides a maximal beneficial effect with as little side effects as possible. Another disadvantage is the unpredictable efficacy of GC therapy in chronic immune disease. There is clinical and laboratory evidence that patients can be divided into steroid sensitive and steroid resistant groups (56). These differences have been documented in the treatment of asthma and rheumatoid artritis as well as in renal grafts recipients, and can be observed every day in clinical settings. Little is known about the role of the structure and function of the glucocorticoid receptor in these circumstances. On the other hand, also little is known about the possible role for GC catabolism. The processes that regulate the physiological actions of steroids in target tissues are not completely known (57, 58). But catabolism of the biological active hormone to inactive forms in the liver is one of the ways this can be achieved. A number of factors like hormonal factors (59), intercurrent disease states (60), obesity (61) and several drugs(62, 63), however, may alter the usual hepatic metabolism of cortisol and other GCs. It is therefore conceivable that differences in GC catabolism might play a role in the different reactions of patients to GC therapy. Nevertheless, all effects of GCs, endogenous or exogenously administered, are mediated via the glucocorticoid receptor. Therefore, it is anticipated that alterations in GR structure or function might at least in part account for differences in GC sensitivity. The next section will discuss the structure and function of the GR, followed by several molecular determinants which could influence GC sensitivity.

2.1. The Glucocorticoid receptor

GCs exert their action on target cells via the glucocorticoid receptor (GR). The GR is a member of the intracellular steroid hormone receptor superfamily. Other members of this family include the receptors for mineralocorticoids, sex steroids, retinoic acid, vitamin D and thyroid hormone (65). These cytoplasmic receptors have a common structure which consists of a characteristic three domain structure: A poorly conserved aminoterminal region contains the transactivation domains responsible for gene activation. In the central part, two highly conserved "zinc-fingers" constitute the DNA-binding domain (66-69). This central region also participates in receptor dimerization (70), nuclear translocation and transactivation (71, 72) (see below). Finally, the relatively well conserved carboxy terminal domain is important for hormone binding. It contains sequences for heat shock protein binding (73-75), nuclear translocation (76), dimerisation (77) and transactivation (78-81). For a schematic structure of the GR protein see Figure 4 (69).

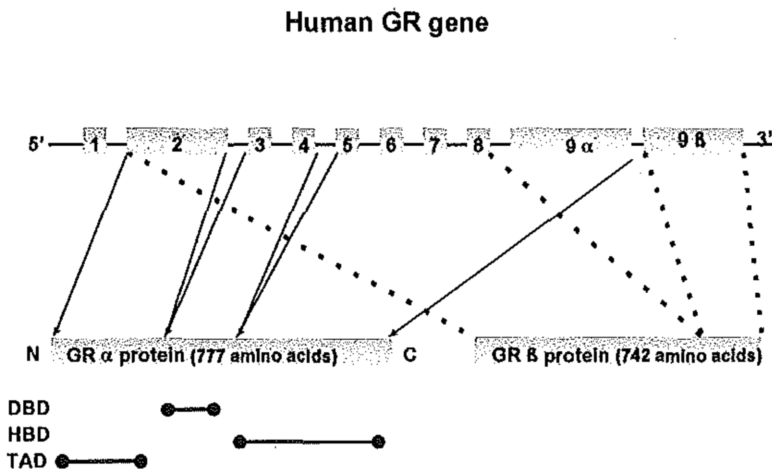

Figure 4. Schematic structure of the human GR-gene, mRNA and receptor protein. Exon 2 codes for the transactivation domain (TAD), exons 3 and 4 for the DNA-binding domain (DBD) and exons 5-9 for the hormone binding domain (HBD).

The structure of the GR gene, located on chromosome 5, consists of 10 different exons, one of which (exon 2) codes for the transactivation domain, two exons (3 and 4) code for the DNA binding domain and four exons (5-9) code for the hormone binding domain (Figure 4). The GR cDNA sequence predicts two forms of the receptor protein, a more abundant 777 amino acid α form and a 742 amino acid β form (Figure 4,) (69).

The inactivated (unliganded) receptor is present in the cytosol as a multiprotein complex with a molecular mass of about 320 kDa (82). The activated (hormone-bound) receptor has a molecular mass of 90 kDa, which means that GR activation involves dissociation of a large multiprotein complex. Using antibodies against the GR it was found that the 320 kDa complex contained only one GR molecule (83). The current understanding is that the complex consists of the receptor, two molecules of heat shock protein (hsp) 90, one molecule each of hsp 70 and hsp 56, and an immunophillin of the FK 506 and rapamycin binding class (69). Binding of hsp90 with the GR is secured by sites in the GR steroid binding domain, and this special interaction seems to facilitate the ligand binding to the receptor protein (84).

The first step in the cascade leading to induction of gene expression by GCs is binding of the hormone to the receptor which is present in the cytoplasm of the target cell (Figure 6) (69). Since the steroid is a lipophilic substance, it is thought to enter the cell by passive diffusion (85). After binding of the GC to the GR-complex, the complex dissociates, hsp90 is released and the domains responsible for nuclear localisation, DNA binding and transactivation are unmasked (73). Furthermore, the partially phosphorylated receptor protein becomes hyperphosphorylated (86-88). The activated GR molecules form homodimers as a result of the interaction of dimerization sequences present in the ligand and DNA binding domains (89). After dimerization, the GR molecules translocate to the nucleus.

Inside the nucleus the activated GR-dimers can act in two ways. The first way represents the classic model for GR action (69). The receptor homodimer binds to short, palindromically arranged DNA sequences in the promoter region of the GC responsive genes, which are called glucocorticoid responsive elements (GREs). The receptor homodimers bind to DNA using their "zinc fingers" (64). These structures of the DNA binding domains form a finger-like loop structure of 12 amino-acids anchored at the base by a zinc ion chelated by two pairs of cysteine residues. These finger-like structures presumably interact with the coils of the DNA double

helix (64). When bound to the GRE, the receptor homodimer can interact with the basic transcription cascade in several ways. One possibility is a direct interaction via contact between the GR transactivation domains and transcription factors (84). Furthermore, binding of the GR homodimer to the GRE can induce a chromatin structure rearrangement allowing other transcription factors to bind to the previously inaccessable DNA (90). In some promoters, the POMC promoter being a prototype example, binding of the activated GR to the GRE induces transcription inhibition rather than activation. These GRE's therefore are called negative GREs (nGREs) (69).

The second way in which activated GR might mediate its function is through direct protein-protein interaction. Many effects of GCs are achieved via inhibition rather than activation. This is especially apparent in the anti-inflammatory actions of GCs which involve negative transcription regulation of genes, coding for different kinds of immunoregulatory cytokines (69). These genes do not contain a nGRE in their promoter regions. It is well known that these genes are positively regulated by activating protein 1 (AP-1), which is a transcription factor that consists of dimers of the Fos and Jun protein family (69, 91, 92). Within responsive promoters, AP-1 binds to specific sequences (93). It is postulated that GR interacts directly with AP-1, thus preventing its activating action. Although AP-1 is the transcription factor that has been studied most extensively, probably many other transcription factors are regulated by the activated GR in this way of direct protein-protein interaction. A similar pattern of GR mediated transrepression has been reported for the transcription factor nuclear factor κB (NF-κB). NF-κB was first identified as a regulator of the expression of the kappa light chain gene in murine B lymphocytes, but has subsequently been found in many different cells (94). The activated form of NF-κB is a heterodimer which consists of two proteins, a p65 (also called relA) subunit and a p50 subunit. The GR mediated transrepression of NF-κB is caused by physical interaction with the p65 subunit of NF-κB. In addition, GR suppresses NF-κB activity by induction of the IκB inhibitory protein, which traps NF-κB in inactive cytoplasmic complexes (94). In Figure 5 the processes involved in the signal transduction of Gcs from the outside of the cell to the target genes are schematically shown.

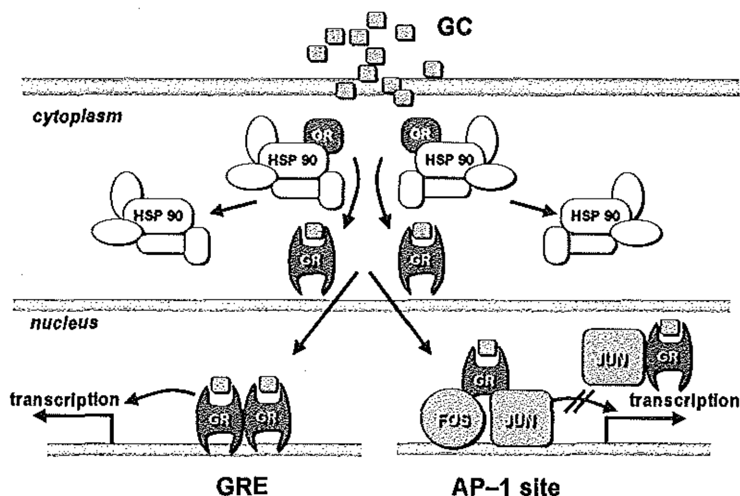

Figure 5. Simplified model of GR-mediated transcriptional modulation. Ligand binding causes dissociation of the GR-hsp multiprotein complex, dimerization of the GR and nuclear translocation. The activated GR-GR complex can either bind to GREs (left side) or inhibit transcription via direct protein-protein interaction (left side).

2.2. Molecular determinants of glucocorticoid sensitivity

All GC effects are mediated by the GR. Therefore, all different steps in the cascade of events from the unliganded silent receptor to the transcription activation or repression of GC responsive genes may be determinants of tissue sensitivity to GCs. Moreover, also post-receptor effects may play a role in the eventual sensitivity to GCs. The next section describes in short the most obvious molecular determinants of GC sensitivity.

GR expression level

It is known that the number of intracellular GRs per cell is closely correlated with the eventual biological GC response by that cell (95). A striking example of this is described in a family with a point mutation in the GR gene, leading to the expression of only one GR allele (96). This family showed signs and symptoms of the syndrome of glucocorticoid resistance (see below),

as they were not able to adequately compensate for the lack of hormone binding capacity. On the other hand, there is no consensus in the literature about the question whether in man there is a receptor up or down regulation under the influence of GCs. Many studies (97-100) show a definite receptor down regulation in reaction to high concentrations of GCs *in vitro*. On the other hand it is clear that patients from the above mentioned family (96) showed only 50 % of the normal receptor number per cell. Apparently they were not able to compensate for the lack of GR expression with an increased transcription from the intact allele of the GR gene. Moreover, the compensation was achieved by re-setting the HPA-axis at a higher setpoint rather than up-regulation the receptor number. For a more detailed description of this topic see section 3.3 of this chapter

Hormone binding affinity

The potency of the GR also closely correlates with the hormone binding affinity. It has been well established that point mutations in the hormone binding domain of the GR gene give rise to the clinical syndrome of cortisol resistance (see below) (101-102).

One of the important functions of the GR-hsp complex is to keep the GR in a "hormone friendly" conformation. As a result, low hsp expression levels or mutated hsp can negatively influence GR ligand binding affinity (103-104). On the other hand high levels of hsp were found in target tissues that are very sensitive to GCs (105).

Ligand induced GR conformation change, dissociation from hsp, phosphorylation and nuclear transformation.

Possibly the most important consequence of GC binding to the silent GR complex is a GR conformation change which causes the dissociation from hsp (116). The exact mechanism of this event is not clear yet. Shortly after or possibly at the time of GR dissociation from hsp, the receptor becomes hyperphosphorylated. It is well known whether phosphorylation influences GR function. It is obvious that these steps in GR activation are influenced by many factors, which all can cause differences in GR sensitivity. The same is true for the factors and mechanisms leading to translocation of the activated GR homodimer to the nucleus.

DNA binding

Once within the nucleus, the activated GR binds to the DNA at specific GREs. It is now known that several proteins assist the GR in DNA binding (107, 108), and that the ability of the GR to bind to DNA can be negatively regulated as well.

It might be predicted that mutations, deletions or insertions in the DNA binding domain of the GR receptor gene can cause an impaired binding ability of the GR to DNA. However, up till now no such alterations in the human GR gene have been described. Differences in GC sensitivity could also be explained by alterations in the GREs in GC responsive cells, but again, this has never been described.

Interaction with other nuclear factors

The final interaction with the transcriptional machinery of the GR responsive cell or interaction with other transcriptional activators or repressors (109) is the last step in GR mediated effects. Many different factors like the exact arrangement of the promoter, the arrangement of chromatin and the presence of other unknown factors may influence the interaction between the GR and nuclear proteins. It is not known, however, how various known mutations in the GR gene affect the interaction with transcription factors such as AP-1 and NF-κB. It has been suggested that cytokines affect the transcription of proinflammatory genes through interaction with cell surface receptors leading to increased transcription of AP-1 and NF-κB.

2.3. Generalized Glucocorticoid Resistance.

The differences in GC sensitivity in the normal population are probably relatively minor as they become only apparent when supraphysiological doses of GC are administered as a therapy for non-endocrinological diseases. An extreme example of decreased sensitivity to GCs is hereditary (generalized) cortisol resistance (CR) in which the relative insensitivity of target tissues to the effects of GCs leads to clinical signs and symptoms. CR was described for the first time in 1976 by Vingerhoeds et al (110), who reported a patient with hypercortisolism without signs and symptoms of Cushing's disease.

The secretion of cortisol by the adrenal gland is regulated by the components of the HPA-axis (4) (see section 1.2.). Of principal importance in this system is the ability of GCs to exert a negative feedback action at both the hypothalamic and the pituitary level, in order to keep a perfect balance between cortisol requirement and cortisol secretion. In cases of generalized CR, patients present with increased cortisol concentrations compared to the normal population. Keeping the structure of the HPA axis in mind, it is clear that in patients with generalized CR, the negative GC feedback on both CRH and ACTH secretion is reduced as a consequence of diminished sensitivity to GC (111, 112). As a result, the HPA axis is set at a higher level: CRH and ACTH secretion increase resulting in higher serum cortisol concentrations. In this way, the body tries to achieve a balance between cortisol requirement and cortisol secretion (11). The increased cortisol concentrations appear to compensate adequately for the reduced sensitivity. It is therefore clear that patients suffering from CR do not show any signs or symptoms of cortisol excess, because they simply need these increased serum cortisol concentrations. Not only at the hypothalamic and the pituitary level, but also at the peripheral target tissue level the sensitivity to GCs is diminished.The clinical symptoms seen in patients with CR are therefore not due to GC excess, but secondary to the activation of the HPA axis, which results in an increased production of ACTH, resulting in the stimulation of mineralocorticoid and androgen secretion. So in case of CR, the increased ACTH secretion will lead to a secondary adrenal overproduction of hormones with mineralocorticoid activity, such as deoxycorticosterone, and with androgen activity such as androstenedione, dehydroepiandrosterone and dehydroepiandrosterone sulfate (11,111,112). The pathophysiologic mechanisms of CR are summarized in Figure 6.

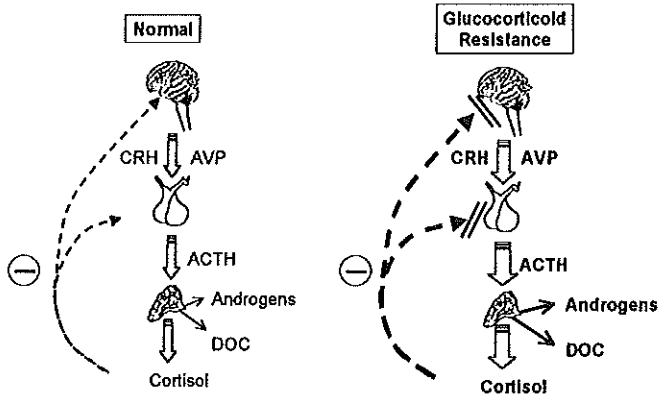

Figure 6. Schematic model for the pathophysiological mechanims in cortisol resistance. As a result of generalized decreased sensitivity to GCs, the HPA-axis is set at a higher level, resulting in adrenal overproduction of cortisol, androgens and mineralocortisoids. The increased GC level has a beneficial effect in patients because it compensates for the GC resistance; the overproduction of androgens and mineralocorticoids leads to the signs and symptoms of the clinical syndrome of CR.

2.3.1. Clinical presentation.

CR is a rare disease, which has been described in only about 30 patients and (a)symptomatic family members since the first description in 1976 by Vingerhoeds et al (110-121). The clinical presentation of the patients is variable. A summary of the different clinical pictures and references is given in Table 1. Generally, symptoms of GC deficiency were not seen in the patients, indicating that they all could compensate adequately for their GC insensitivity by activation of the HPA axis. Moreover, most of the "patients" evaluated in the context of family studies (i.e. affected family members) were asymptomatic despite increased peripheral GC levels. The only complaint of members of one particular affected family (116), however, was chronic fatigue, which might indicate that there was an insufficient compensation by

increased GC secretion in certain target tissues, like for example the central nervous system.
As a result of the increased secretion of steroids with mineralocorticoid activity, patients may present with hypertension or even hypokalemic alkalosis (110, 112). In women with CR, the secondary overproduction of adrenal androgens was reported to result in acne, hirsutism, male pattern baldness (geheimratsecken), and manifestations affecting the reproductive system like oligomenorrhoea and infertility (111, 112). Malchoff and colleagues described a seriously affected boy, who presented with precocious puberty resulting from adrenal androgen overproduction (120).
As mentioned before, the clinical presentation of CR is diverse. The manifestations described were not present in all symptomatic patients, several biochemically diagnosed patients were asymptomatic, and even within families, clinical presentation varied. A possible explanation might be that there are different degrees of resistance to cortisol among patients, resulting in different degrees of overproduction of mineralocorticoid and androgens. Moreover, the sensitivity of target tissues to mineralocorticoids and androgens might differ among patients, resulting in variable responses between individuals to the same concentrations of steroids (11).

2.3.2.Diagnostic evaluation

Biochemically, the disease is characterized by increased concentrations of plasma cortisol and increased 24 hour urinary free cortisol excretion, a normal circadian pattern of ACTH and cortisol secretion and resistance to adrenal suppression by dexamethasone (DEX), without signs or symptoms of Cushing's syndrome. In the 1 mg overnight DEX suppression test (DST), the patient is given one mg of DEX at 11.00 pm. DEX, which is a powerful synthetic corticosteroid, suppresses endogenous cortisol secretion almost completely by negative feedback action on the HPA axis. In cases of CR, the negative feedback of DEX is diminished, so the endogenous cortisol concentration remains high compared to that in controls. It is known that in cases of Cushing's syndrome, resistance to adrenal suppression by DEX exists as well (122). Therefore, clinical evaluation for signs and symptoms of Cushing's syndrome is of crucial diagnostic importance once hypercortisolism has been biochemically established. Nevertheless, in most patients with hypercortisolaemia due to Cushing's syndrome, the circadian pattern of cortisol secretion is lost, which therefore is another important differential

diagnostic parameter. Because the effects of GCs are exerted by the GR, the next step in the diagnostic evaluation of patients with hypercortisolism without Cushing's syndrome is the evaluation of the GR in these patients. As described above, ligand binding capacity and affinity are important determinants of GC sensitivity. GR characteristics can be adequately determined in whole mononuclear leukocytes as described by Lamberts et al (112). Furthermore, *in vitro* bioassays determining the GC induced inhibition of [^{3}H]-thymidine incorporation in activated mononuclear leukocytes give an impression of GC sensitivity (112). Table 1 summarizes the GR characteristics and outcomes of bioassays in all patients with clinical and biochemical CR reported up till now.

Apart from generalized (hereditary) CR, in which clinical symptoms are related to the secondary overproduction of mineralocorticoids and androgens, Norbiato et al (123) described a number of aquired immuno deficiency syndrome (AIDS) patients, who presented with signs of hypocortisolism despite elevated cortisol serum concentrations. It appeared that the GR in these patients had a lowered affinity for cortisol, whereas the number of receptors per cell was elevated. Despite this increment in receptor number, there seemed to be insufficient compensation for the diminished ligand affinity with the clinical picture of hypocortisolism as a result. These cases probably represent a form of acquired, generalised CR. Another example of CR without the classical stigmata was described by Sher, who reported two patients with severe asthma which did not respond to steroid therapy (124). The patients were asymptomatic with respect to the classical syndrome of CR, but seemed to be resistant to GC therapy. On a biochemical level, it became apparent that these patients had a decreased receptor number per cell. This probably generalized CR, presenting as steroid resistant asthma, should be separated from a form of steroid resistant asthma which is induced by local cytokine production (see section 3.1)

Table 1. Clinical presentation, receptor alterations and molecular characterization in generalized CR.

Authors	year	patients	clinical features	receptor alterations	molecular characterization
Vingerhoeds	1976	father and son	hypertension, hypokalemic alkalosis, asymptomatic	not determined	D641V
Chrousos	1982	ten affected family members	asymptomatic	decreased affinity	
Iida	1985	mother and son	mild hypertension	decreased number	
Brönnegard	1986	mother and son	fatigue	thermolability	
Lamberts	1986	one sister, three brothers	hyperandrogenism, asymptomatic	decreased number	4 base pair deletion in intron flanking exon 6
Nawata	1987	woman	asymptomatic	decreased affinity, decreased DNA binding	
Jaursch-Hancke	1989	woman	hyperandrogenism	decreased number	
Vercei	1989	woman	mild hypertension, hyperandrogenism	not determined	
Malchoff	1990	boy	isosexual precocity	decreased affinity	V729I
Lamberts	1992	two men, three women, unrelated	mild hypertension, hyperandrogenism	decreased number, decreased affinity	
Werner	1992	six women, unrelated	fatigue, hyperandrogenism, obesity	decreased number, decreased affinity, thermolability	
Norbiato	1992	nine HIV patients	hypocortisolism	decreased affinity, increased number	
Sher	1994	two asthma patients	steroid resistant asthma	decreased number	

2.3.3.Molecular characterization

As early as 1980, it was suggested that CR was the result of a defect in the intracellular cascade of events from the entrance of GCs into the cell to their final effect on cellular function. Even before the structure of the GR was known, studies performed in patients with CR showed alterations in receptor number or ligand binding affinity of the GR. Following cloning of the GR gene and subsequent studies of the genomic structure (125), PCR amplification and sequencing of genomic DNA have been possible.This strategy has revealed point-mutations and/or microdeletions in the GR gene in patients with familial CR (96, 101, 102). Circulating peripheral mononuclear leukocytes from affected members from a family previously described by Lamberts et al (111), had only 50 % of the normal number of receptors. The proband of this kindred, a 26 years old woman, presented with hirsutism, male pattern baldness and menstrual irregularities. She had no hypertension and normal serum potassium concentrations. It appeared that the patient had marked elevated levels of adrenal androgens, explaining the clinical symptoms. Furthermore, she had greatly elevated plasma cortisol levels, which were insufficiently suppressed in a 1 mg overnight DEX suppression test. The father and two of the brothers of the patient had elevated basal plasma cortisol levels, which were insufficiently suppressed by 1 mg overnight DEX suppression, but they were clinically unaffected with normal blood pressure and normal serum potassium levels. In contrast to the proband of this family, the father and affected brothers had normal androgen concentration. This was to be expected because of the much higher gonadal androgen production in males compared to females. Therefore, the overproduction of adrenal androgens gives rise to signs of hyperandrogenism in females only, and is mostly asymptomatic in males. Karl and colleagues (96) identified a 4 base pair deletion at the 3' boundary of exon and intron 6. Mutations or deletions involving the first two nucleotides of an intron disrupt normal splicing (11), generating aberrant mRNA variants. These variants are probably more susceptible to nuclease digestion, which precludes the production of mature mRNA, and may therefore exclude the expression of the encoded protein. This could explain why the receptor number on peripheral mononuclear leukocytes was reduced by 50 % in the affected members of this family.Vingerhoeds et al (110) described CR in 1976 for the first time in a father (and an asymptomatic son) who presented with severe hypertension and hypokalemia. In the

biochemical analysis, it appeared that the ligand affinity of GR in peripheral mononuclear leukocytes of this patient was decreased by a factor three. Molecular analysis revealed a homozygous (father) and heterozygous (son) aspartic acid to valine change at codon 641 of the GR. An amino acid change like this in the hormone binding domain of the GR might explain the lowered ligand affinity of the receptor in these patients. Finally, Malchoff described a young boy who presented with isosexual precocity, caused by increased adrenal androgen production associated with CR (102, 120). Biochemical analysis showed a two fold higher dissociation constant for the GR in peripheral mononuclear leukocytes of the boy. Molecular analysis of the GR gene revealed a homozygous valine to isoleucine change at codon 729 of the GR protein. In Table 1 and Figure 7, these three different previously described molecular abnormalities are summarised. The molecular basis of the only patient ever described with cortisol hypersensitivity (126) has not been elucidated yet.

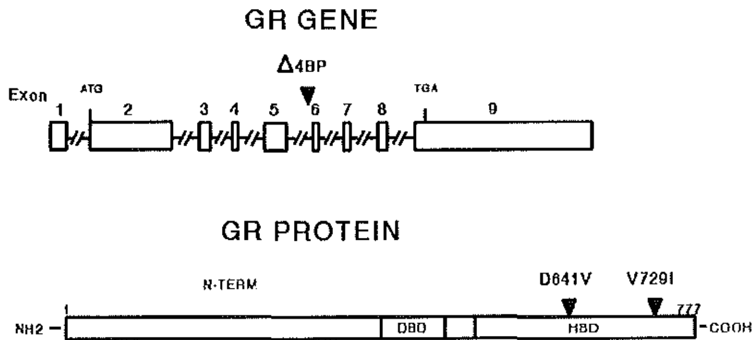

Figure 7. Schematic model of GR gene mutations in patients with heriditary GR. DBD: DNA binding domain. HBD: hormone binding domain.

3. TISSUE SPECIFIC GLUCOCORTICOID SENSITIVITY

3.1 Transient and /or acquired glucocorticoid resistance

In most cases, tissue specific CR becomes clinically apparent, because it is not compensated for by increased cortisol concentrations. This type of resistance is thought to occur in inflammatory diseases such as asthma or rheumatoid arthritis, because GCs cannot exert their physiological antiinflammatory actions. CR in asthma and rheumatoid arthritis has been reported to be accompanied by a reduction in the number of GRs in circulating leukocytes (127), and /or a reversible decrease in the ligand affinity of GR in T-lymphocytes (124), respectively. GCs are known to be powerfull suppressors of the activity of the immune system. Inhibition of chemotaxis and bactericidal activity in neutrophils and monocytes, lymphopenia, decreased macrophage function and disturbed complement function are well known effects of GC administration (25). Most effects of GCs on the immune system are thought to be mediated via inhibition of transcription of various cytokine genes, particularly those coding for IL-1 and IL-6 in macrophages (128) and IL-2 in lymphocytes (41, 42). However, during inflammation, higher concentrations of cytokines, especially IL-2, antagonize the suppressive effects of GCs in a dose dependent manner, thus counteracting these transcriptional effects (129). The balance between GC actions and the production of interleukins in mitogen-stimulated immune cells is in favor of the actions of GCs in most cases, resulting in suppression of the activity of the immune cells. In contrast, the results of a number of studies suggest that in cases of rheumatoid arthritis, asthma and sepsis, high local concentrations of cytokines in fact induce a localized CR at the site of inflammation, which cannot be overcome by excess exogenous GCs (124, 129-131). This reversible cytokine-induced form of CR should be differentiated from the situation in the patients with steroid resistant asthma described by Sher et al (124), which was due to an irreversible (probably generalized) reduction in GR number (see also Table 1.).

GCs are, in combination with chemotherapy, widely used in the therapeutic regimen of hematologic malignancies, because of their apoptotic effect on lymphoid cells. One of the best known examples of acquired CR is seen in neoplastic cells of human hematologic malignancies, which fail to respond to these apoptotic effects. The sensitivity of the malignant cells to the

apoptotic effect of GCs plays an important role in the ultimate prognosis of patients with acute leukemia or malignant lymphomas. GR alterations as wel as post-receptor abnormalities have been described as possible explanations for the CR seen in these cells (132-134).
As described before, in some patients with AIDS, GRs in peripheral leukocytes show a marked decrease in their affinity for cortisol (123). These patients are clinically characterized by features that mimic an Addisonian picture in combination with increased serum and urinary cortisol concentrations. So in some cases of AIDS, a form of aquired CR might be the cause of this clinical picture. The molecular basis of the decreased ligand affinity of the GR has not been elucidated yet.
Finally, ectopic tumors secreting ACTH and pituitary corticotropinomas form a very interesting group of tumors demonstrating localized CR. The following section will focus on the clinical, biochemical and molecular biological aspects of Cushing's disease and ACTH producing tumors.

3.2 Cushing's Disease

In 1912 Harvey Cushing described a woman with a syndrome of painfull obesity, hypertrichosis and amenorrhoea. Twenty years later, he brought together a series of 12 patients with a similar "polyglandular syndrome". Six of these patients had proven pituitary adenomas, and Cushing suggested the pituitary dependence of the disease that now bears his name (135). The clinical manifestations of chronic cortisol excess irrespective of its cause, are referred to as Cushing's syndrome (135). In classical cases, the clinical features of Cushing's syndrome are the triad of hypertension, obesity and diabetes mellitus (122). Weight gain is the most common presenting feature, and classically, the patient develops a fat redistribution to the trunk and abdominal, supraclavicular, and cervicodorsal (buffalo hump) regions, with a round "moon-face". Over time, the extremities become thinner, and the thigh and shoulder musculature becomes weaker.The skin gets thin and easily bruised, and poor wound healing may occur. Thinning of facial skin with increased visibility of the underlying blood vessels in combination with an increased hemoglobin concentration contribute to the red or plethoric facial appearance often seen in patients suffering from this disease. More than half of the patients develop wide, red striae, which should be distinguished from the milder, paler "stretch marks"

seen in obese people and pregnant women. Increased adrenal androgen production may lead to acne and folliculitis in both sexes, and to hirsutism and menstrual irregularities in women (122). Administration of exogenous GCs is the most frequent cause of Cushing's syndrome. Spontaneous Cushing's syndrome due to the overproduction of endogenous cortisol occurs in three distinct pathogenetic states: 1. Excessive pituitary ACTH secretion (Cushing's disease), 2. Autonomous cortisol production by adrenal tumors. 3. Ectopic ACTH production by a variety of extra-hypophyseal tumors. Cushing's disease accounts for about two thirds of all cases of endogenous hypercortisolism (135).

Cushing's disease is characterized by hypercortisolism caused by semiautonomous ACTH secretion by a pituitary corticotropinoma, in most cases a microadenoma.Corticotropinomas are characterized by a decreased sensitivity of the tumor cells to the negative feedback action of cortisol on ACTH secretion (136, 137), which is mediated by the GR. As described in section 2.1, hormone-bound GRs regulate the expression of glucocorticoid responsive genes by activating or repressing the transcription of glucocorticoid regulated genes after binding to "positive" or "negative" glucocorticoid response elements (GREs or nGREs) (138). The POMC gene has been shown to contain a nGRE. With respect to the diminished sensitivity to the inhibitory feedback action of cortisol in corticotrophinomas, in combination with excess cortisol secretion resulting in deleterious effects on the target tissues, Cushing's disease can be seen as a localized, acquired form of cortisol resistance. Also most tumors producing and secreting ACTH ectopically (eg small cell lung carcinomas), are characterized by a high degree of CR. This has been demonstrated to be accompanied by a reduction in the number of GR in several human small cell lung cancer cell lines (139). However, up till now, the molecular background of this form of CR has not been elucidated. Another example in this setting is the Nelson tumor, which is a fast growing ACTH producing pituitary adenoma that appears in 30% of the patients who underwent bilateral adrenalectomy for Cushing's disease (140).These tumors also are relatively insensitive to the negative feedback action of cortisol on ACTH secretion. Cells of one expanding, infiltrating Nelson tumor have been demonstrated to have a somatic frame shift mutation in the GR gene, thus explaining their GC insensitivity (140). Little is known about alterations in the GR in ACTH producing adenomas in Cushing's disease. Dahia et al (141) studied ACTH secreting tumors with respect to GR isoforms and mutations

in the GR gene. They found no significant abnormalities in the relative expression of the two main GR isoforms in these tumors. Moreover, mutations of critical regions of the GR gene did not seem to be a frequent event in studied adenomas.

3.3. Adaptation to hypercortisolemia

It has been well established that the ability of GCs to act on target tissues requires the presence of sufficient numbers of intact GRs (96). With respect to regulation of GC action, many studies investigating GR number were performed, hypothesizing that receptor down-regulation might be an additional form of negative feedback, protecting the cell from the continued signal elicited by the ligand in cases of hypercortisolism or other forms of GC excess (142). A number of studies demonstrated a direct correlation between GR number and the cell's sensitivity to GCs (95). Furthermore, a receptor down-regulation in reaction to GC therapy was demonstrated in cell cultures and animals including humans (100, 143). However, the possible mechanism of receptor down regulation is poorly understood. There is evidence for an enhancement of receptor degradation (144, 145), but these are *in vitro* data, and nothing is known about accelerated GR turnover *in vivo*. Furthermore, many investigations were performed on GR mRNA levels (98). There is evidence that GC treatment modulates GR expression levels in a number of tissues and cell types, and that down-regulation occurs at both transcriptional, post-transcriptional and/or post-translational levels (97-99). Most of the data available at present concern *in vitro* data or *in vivo* data obtained after the administration of pharmacological amounts of exogenously GC. Little is known about the physiological actions of GC on receptor number or affinity. An elegant example in this respect would be the syndrome of generalized GC resistance. It is striking that in all patients described up till now, the HPA-axis was set at a higher level, resulting in higher ACTH and cortisol concentrations. In none of these patients a receptor up-regulation was demonstrated, especially not in the patient with only half the number of receptors as a result of a splice site deletion (96). In these cases, it can be concluded that receptor up-regulation is not an additional feedback system in cases of relative cortisol shortage. Another question that could be asked with respect to receptor down regulation, is why people with any form of GC excess develop Cushing's syndrome. It could be imagined that a sufficient receptor down-regulation should protect a

patient from developing side-effects. Nevertheless, many patients treated with GC have serious adverse effects. Up till now, GR characteristics in patients with Cushing's syndrome have not been studied.

4. SCOPE OF THIS THESIS.

Within the normal population, there is a considerable variability in the sensitivity to GCs. On the other hand, little is known about the individual stability of cortisol concentrations. In **Chapter 2** a study on the reproducibility of cortisol concentrations and their relation to the feed back sensitivity of the HPA axis to a low dose of DEX is described.
In extreme cases the abnormal GC sensitivity may lead to clinical syndromes like cortisol resistance or the cortisol hyperreactivity syndrome. Ever since the first description 1979, only about 20 patients and (asymptomatic) family members with CR have been described. Up till now, the molecular basis for CR has been elucidated in only three patients and affected family members. In all three cases, mutations in the hormone binding domain of the GR gene were responsible for the clinical manifestation of cortisol resistance. Recent reports of a substantial prevalence of abnormalities in the GR in patients attending the endocrine clinic for hypokalemia, hypertension, acne, hirsutism and menstrual disorders (112, 120), prompted us to carry out a cross sectional study on the prevalence of GR abnormalities in the normal population. This study is described in **Chapter 3**. It was hypothesised that point mutations or polymorphisms in the GR gene might be reponsible for the differences in sensitivity to GCs in the normal population. **Chapter 4** describes a polymorphism in the GR gene that seems to be associated with increased sensitivity to GC *in vivo*. Moreover, as it became apparent that CR might be a less rare disease than previously thought, 6 patients attending the endocrine clinic for a broad spectrum of complaints were thouroughly investigated with respect to GC sensitivity and GR characteristics. **Chapter 5** describes their variable clinical histories in combination with endocrine testing and biochemical- and molecular characterisation of the GR in these patients and their family members.
Chapter 6 provides the extensive history of a patient with severe cortisol resistance, who finally developed Cushing's disease. It was investigated which molecular defects were the cause of this complicated clinical case. On basis of the findings described in Chapter 6 it was hypothesised that point mutations in the GR gene could play a role in the pathogenesis of Cushing's disease, or that point mutations in the GR gene in corticotropinomas were responsible for the relative CR observed in these tumors. **Chapter 7** describes the results of a

study on the prevalence of point mutations in the GR gene in corticotropinomas, and provides a model for the development of these tumors.

Chapter 8 describes the possible adaptation of glucocorticoid receptors in peripheral mononuclear leukocytes in patients with Cushing's disease as a reaction to hypercortisolism.

Finally, **Chapter 9** provides the general discussion of the data obtained from this thesis .

List of references

1. Borkowski AJ, Levin S, Delcroix C et al. 1967 Blood cholesterol and hydrocortisone production in man: Quantitative aspects of the utilization of circulating cholesterol by the adrenals at rest, and under adrenocorticotropin stimulation. J. Clin. Invst. 46:797-811.
2. Bolt E, Coudert S, Lefebvre Y. 1967 Steroid production from plasma cholesterol. II In vivo conversion of plasma cholesterol to ovarian progesterone and adrenal C19 and C21 steroids in humans. J. Clin Endocrinol Metab 38:394-400.
3. Gwynne JT, Strauss JF. 1982 The role of lipoproteins in steroid genesis and cholesterol metabolism in steroidogenic glands. Endocrine Rev 3:299-329.
4. Chrousos GP. 1995 The hypothalamus-pituitary-adrenal axis and immune mediated inflammation. N Engl J Med 332:1351-1362
5. Antoni FA. 1986 Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin releasing factor. Endocrine Rev 7:351-378.
6. Taylor AL, Fishman LM. 1988 Corticotropin releasing hormone. N Engl J Med 319:213-222.
7. Schurmeyer TH, Avgerinos PC, Cold PW et al. 1984 Human corticotropin releasing factor in man: Pharmacokinetic properties and dose-response of plasma adrenocorticotropin and cortisol secretion. J Clin Endocrinol Metab 59:1103-1108.
8. DeBold CR, Sheldon WR, DeCherney GS et al. 1984 Arginine vasopressin potentiates adrenocorticotropin release induced by ovine corticotropin releasing factor. J Clin Invest 73:533-538.
9. Lundblad JR, Roberts JL. 1988 Regulation of proopiomelanocortin gene expression in pituitary. Endocrine Rev 9:135-158.
10. Dickerman Z, Grant DR, Faiman C et al. 1984 Intraadrenal steroid concentrations in man: Zonal differentiations and developmental changes. J Clin Endocrinol Metab 59:1031-1036.
11. Chrousos GP. 1993 Syndromes of glucocorticoid resistance. Ann Internal Med 119:1113-1124.
12. Gill GN. 1972 Progress in endocrinology and metabolism. Mechanism of ACTH action. Metabolism 21:571-588.
13. Davis LG, Arentzen R, Reid JM et al. 1984 Glucocorticoid sensitivity of vasopressin mRNA levels in the paraventricular nucleus of the rat. Proc Natl Acad Sci USA 5:1145-1149.
14. Itoi K, Mouri T, Takahashi K et al. 1987 Suppression by glucocorticoid of the immunoreactivity of corticotropin releasing factor and vasopressin in the paraventricular nucleus of the rat hypothalamus. Neurosci Lett 73:231-236.
15. Beyer HS, Matta SG, Sharp BM. 1988 Regulation of the mRNA for corticortopin releasing factor in the paraventricular nucleus and other brain sites of the rat. Endocrinology 123:2117-2113.
16. Orth DN, Island DP. 1969 Light synchronization of the circadian rhythm in plasma cortisol (17-OHCS) in man. J Clin Endocrinol Metab 29:479-486.

17. Orth DN, Bresser GM, King PH et al. 1979 Free running circadian plasma cortisol rhythm in a blind human subject. Clin Endocrinol 10:603-617.
18. Moore-Ede MC, Czeisler CA, Richardson DS. 1983 Circadian time keeping in health and disease. N Engl J Med. 309-469-476.
19. Parker LN, Levin ER, Lifrak ET. 1985 Evidence for adrenocortical adaptation to severe illness. J Clin Endocrinol Metab 60:945-952.
20. Drucker D, Shandling M. 1985 Variable adrenocortical function in acute medical illness. Crit Care Med 13:477-479.
21. Drucker D, McLaughlin J. 1986 Adrenocortical dysfunction in acute medical illness. Crit Care Med 14:789-791.
22. Jurney TH, Cockrell JR, Lindberg JS et al. 1987 Spectrum of serum cortisol response to ACTH in ICU patients: correlation with degree of illness and mortality. Chest 92:292-295
23. Chopra MP, Thadani U, Aber CP et al. 1972 Plasma cortisol, 17OH corticoids and urinary vanilyl mandelic acid after acute myocardial infarction. Br Heart 34:992-995.
24. Reincke M, Allolio B, Wurth G et al. 1993 The hypothalamic pituitary adrenal axis in critical illness: Response to dexamethasone and corticotropin releasing hormone. J Clin Endocrinol Metab 77:151-156.
25. Munck A, Guyre PM, Holbrook NJ. 1984 Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocrine Rev. 5:25-44.
26. Lamberts SWJ, Bruining HA, de Jong FH. 1997 Glucocorticoid therapy in severe illness. N Engl J Med 337:1285-1292.
27. Hornbrook KR, Burch HB, Lowry OH. 1966 The effects of adrenalectomy and hydrocortisone on rat liver metabolilites and glycogen synthetase activity. Mol Pharmacol 2:106-116.
28. Stalmans W, Laloux M. 1979 Glucocorticoid and hepatic glycogen metabolism. In: Baxter JD, Rousseau CG, Eds. Glucocorticoid homone action. New York: Springer Verslag 518-533.
29. Exton JH. 1979 Regulation of gluconeogenesis by glucocorticoids. In: Baxter JD, Rousseau CG, Eds. Glucocorticoid homone action. New York: Springer Verslag 535-546.
30. Friedmann N, Exton JH, Park CR. 1967 Interaction of adrenal steroids and glucagon on gluconeogenesis in perfused rat liver. Biochem Biophys Res Commun 29:113-119.
31. Olefsky JM. 1975 Effect of dexamethasone on insulin binding, glucose transport and glucose oxidation of isolated rat adipocytes. J Clin Invest 56:1499-1508.
32. Livingston JN, Lockwood DH. 1975 Effect of glucocorticoids on the glucose transport system of isolated fat cells. J Biol Chem 250:8353-8360.
33. Fain JH. 1979 Inhibition of glucose transport in fat cells and activation of lipolysis by glucocorticoids. In: Baxter JD, Rousseau CG, Eds. Glucocorticoid homone action. New York: Springer Verslag 547-560.
34. Goodman HM, Knobil E. 1961 Some endocrine factores in the regulation of fatty acids mobilazation during fasting. Am J Physiol 201:1-3.

35. Rudman D, Di Girolamo M. 1971 Effects of adrenal cortical steroids on lipid metabolism. In: Christy NP, Ed. The human adrenal cortex. New York: Harper & Row. 241-255.
36. Wilckens T, de Rijk R 1997 Glucocorticoids and the immune function. Unknown dimensions and new frontiers. Immunol Today 18:418-424.
37. Akira S, Hirano T, Taga T et al. 1990 Biology of multifunctional cytokines: IL6 and related molucules (IL1 and TNF). FASEB j 4:2860-2869.
38. Hesse DG, Tracey KJ, Fong Y et al. 1988 Cytokine appearance in human endotoxemia and primate bacteremia. Surg Gynecol Obstet 166:147-153.
39. van Deventer SJH, Buller HR, ten Cate JW et al. 1990 Experimental endotoxemia in humans: analysis of cytokine release and coagulation, fibrinolytic and complement pathways. Blood 76:2520-2526.
40. Almawi WY, Lipman ML, Stevens AC et al. 1991 Abrogation of glucocorticoid mediated inhibition of T-cell proliferation by the synergistic action of IL-1, IL-6 and TNF-y. J Immunol 146:3523-3527.
41. Gilles S, Crabtree CR, Smith KA. 1979 Glucocorticoid induced inhibition of T cell growth factor production. J Immunol 123:1624-1631.
42. Boumpas DT, Anastassiou ED, Older SA et al. 1991 Dexamethasone inhibits human IL-2 but not IL-2 receptor gene expression in vitro at the level of nuclear transcription. J Clin Invest 87:1739-1747.
43. Canalis E. 1996 Mechanisms of glucocorticoid action in bone: Implications to glucocorticoid induced osteoporosis. Endocrine Rev 81:3441-3447.
44. Morris HA, Need AG, O'Loughlin PD et al. 1990 Malabsorption of calcium in corticosteroid induced osteoporosis. Calcif Tissue Int 46:305-308.
45. Mattersley AT, Meeran K, Burrin J et al. 1994 The effect of long- and short-term corticosteroids on plasma calcitonin and parathyroid hormone levels. Calcif Tissue Int 54:198-202.
46. Blodget FM, Burgin L, Iezzou D et al. 1956 Effects of prolonged cortisone therapy on the statural growth, skeletal maturation, and metabolic status of children. N Engl J Med 254:636-641.
47. Martial JA, Seeburg PA, Matulich DT et al. 1979 Regulation of growth hormone mRNA. In: Baxter JD, Rousseau CG, Eds. Glucocorticoid homone action. New York: Springer Verslag 279-289.
48. Stricklan AL, Underwood LE, Voina SJ. 1972 Growth retardation in Cushing's Syndrome. Am J Dis Child 123:207-213.
49. Wajchenberg BL, Pereira VG, Kieffer J et al. 1969 Effect of dexamethasone on calcium metabolism and calcium kinetics in normal subjects. Acta Endocrinol 61:173-192.
50. Lukert BP, Adams JS. 1976 Calcium and phosphorus homeostasis in man: effects of corticosteroids. Arch Intern Med 136:1249-1253.
51. Klein RG, Arnaud SB, Gallagher JC et al. 1977 Intestinal calcium absorption in exogenous hypercortisolism: role of 25-OH vitamin D and corticosteroid dose. J Clin Invest 60:253-259.
52. Ballard PL. 1987 Glucocorticoid regulation of lung maturation. Mead Jhonnon Symp Perinat Dev Med; 22-27.

53. Boumpas DT, Chrousos GP, Wilder RL et al. 1993 Glucocorticoid therapy for immune mediated diseases: Basic and clinical correlates. Ann Intern Med 119:1198-1208.
54. Bollet AJ, Black R, Bunim JJ. 1955 Major undesirable side effects resulting from prednisolone and prednisone. JAMA 158:459-463.
55. Saag KG, Oehnke R, Caldwell JR et al. 1994 Low dose long term corticosteroid therapy in rheumatoid arthritis: an analysis of serious adverse effects. Am J Med 96:115-123.
56. Lamberts SWJ, Huizenga NATM, de Lange P et al. 1996 Clinical aspects of glucocorticoid sensitivity. Steroids 61:157-160.
57. Peterson RE. 1971 Metabolism of adrenal cortical steroids. In: Christy NP, Ed. The human adrenal cortex. New York: Harper & Row. 241-255.
58. Monder C, Bradlow LH. 1980 Cortoic acids: explorations at the frontier of corticosteroid metabolism. Recent Prog Horm Res 36:345-400.
59. McGuire JS, Tomkins GM. 1959 The effects of thyroxine administration on the enzymatic reduction of delta-3-ketosteroids. J. Biol. Chem. 234:791-794.
60. Peterson RE. 1972 Metabolism of adrenocortical steroids. In: Christy NP Ed. The human adrenal cortex. New York: Harper & Row, 87-189.
61. Migeon CJ, Green OC, Eckert JP. 1963 Study of the adrenocortisac function in obesity. J Clin Endocrinol Metab 12:718-739.
62. Werk EE, McGee J, Sholiton LJ. 1964 Effect of diphenylhydantoin on cortisol metabolism in men. J. Clin. Invest. 43:1824-1853.
63. Burnsein S, Klaiber EL. 1965 Phenobarbital induced increase in 6ß-hydroxycortisol excretion: clue to its significance in human urine. J Clin Endocrinol Metab 25:293-296.
64. Wright AP, Zilliacus J, McEwan IJ et al. 1993 Structure and function of the glucocorticoid receptor. J Steroid Biochem Mol Biol. 47:11-19.
65. Evans ME. 1988 The steroid and thyroid hormone receptor superfamily. Science 40:889-895.
66. Giguere V, Hollenberg SM, Rosenfeld MG et al. 1986 Functional domains of the human glucocorticoid receptor. Cell. 46:645-652.
67. Frredman LP, Luisi BF, Korszun ZN et al. 1988 The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. Nature 334:543-546.
68. Zilliacus J, Wright APH, Calrstedt-Dule J et al. 1995 Structural determinants of DNA binding specificity by steroid receptors. Mol Endocrinol 9:389-400.
69. Bamberger CM, Schulte HM, Chrousos GP. 1996 Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocrine Rev 17:245-261.
70. Tsai SY, Carlstedt-Duke J, Weigel NL et al. 1988 Molecular interactions of steroid hormone receptor with its enhancer element: Evidence for receptor dimer formation. Cell 55:361-369.

71. Hollenberg SM, Weinberger C, Ong ES et al. 1985 Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 318:635-641.
72. Lefstin JA, Thomas JR, Yamamoto KR. 1994 Influence of a steroid receptor DNA binding domain on transcriptional regulatory functions. Genes Dev 8:2842-2856.
73. Dalman FC, Scherrer IC, Taylor LP et al. 1991 Localization of the 90 kDa heat shock protein binding site within the hormone binding domain of the glucocorticoid receptor by peptide competition. J Biol Chem 266:3482-3490.
74. Hutchkinson KA, Scherre LC, Czar MJ et al. 1993 Regulation of glucocorticoid receptor function through assembly of a receptor heat shock protein complex Ann NY Acad SCI 648:35-48.
75. Pratt WB. 1993 The role of heat shock proteins in the regulation of the function, folding and trafficing of the glucocorticoid receptor. J Biol Chem 268:21455-21458.
76. Picard D, Yamamoto KT. 1987 Two signals mediate hormone dependent nuclear localization of the glucocorticoid receptor. EMBO 6:3333-3340.
77. Dahlman-Wright K, Wright APH, Gustafsson J-A. 1992 Determinants of high-affinity DNA binding by the glucocorticoid receptor: Evaluation of receptor domains outside the DNA-binding domain. Biochemistry 31:9040-9044.
78. Hollenberg S, Evans RM. 1988 Multiple cooperative transactivation domains in the human glucocorticoid receptor. Cell 55:899-906.
79. Webster NJG, Green S, Jin JR et al. 1988 The hormone binding domain of estrogen and glucocorticoid receptors contain an inducable transcription activation function. Cell 54:199-207.
80. Danielian PS, White R, Lees JA et al. 1992 Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. EMBO J 11:1025-1033.
81. Lanz RB, Rusconi S. 1994 A conserved carboxy terminal subdomain is important for ligand interpretation and transactivation by nuclear receptors. Endocrinology 135:2183-2195.
82. Vedeckis WV. 1983 Biochemistry 22:1983-1984
83. Okret S, Wikstrom A-C, Gustafsson J-A. 1985 Biochemistry 24:6581-6586.
84. Muller M, Renkawitz R. 1990 Biochim Biophys Acta 1088:171-182.
85. Harrison RW, Fairfield S, Orth DN. 1974 Evidence for glucocorticoid transport through the target cell membrane. Biochem Biophys Res Commun 61:1262-1267.
86. Hu L-M, Bodwell J, Hu J-M et al. 1994 Glucocorticoid receptors in ATP depleted cells. J Biol Chem 269:6571-6577.
87. Orti E, Bodwell JE, Munck A. 1992 Phosphorilation of steroid hormone receptors. Endocrine Rev 13:105-128.
88. Bodwell JE, Hu L-M, Hu J-m et al. 1993 Glucocorticoid receptors: ATP dependent cycling and hormone dependent hyperphosphorilation J Steroid Biochem Mol Biol 47:31-38.
89. Nigg EA, Bauerle PA, Luhrmann R. 1991 Cell 66:15-22.

90. Bresnick EH, John S, Berard DS et al. 1990 Glucocorticoid dependent receptor disruption of a specific nucleosome on the MMTV promotor is prevented by sodium butyrate. Proc Natl Acad Sci USA 87:3977-3981.
91. Ransone LJ, Verma IM. 1990 Nuclear proto oncogenes fos and jun. Annu Rev Cell Biol 6:539-557.
92. Angel P, Karin M 1991 The role of jun, fos and AP-1 complex in cell proliferation and transformation. Biochim Biophys Acta 1072:129-157.
93. Lee W, Mitchell P, Tjian R 1987 Purified AP-1 interacts with TPA-inducible enhancer elements. Cell 49:741-752.
94. Barnes PJ, Karin M. 1997 Nuclear factor kB - A pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336:1066-1071.
95. Vanderbilt JN, Miesfeld R, Maler BA et al. 1987 Intra-cellular receptor concentration limits glucocorticoid-dependent enhancer activity. Mol Endocrinol 1:68-74.
96. Karl M, Lamberts SWJ, Detera-Wadleigh SD et al. 1993 Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. J Clin Endocrinol Metab 76:683-689
97. Burnstein KL, Bellinham DL, Jewell CM et al. 1991 Autoregulation of glucocorticoid receptor gene expression. Steroids 56:52-58.
98. Burnstein KL, Cidlowski JA. 1992 The down side of glucocorticoid receptor regulation. Mol Cell Endocrinol 83:C1-C8.
99. Burnstein KL, Jewell CM, Sar M et al. 1994 Intragenic sequences of the human glucocorticoid receptor complement DNA mediate hormone inducable receptor messenger RNA down regulation through multiple mechanisms. Mol Endocrinol 8:1770-1773.
100. Silvia CM, Powell-Oliver FE, Jewell CM et al. 1994 Regulation of the human glucocorticoid receptor by long term and chronic treatment with glucocorticoids Steroids 59:436-442.
101. Hurley DM, Accili D, Stratakis CA et al. 1991 Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucocorticoid resistance. J Clin Invest 87:680-686
102. Malchoff DM, Brufsky A, Reardon G et al. 1993 A mutation of the glucocorticoid receptor in primary cortisol resistance. J Clin Invest 91:1918-1925
103. Picard D. 1990 Reduced levels of hsp90 compromize steroid receptor action in vivo. Nature 348:166-168.
104. Nathan DF, Lindquist S. 1995 Mutational analysis of hsp90 function: Interactions with a steroid receptor and a protein kinase. Mol Cell Endo 15:3917-3925.
105. Vamvakopoulos NO. 1993. Tissue specific expression of hsp70 and 90: potential implication for differential sensitivity to glucocorticoids. Mol Cell Endocrinol 98:49-54.
106 Tsai MJ, O'Malley BW. 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. 63:451-486.

107. Cavanaugh AH, Simons SSJ. 1994 Factor assisted DNA binding as possible general mechanism for steroid receptors. Functional heterogenity among activated receptor steroid complexes. J Steroid Biochem Mol Biol 48:433-446.
108. De Vos P, Claessens F, Celis L et al. 1994 Nuclear extracts enhance the interaction of fusion proteins containing the DNA binding domain of the androgen and glucocorticoid receptor with androgen and glucocorticoid response elements. J Steroid Biochem Mol Biol 48:317-323.
109. Katzenellenbogen JA, O'Malley BW, Katzenellenbogen BS. 1996 Tripartite steroid hormone receptor pharmacology: Interaction with multiple effector sites as a basis for the cell-promotor-specific action of these hormones. Mol Endocrinol 10:119-131.
110. Vingerhoeds AC, Thijssen JH, Schwarz F. 1976 Spontaneous hypercortisolism without Cushing's syndrome. J Clin Endocrinol Metab 43:1128-1133
111. Lamberts SWJ, Poldermans D, Zweens M et al. 1986 Familial cortisol resistance: differential diagnostic and therapeutic aspects. J Clin Endocrinol Metab 63:1328-1333
112. Lamberts SWJ, Koper JW, Biemond P et al. 1992 Cortisol receptor resistance: the variability of its clinical presentation and response to treatment. J Clin Endocrinol Metab 74:313-321
113. Chrousos GP, Vingerhoeds AC, Brandon D et al. 1982 Primary cortisol resistance in man: a glucocorticoid receptor mediated disease. J Clin Invest 69:1261-1268.
114. Chrousos GP, Vingerhoeds AC, Loriaux DL et al. 1983 Primary cortisol resistance: a family study. J Clin Endocrinol Metab 56:1243-1245.
115. Lida S, Moriwaki K, Itoh Y et al. 1985 Primary cortisol resistance accompanied by a reduction in glucocorticoid receptors in two members of the same family. J Clin Endocrinol Metab 60:967-971.
116. Bronnegard M, Werner S, Gustafsson JA. 1989 Primary cortisol resistance associated with a thermolabile glucocorticoid receptor in a patient with fatigue as the only symptom. 78:1270-1278.
117. Nawata H, Sekiya K, Higuchi K et al 1987 Decreased DNA binding of glucocorticoid receptor complex in cultured skin fibroblasts from a patient with the glucocorticoid resistance syndrome. J Clin Endocrinol Metab 65:219-226.
118. Vercei P, Frank K, Haack D et al. 1989 Primary glucocorticoid receptor defect with likely familial involvement. Cancer Res 49 (supp) 2220s-2221s.
119. Jaursch-Hancke C, Allolio B, Reincke M et al. 1989 Primary cortisol resistance as a differential diagnosis of Cushing's syndrome. Acta Endocrinol Copenh 120 (supp) 113.
120. Malchoff CD, Javier EC, Malchoff DM et al. 1990 Primary cortisol resistance presnting as isosexual precocity. J Clin Endocrinol Metab 70:503-507.
121. Werner S, Thoren M, Gustafsson JA et al. 1992 Glucocorticoid recptor abnormalities in fibroblasts from patients with idiopathic resistance to dexamethasone diagnosed when evaluated for adrenocortical disorders. J Clin Endocrinol Metab 75:1005-1009.
122. Orth DN. 1995 Cushing's syndrome. N Engl J Med 332:791-803.

123. Norbiato G, Bevilaqua M, Vago T et al. 1992 Cortisol resistance in acquired immunodeficiency syndrome. J Clin Endocrinol Metab 74:608-613.

124. Sher ER, Leung DYM, Surs W et al. 1994 Steroid resistant asthma. Cellular mechanisms contributing to inadequate response to glucocorticoid therapy. J Clin Invest 93:33-39.

125. Enico IJ, Detero-Wadleigh SD. 1991 The genomic structure of the human glucocrticoid receptor. J Biol Chem 266:7182-7188.

126. Lida S, Nakamura Y, Fujii H et al. 1990 A patient with hypocortisolism and Cushing's syndrome-like manifestations: cortisol hyperreactive syndrome. J Clin Endocrinol Metab 70:729-737.

127. Chikanza IC, Panayi GS. 1993 The effects of hydrocortisone on in vitro lymphocyte proliferation and interleukin-2 and -4 production in corticosteroid sensitive and resistant subjects. Eur J Clin Invest 23:845-850.

128. Almawi WY, Lipman ML, Stevens AC et al. 1991 Abrogation of glucocorticoid mediated inhibition of T cell proliferation by the synergistic action of IL-1, IL-6 and IFN gamma. J Immunol 146:3523-3537.

129. Molijn GJ, Spek JJ, van Uffelen JCJ et al. 1995 Differential adaptation of glucocorticoid sensitivity of peripheral blood mononuclear leukocytes in patients with sepsis or septic shock J Clin Endocrinol Metab 80:1799-1803.

130. Corrigan CJ, Brown JPH, Barnes NC et al. 1991 Glucocorticoid resitance in chronic asthma: glucocorticoid pharmacokinetics, glucocorticoid receptor characteristics and inhibition of peripheral blood T cell proliferation by glucocorticoid in vitro. Am Rev Respir Dis 144:1016-1025.

131. Corrigan CJ, Brown JPH, Barnes NC et al. 1991 Glucocorticoid resistance in chronic asthma: peripheral blood T lymphocyte activation and comparison of the T lymphocyte inhibitory effects of glucocorticoids and cyclosporin A. Am Rev Respir Dis 144:1026-1032.

132. Lippman ME, Halterman RH, Leventhal BG et al. 1973 Glucocorticoid binding proteins in human acute lymphoblastic leukemic blast cells. J Clin Invest 52:1715-1726.

133. Moalli P, Rosen S. 1994 Glucocorticoid receptors and resistance to glucocorticoids in hematologic malignancies. Leukemia Lymphoma 15:363-374.

134. Strasser Wozak E, Hattmann-Storfer R, Hala M et al. 1995 Splice site mutations in the glucocortiod receptor gene cause resistance to glucocorticoid induced apaptosis in a human acute leukemic cell line. Cancer Res 55:348-353.

135. Howlett TA, Rees LH, Besser GM. 1985 Cushing's syndrome. Clin Endocrinol Metab 14:911-943.

136. Liddle GW. 1960 Tests of pituitary-adrenal suppressibility in the diagnosis of Cushing's syndrome. J Clin Enocrinol and Metab. 20:1539-1560.

137. James VHT, Landon J, Wynn V. 1965 Oral and intravenous suppression tests in the diagnosis of Cushing's syndrome. J Clin Endocrinol and Metab. 33:515-524.

138. Drouin J, Trifiro MA, Plante RK et al. 1989 Glucocorticoid receptor binding to a specific DNA sequence is required for hormone-dependent repression of proopiomelanocortin gene transcription. Mol Cell Biol 9:5305-5314.

139. Ray DW, Littlewood AC, Clark AJL et al. 1994 Human small cell lung cancer cell lines expressing the proopiomelanocortin gene have aberrant glucocorticoid receptor function. J Clin Invest 93:1625-1630.

140. Karl M, Von Wichert G, Kempter E. et al. 1996 Nelson's syndrome associated with a somatic frame shift mutation in the glucocorticoid receptor gene. J Clin Endocrinol Metab. 81:124-129.

141. Dahia PLM, Honneger J, Reincke M et al. 1997 Expression of glucocorticoid receptor gene isoforms in corticotropin secreting tumors. J Clin Endocrinol Metab. 82:1088-1093.

142. Bronnegard M, Stierna P, Marcus C. 1996 Glucocorticoid resistant syndromes - Molecular basis and clinical presentation. J Neuroendocrinol 8:405-415.

143. Knutsson U, Stierna P, Carlstedt-Duke J et al. 1995 Effects of intranasal glucocorticoids on endogenous glucocorticoid peripheral and central function. J Endocrinol 144:301-310.

144. Dong Y, Poelinger L, Gustafsson JA et al. .1988 Regulation of glucocorticoid receptor expression: evidence for transcriptional and posttranslational mechanisms. Mol Endocrinol 2:1256-1264.

145. Okret S, Dong Y, Brönnegård M et al. 1991 Regulation of glucocorticoid receptor expression. Biochimie 73: 51-59.

CHAPTER 2

INTER-PERSON VARIABILITY, BUT INTRA-PERSON STABILITY OF BASELINE PLASMA CORTISOL CONCENTRATIONS AND THEIR RELATION TO THE FEEDBACK SENSITIVITY OF THE HYPOTHALAMO-PITUITARY-ADRENAL-AXIS TO A LOW DOSE OF DEXAMETHASONE IN ELDERLY INDIVIDUALS.

Nannette A.T.M. Huizenga, Jan W. Koper, Pieter de Lange, Huibert A.P. Pols, Ronald P. Stolk, Diederick E. Grobbee, Frank H. de Jong and Steven W.J. Lamberts.

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ABSTRACT

In the present study, we investigated whether the negative feedback action of glucocorticoids (GCs) upon the HPA-axis changes with age. We performed a 1 mg dexamethasone suppression test (DST) in 216 healthy elderly individuals. In order to investigate individual variability of feedback sensitivity in more detail, two and a half years later a 0.25 mg DST was carried out in 164 of them. It was investigated whether there was an effect of age or gender on both basal and post dexamethasone (DEX) cortisol levels, as well as on the concentration of DEX. Furthermore it was examined whether the reactions to the two doses of DEX differed, and whether indications for an intraperson stability of baseline cortisol levels could be found.
Neither the pre- nor the post- 1 mg DEX plasma cortisol concentrations showed a correlation with age, while there were no differences between men and women. The same was true for the pre- and post 0.25 mg DEX cortisol concentrations.
In reaction to 1 mg DEX, over 90% of the subjects investigated showed a cortisol suppression to levels below 50 nmol/l. After the administration of 0.25 mg DEX, there was a much wider range in post DEX cortisol concentrations. After the administration of 1 mg DEX there was a significant correlation between liver function parameters and plasma DEX concentrations in males, while there was a correlation between body mass index (BMI) and plasma DEX concentration in females. Plasma DEX concentrations after the administration of 1.00 and after 0.25 mg DEX were closely correlated within subjects ($P<0.001$).
There was an intra-individual stability of serum cortisol levels, determined at an interval of two and a half years. Furthermore, the individuals with the highest baseline cortisol concentrations also had the highest post 0.25 mg DEX cortisol concentrations, indicating a close relationship between basal cortisol levels and the feedback sensitivity of the HPA-axis to a low dose of DEX.These observations suggest a genetic influence on the setpoint of the HPA-axis. Aging does not seem to lead to a change in HPA activity as measured by early morning total cortisol levels. Also, no changes in the sensitivity of the feedback system to DEX were observed with age. DEX metabolism is influenced by liver function (in males) and by BMI (in females).

INTRODUCTION

Glucocorticoids (GCs) are necessary for the normal functioning of most tissues. Even small changes in the concentrations of circulating GCs may result in a wide spectrum of physiological and biochemical changes throughout the body. Under basal conditions, the concentration of plasma cortisol and the secretion of pituitary ACTH constitute a negative feedback system. Because of the diurnal rhythm in the rate of ACTH secretion, plasma cortisol levels are higher in the morning than at night. Both negative feedback and diurnal rhythm may be overcome by stress which causes increases in ACTH output resulting in plasma cortisol levels considerably above those found in basal conditions.

Basal concentrations of plasma cortisol exhibit a wide variation between normal subjects (1). Little is known concerning the question whether cortisol levels are stable within subjects. Interperson variation and intraperson stability of plasma cortisol levels have not been extensively investigated. For the intraperson constancy, as reported in twins, genetic factors may be of importance (2, 3). The variation in hypothalamo-pituitary-adrenal (HPA) activity between persons is thought to be a function of many influences including the individual feedback sensitivity, circadian rhythm and episodic secretion, whereas cortisol binding globulin (CBG) levels also affect cortisol concentrations (3). Over the years the effects of age and gender on the function of the pituitary-adrenocortical axis have been extensively investigated. These studies, however, have resulted in contradictory results. Both circadian patterns of cortisol and ACTH secretion and random morning plasma cortisol concentrations were reported to be unaltered in the elderly (4-7). On the other hand, an age-related trend towards increased levels of evening plasma cortisol was reported (8), and increased mean cortisol levels derived from 24-h frequent sampling were found in elderly men but not in women (9). Finally, Waltman et al (10) reported that aging in humans is not accompanied by a significant increase in spontaneous ACTH or cortisol secretion or by a decrease in HPA sensitivity to GC feedback suppression.

Another controversy in the literature concerns the influence of age and gender on the dexamethasone (DEX) suppression test (DST). The 1 mg overnight DST is a convenient screening procedure for patients with Cushing's syndrome. Administration of 1 mg of DEX at

11.00 pm to normal subjects suppresses the nocturnal surge in ACTH production and as a consequence, cortisol levels are low when measured the next morning (11). In patients suffering from endogenous Cushing's syndrome of any etiology, cortisol levels are less sensitive to DEX. Another application for this test was found in depressed patients, who may show an insufficient cortisol suppression in reaction to the 1 mg DST as well (12). To interpret this test in geriatric patients, many studies were done to investigate the effect of age per se on the duration and pattern of cortisol suppression by DEX. Also, many drugs and medical conditions may affect the outcome of the DST (13). In some studies (14, 15) there were significant correlations between age and post DEX cortisol concentrations whereas in other studies no such correlations could be demonstrated (16, 17). Irrespective of psychiatric disturbances, a decreased sensitivity to GC feedback may be prevalent in the very elderly (>80 years) (18).

In the present study we investigated whether the autoregulatory negative feedback action of GCs upon the HPA-axis changes with age. We performed a 1 mg DST in 216 healthy elderly individuals, and two and a half years later a 0.25 mg DST was carried out in 164 out of these 216 subjects. In addition, it was investigated whether there was an effect of age or gender on basal and post DEX cortisol, as well as on the metabolism of DEX. Furthermore it was examined whether there were differences in response to the two doses of DEX administered, and whether the suppression of cortisol was related to baseline cortisol levels. Finally, the two baseline cortisol concentrations in the 164 individuals who underwent two examinations were compared, in order to get an impression concerning the intraperson stability of these concentrations.

SUBJECTS, METHODS AND MATERIALS.

For the present study, a sample of participants from the Rotterdam Study was invited for an additional examination. The Rotterdam study is a population-based cohort study of the determinants of chronic disabeling diseases in the elderly. All approximately 10,000 inhibitants of a suburb of Rotterdam, aged 55 years and over were invited to participate as described elsewere (19). The population for the present study included 216 persons aged 55 to 80 years

(102 men and 114 women with mean ages of 67.7 ± 5.6 and 65.8 ± 6.1 years, respectively) who had completed the baseline visit for the Rotterdam Study not more than six month earlier. Subjects with acute, psychiatric or endocrine diseases, including diabetes mellitus treated with medication, were not invited.Three of the patients used anti epileptic drugs, whereas one, who showed the lowest pre-dexamethasone cortisol concentration, was treated with glucocorticoids. None of them was treated with estrogens.Compared to the other participants of the Rotterdam Study of the same age without diabetes mellitus, there were no differences in age and gender distribution. From all subjects written informed consent was obtained and the study was approved by the medical ethics committee of the Erasmus University Medical School.

1 mg dexamethasone suppression test.

Participants were seen at the research centre after an overnight fast. Between 8.00 and 9.00 am. blood was drawn by venapuncture in order to determine the serum cortisol concentrations (pre DEX concentrations), using RIA-kits obtained from DPC (Los Angeles, CA). Intra- and interassay variations were below 8.0% and 9.5%. Also, serum concentrations of the liver enzymes alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and gamma glutamyltransferase (γGT) were determined, using standard methods. At the same time, the body weight and height of the volunteers were measured, and the body mass index (BMI,kg/m^2) was calculated to be 26.4±0.29 for the men and 26.4±0.39 for the women (mean±SE).

In the evening following the first visit to the research centre a 1 mg DST was carried out. Participants were given a tablet of 1.00 mg of dexamethasone (DEX) and instructed to ingest these at 11.00 pm. The next morning, fasting blood samples were obtained by venapuncture at the same time as the previous morning. In these samples, cortisol and DEX concentrations were measured. DEX levels were estimated by RIA using an anti serum supplied by IgG Corporation (Nashville, TN) and [^{3}H]-DEX from Amersham (Little Chalfont, UK). Intra- and interassay variations were below 8.5 and 14.2%, respectively. Sensitivity of this assay defined as mean result of assay of non-DEX containing plasma + 3SD was 0.13 nmol/l.

0.25 mg dexamethasone suppression test (DST).

In order to get more insight into individual variability of feedback sensitivity to GC's, all 216 participants were invited for a second DST two and a half years later. Out of this original group, 11 subjects had died and 6 subjects could not be reached. Eventually, 164 individuals (76 men and 88 women with mean ages of 69.1 ± 5.9 and 67.6 ± 5.6, respectively), agreed to participate in a second test. The same procedures were used as described for the 1 mg DST, now using 0.25 mg of DEX. The samples obtained from the first and the second baseline visit as well as the pre- and post 1 and 0.25 mg DEX samples were determined in separate assays. To investigate possible differences in cortisol concentrations due to interassay variation, baseline samples obtained at the first and the second examination from 20 randomly selected subjects were determined in a single cortisol assay.

0.25, 0.50 and 1 mg DST in healthy laboratory personel

A group of 9 healthy laboratory workers (aged 26 - 49 years) agreed to participate in a series of DEX suppression tests with increasing doses of DEX. At intervals of two weeks to ensure the absence of interference with DEX effects from the previous test, we performed three DEX suppression tests, using 0.25, 0.50 and 1 mg of DEX as described above. None of these individuals were using medication or oral contraceptives.

Statistical analysis

All results are reported as the mean ± SE. Serum cortisol concentrations in men and women were compared using the two sample Wilcoxon rank-sum test. The Wilcoxon matched pairs signed rank sum test was used to compare serum cortisol concentrations before and after DEX administration and between the two examinations in each person.

To assess the relationships between cortisol and age, and between DEX and liver enzymes, BMI and age, linear regression analysis was used. To calculate the degree of association between these parameters, the Pearson correlation coefficient r was calculated. Partial correlation coefficients were used in order to adjust for several parameters.

Spearman's rank correlation coefficient r_s was used to assess the association between the DEX concentrations after the administration of the two different doses and between basal cortisol concentrations at the two examinations.

RESULTS

Baseline hormone determinations.

Table 1 shows the baseline concentrations of serum cortisol in the whole group of 216 elderly subjects, and in males and females separately. There was no difference in cortisol concentrations between men and women.Using linear regression analysis, no significant correlation could be demonstrated between the baseline serum cortisol concentration and age (P>0.2).

Table 1. Cortisol concentations (nmol/l) before and after the administration of 1 mg of DEX in 216 elderly individuals (114 females and 102 males).

	total		females		males		
	mean	**SE**	**mean**	**SE**	**mean**	**SE**	**P-value***
before DEX	521.7	10.4	524.8	14.5	517.4	15.4	0.89
after DEX	35.5	4.40	32.0	4.70	38.9	8.1	0.55

* Test for the difference between males and females.

1 mg dexamethasone suppression test

Figure 1A shows the distribution of cortisol concentrations before and after 1 mg of DEX. Out of the 216 healthy individuals, 196 (90.7%) showed a post DEX cortisol concentration below 50 nmol/l. Only five individuals (2.3%) had post DEX cortisol concentrations above 140 nmol/l, which is the cut-off point for a normal test result when the DST is used as a screening procedure for Cushing's syndrome in our clinic (20).These five individuals have been further investigated for cortisol resistance and are reported separately (21)

As shown in table 1 there was no difference between men and women with regard to their post DEX cortisol concentrations. There was no correlation between post DEX cortisol concentration and age.

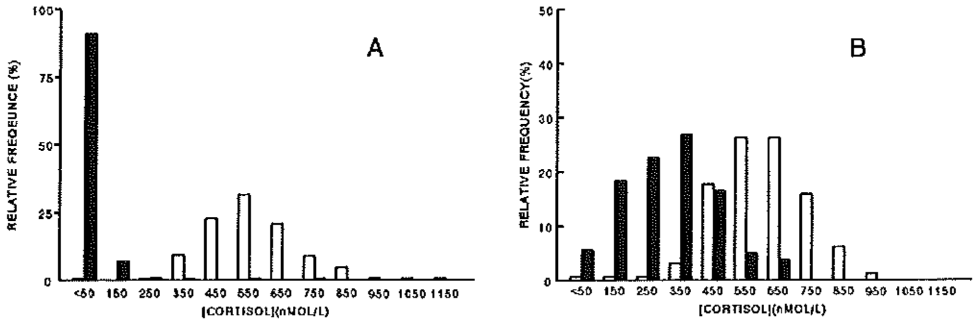

Figure 1. Cortisol concentrations before and after dexamethasone administration.

(A) The relative distribution of cortisol concentrations before (open bars) and after (black bars) 1 mg of DEX in 216 healthy elderly individuals. **(B)** The relative distribution of cortisol concentrations before (open bars) and after (black bars) 0.25 mg of DEX in 164 healthy elderly individuals. Blood samples were drawn between 8.00-9.00 a.m. after 1 or 0.25 mg of DEX was administered at 11.00 p.m. the previous night. The period between the two doses was approximately two and a half years.

Figure 2A shows the distribution of the early morning plasma DEX concentrations after the administration of 1 mg DEX. None of the individuals investigated had an unmeasurably low circulating DEX level, indicating that all participants indeed had ingested the DEX tablets. Six individuals had DEX concentrations below 1.8 nmol/l. Three of them were using anti-epileptic drugs.

Table 2 shows the results of the linear regression analysis for the relationship between DEX and several factors which might influence the metabolism of DEX. In males, there was a positive correlation between concentrations of DEX and the liver enzymes ALAT, ASAT and γGT. After deletion of the results of the 35 men, who had suprenormal levels of at least one of the enzymes, the relationship was no longer significant. In females, these correlations were not significant, despite the fact that in 21 of them increased levels of at least one of the enzymes was found. In women there was a significant correlation between DEX concentrations and BMI which was not present in men. Neither in men nor in women there was a relation between the DEX concentration and age. Baseline cortisol concentrations were not correlated with liver function tests in either sex.

Table 2. Relations between DEX concentrations and liver enzymes, BMI and age.

	total		females		males	
	r	P-value	*r*	P-value	*r*	P-value
ALAT(U/l)	0.19	<0.01	0.08	0.38	0.28	<0.01
ASAT(U/l)	0.22	<0.01	0.14	0.13	0.25	<0.02
γGT(U/l)	0.06	0.36	-0.1	0.33	0.24	<0.02
BMI(kg/m²)	0.12	0.07	0.24	<0.01	-0.03	0.78
age(years)	0.09	0.15	0.14	0.14	0.03	0.78

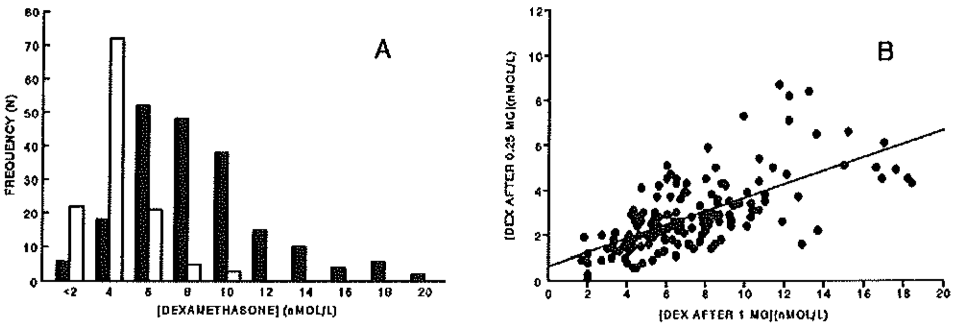

Figure 2. DEX concentrations after 1 or 0.25 mg of DEX.

(A) Distribution of DEX concentrations after 1 mg (n=216, black bars) or 0.25 mg (n=164, open bars) of DEX.

(B) Relationship between between DEX concentrations after 1 mg and 0.25 mg of DEX. r_s=0.66, P<0.001, in 164 subjects in whom both doses were tested. Blood samples were drawn between 8.00-9.00 a.m. after 1 or 0.25 mg of DEX were administered at 11.00 p.m. the previous night. The period between the two doses was approximately two and a half years.

There was no significant relation between cortisol concentrations before and after 1 mg DEX (P>0.1). The post DEX cortisol concentration and the DEX concentration itself demonstrated a low but significant negative relationship (*r*=-0.17, P<0.01). This was due to the six

individuals with the lowest DEX concentrations (<1.8 nmol/l); when these individuals were excluded from the analysis, the association was no longer significant. Also, there was no significant relationship

between the decrease in cortisol concentrations in reaction to DEX administration (Δ cortisol) and the DEX concentration (P>0.4).

0.25, 0.50 and 1 mg DST in healthy laboratory personel

From the results of the 1 mg DST, it became clear that more than 90% of the subjects investigated demonstrated a suppression of cortisol to levels below 50 nmol/L. Therefore 1 mg of DEX might be too high a suppressive dose to allow the detection of individual differences in sensitivity of the HPA-axis to glucocorticoids. A pilot study with several doses of DEX was subsequently carried out among 9 healthy volunteers. Figure 3A shows the cortisol and DEX levels after the administration of 0.25, 0.50 and 1 mg of DEX.

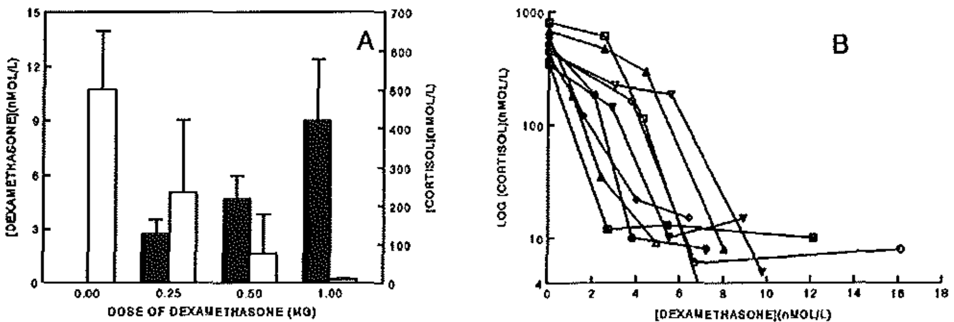

Figure 3. Effects of various doses of DEX on cortisol suppression.

(A) The cortisol (open bars) and DEX (black bars) concentrations after the administration of 0.25, 0.50 and 1 mg of DEX in 9 healthy individuals. Blood samples were drawn between 8.00-9.00 a.m. 0.25, 0.50 or mg of DEX were administered at 11.00 pm. the previous night at intervals of two weeks. Data are means and SD of the concentrations in 9 the individuals. **(B)** Individual relations between the logarithm of the cortisol concentration and the DEX concentration.

There was a considerable variability in baseline serum cortisol concentrations. The administration of increasing doses of DEX, resulted in a dose dependent increase in mean circulating DEX levels and in a dose dependent decrease of cortisol levels. The administration of 0.25 mg of DEX even enlarged the range of serum cortisol levels: in one subject the serum cortisol level was suppressed to a level below 50 nmol/l, whereas in three individuals post DEX cortisol levels remained above 200 nmol/l. After 0.50 mg of DEX the range became more narrow and after the administration of 1 mg of DEX, all persons had a cortisol concentration below 50 nmol/l.

As shown in Figure 3B, there was a considerable individual variability in cortisol suppression in reaction to a given DEX concentration. This variability was not due to individual differences in CBG concentrations (data not shown). Using linear regression analysis, there was no significant relationship between the individual DEX concentrations and the individual degree of cortisol suppression or the actual post DEX cortisol levels (all P-values > 0.8). This suggests that different individuals suppress the HPA-axis to a variable degree in reaction to similar circulating DEX concentrations.

0.25 mg dexamethasone suppression test

Anticipating that a lower dose of DEX would indeed allow a better insight in the individual variability of the feedback sensitivity of the HPA-axis, all subjects who originally participated in the 1 mg DST were invited to join in a 0.25 mg DST approximately two and a half years later.

Table 3 Cortisol concentrations (nmol/l) before and after the administration of 0.25 mg of DEX in 164 elderly individuals (88 females and 76 males).

	total		females		males		
	mean	**SE**	**mean**	**SE**	**mean**	**SE**	**P-value***
before DEX	547.8	10.9	568.4	14.2	523.6	17.2	<0.04
after DEX	260.5	10.9	243.5	15.2	281.3	16.0	0.08

* Test for the difference between males and females

Table 3 shows the mean baseline levels of serum cortisol in 164 elderly subjects. The second baseline mean serum cortisol concentrations turned out to be significantly higher than those before the 1 mg DST. The original cortisol levels of these 164 subjects amounted to 503.0 ± 11.3, while the follow up baseline cortisol levels were 547.8 ± 1.9 (P<0.001). Furthermore, in these 164 individuals, there was a significant difference in cortisol levels between men and women (P<0.04). Linear regression analysis for baseline cortisol levels and age showed no significant correlation (P>0.06). Random samples from 20 individuals out of both groups of baseline samples were determined in a single assay. Mean cortisol concentrations in both groups were identical and there was no difference between the sexes (data not shown). This suggests that there was no age-related change in cortisol concentrations, but that the differences found should be explained by the inter-assay variation.

Figure 1B shows the distribution of cortisol concentrations before and after 0.25 mg DEX. There was a much wider range in post DEX cortisol concentrations than observed after 1 mg DEX (cf. figure 1A). A small number of individuals suppressed their cortisol levels to below a level of 140 nmol/l (n=34, 20.7%), while only 9 individuals (5.5%) suppressed to levels below 50 nmol/l.

Table 3 shows the serum cortisol concentrations after the administration of 0.25 mg of DEX. There are no differences between men and women.

The distribution of DEX concentrations after 0.25 mg DEX is shown in Figure 2A. Mean DEX concentrations after this low dose of DEX were significantly lower than after 1 mg DEX (P<0.001). On the other hand, as shown in Figure 2B, subjects who showed the highest DEX concentrations after the administration of 1 mg DEX, also showed the highest levels after the administration of 0.25 mg DEX (r_s=0.66, P<0.001). Figure 2B shows only 2 out of the six individuals with DEX concentrations below 1.8 nmol/l, because 4 of those showing the lowest DEX concentrations after 1 mg DEX did not participate in the 0.25 mg DST.

Figures 4A and 4B show the linear regression analysis for the relationship between pre- and post 0.25 mg DEX cortisol concentrations (r=0.39, P<0.001), and the DEX concentration and the post DEX cortisol concentrations (r=-0.24, P<0.002). Adjusting the relationship between pre- and post DEX cortisol concentrations for the DEX concentration gives rise to a partial correlation coefficient of 0.43, P<0.001.

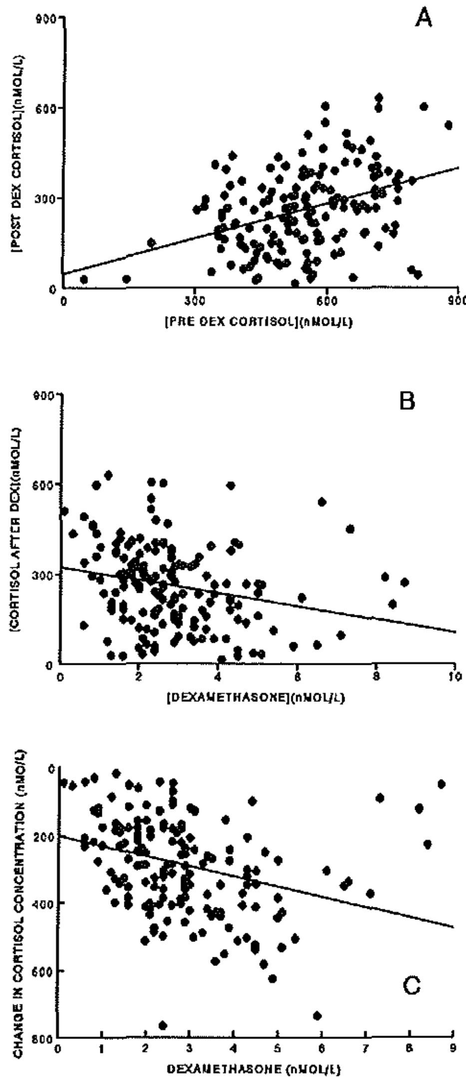

Figure 4. Relationships between cortisol and DEX concentrations in the 0.25 mg dexamethasone suppression test. **(A)** Linear regression analysis for the relationship between pre- and post 0.25 mg DEX cortisol concentrations in 164 elderly subjects. r=0.39, P<0.001. **(B)** Linear regression analysis for the relationship between DEX concentrations and the post 0.25 mg DEX cortisol concentrations in 164 elderly subjects. r=-0.24, P<0.002. **(C)** Linear regression analysis for the relationship between the DEX concentrations and the change in cortisol concentration in response to the administration of 0.25 mg DEX in 164 elderly subjects. r=0.31, P<0.001.

This indicates that those individuals with the highest baseline cortisol levels have also higher post 0.25 mg DEX cortisol levels in response to a given DEX concentration. Figure 4C shows the linear regression analysis for the DEX concentration and Δ cortisol (decrease in cortisol concentration after 0.25 mg of DEX), (β=30.7, r=0.31, P<0.001). Although there was no correlation between delta cortisol and the DEX concentration in the pilot study (n=9), there was an association between these parameters in this much larger group of individuals. The finding of a regression coefficient of 30.7 confirms that the biological activity of DEX is about 30 times higher than the biological activity of cortisol. Figure 4C also shows that in 4 subjects there is a low delta cortisol (<230 nmol/l), at DEX levels above 7 nmol/l. All 4 subjects showed post 1 mg DEX cortisol concentrations below 50 nmol/l. This indicates again that the 0.25 mg DST gives indeed a better insight into individual sensitivity to GCs than the 1 mg DST.

Individual stability of serum cortisol and CBG concentrations

In order to investigate wether plasma cortisol concentrations were stable within each person after an interval of two and a half years, Spearman's rank correlation coefficient r_s was calculated for the baseline cortisol concentrations that were obtained at the two examinations. Table 4 shows that there are highly significant correlations between the concentrations determined at an interval of two and a half years in both sexes. It can be concluded that subjects with high cortisol levels at the first examination, also had a high level two and a half years later. The small size of the coefficient is probably due to the inter assay variation. The coefficient within the 20 samples measured in one assay was much larger (r_s=0.59).Therefore there is indeed an intra-person stability in the baseline early morning concentrations of these parameters.

Table 4. Spearman's rank correlation coefficient between cortisol concentrations determined at an interval of two and a half years.

	total		females		males	
	r_s	P-value	r_s	P-value	r_s	P-value
cortisol (nmol/l)	0.45	<0.001	0.34	<0.001	0.53	<0.001

DISCUSSION

Individual feedback sensitivity of the HPA-axis

1 mg DST

In man, the major circulating glucocorticoid hormone is cortisol. Cortisol production in an unstressed individual follows a nycthemeral rhythm with peak cortisol concentrations shortly before waking and a decline through the day reaching a nadir at bedtime. Large individual variations both in baseline and stimulated cortisol levels have been reported (1). One of the goals of the present study was to get more insights in the inter-individual variability of feedback sensitivity of the HPA-axis, as well as in the intra-individual stability of circulating cortisol values. In 216 healthy elderly individuals, a 1 mg overnight DST was carried out in order to investigate the variability in feedback sensitivity. Only five out of the 216 individuals (2.3 %) showed post DEX cortisol concentrations above 140 nmol/l, which is the cut-off point for a normal test result in our clinic when the DST is used as a screenings procedure for Cushing's syndrome (20). These results are in accordance with previous studies where non-suppression was reported in 4, 9.1 and 5%, respectively (14, 16, 17). Our data demonstrate that 1 mg of DEX is too high a dose to detect individual differences in feedback sensitivity within a normal population, as near total suppression of cortisol levels was observed in most individuals.

Another aspect of this study was to get more insight in the role of the metabolism of DEX in its suppressive effects on the HPA-axis. Synthetic steroid hormones are metabolized in the liver before being eliminated from the organism (22). It is well known that a number of drugs influence the outcome of a DST, by altering the metabolism of DEX: e.g. sodium phenytoin induces hepatic enzyme activity, thereby increasing the metabolic clearance of glucocorticoids including DEX (23). In our study population, there were six subjects with a DEX concentration below 1.8 nmol/l, three of whom were indeed on anti-epileptic drugs. In the other subjects, there was a wide variation in DEX levels (ranging from 2.0 to 18.4 nmol/l). Also, obesity might influence the outcome of a DST. In a study by Crapo et al.(24), non suppression of cortisol after 1 mg DEX was reported in 1.1% of lean out-patient controls and

in 13% of obese controls. Cronin et al (25) reported that the degree of adiposity did not differ between patients with false positive responses in a DST and patients with normal responses. The data from our study show that in males, the capacity of the liver to metabolize DEX is related to liver function parameters (ALAT, ASAT and γGT). In women, DEX metabolism was not related to liver enzyme levels. This sex difference may be caused by the larger proportion of men with liver function disturbances, and the larger range of BMI values in women. Baseline and post DEX cortisol levels were not related to liver function parameters and/or BMI in either sex. Still, the influence of liver function disturbances and BMI on the metabolism of DEX might contribute to the occurrence of false positive or false negative outcomes of the 1 mg DST in the endocrine clinic in more severely affected individuals. In studies performed by Meikle (26) and by Weiner (27), it was found that plasma DEX concentrations of respectively 5.6 nmol/l and 5.1 nmol/l at 8.00h post administration are required to reliably suppress HPA function. In another study (28) in psychiatric patients, abnormal results of the DST were closely associated with low plasma levels of DEX. In a study comparing patients with endogenous depression and normal controls, it was shown that serum DEX levels were significantly lower in nonsuppressors than in suppressors (29).

0.25, 0.50 and 1 mg DST in healthy laboratory personel

From the data obtained from the 1 mg DST, it became clear that this dose might suppress the HPA-axis too much to allow the detection of individual differences of its feedback regulation. A pilot study with 0.25, 0.50 and 1 mg of DEX in 9 healthy laboratory workers showed that these lower doses indeed allowed a better discrimination in HPA-axis feedback sensitivity. The broadest range of cortisol concentrations was present after the administration of 0.25 mg DEX. This suggests that the 0.25 mg DST may give a good insight into the individual feedback sensitivity of the HPA-axis.

0.25 mg DST.

Out of the original group of 216 subjects, 164 persons participated in a 0.25 mg DST. The results from the 0.25 mg DST showed a wide range of post DEX cortisol concentrations from 14 - 630 nmol/l. It also became clear, that there was a close relationship between pre and post

DEX cortisol concentrations, a relation which was absent in the 1 mg DST. The association became even stronger when, by calculating a partial correlation coefficient, it was adjusted for the actual DEX concentration. These results show that subjects with the highest baseline cortisol concentrations also have the highest post 0.25 mg DEX cortisol concentrations for a given DEX concentration, and indicate that the sensitivity of the feedback regulation of the HPA-axis is related to the baseline cortisol concentration of the individual. This means that the "set-point" of the HPA-axis baseline levels is related to its feedback sensitivity.

Individual stability of cortisol concentrations

In this study, we were able to measure early morning cortisol concentrations in a large group of healthy subjects twice, at an interval of approximately two and a half years. Serum cortisol showed an individual stability, indicating that the HPA-axis is set at a stable and reproducible setpoint for a given individual. As described above, it became clear from the data of the 0.25 mg DST that subjects with the highest baseline cortisol concentrations also had the highest post DEX cortisol concentrations. It seems that in one individual, there is a setpoint for HPA activity, which is defined before as well as after a low dose of DEX. A dose of 1 mg DEX has too much suppressive effect to demonstrate this phenomenon, but a dose of 0.25 mg DEX, which results in a subtotal suppression of cortisol levels, leaves the influence of the individual's setpoint of the HPA axis intact. Previous studies showed that genetic factors may play a role in the regulation of cortisol levels. Maxwell et al (2) reported similar unstimulated plasma cortisol levels in a sample of female monozygotic twins. Nevertheless, this was not found in male monozygotic twins. Meikle et al.(3) observed evidence for a moderate genetic impact on early morning plasma cortisol levels. A study by Kirschbaum et al (30) investigated cortisol responses to three different stimulation procedures (a CRH test, a physical stimulation procedure and a psychological stress test), with the focus on the contribution of genetic factors. A decided influence of genetic factors was observed for baseline cortisol levels, as well as for the response to CRH, but heredity appeared to play a minor role in the adrenocortical response to physiological stress. One of the differences between this last study and the present results is, that Kirschbaum et al. demonstrated a significant intraindividual stability of baseline cortisol concentration in females, whereas levels in males were much less stable. In our study,

however, there was a highly significant individual stability of baseline cortisol concentrations in both sexes. Furthermore, the post 0.25 mg DEX cortisol levels were significantly correlated to the baseline cortisol concentrations, both in male and in female elderly individuals.

Effect of age on pre and post DEX cortisol concentrations

Studies concerning age related changes in basal, nonstimulated HPA function have produced varying results (31). Most studies have demonstrated no change or non-significant increases in mean baseline ACTH (6, 8, 10) and serum cortisol levels at older age (32, 33). The consistent lack of age and gender differences in basal cortisol levels across these various studies strengthens the conclusion that age does not have a major impact on basal nonstimulated cortisol concentrations (31). In the present study there was no relation between age or gender and basal total cortisol concentrations, neither in the first nor in the second examination. Furthermore, there was no difference between these baseline cortisol concentrations which were determined at an interval of approximately two and a half years. However, it has to be stated that our study population included elderly people only, so we were not able to compare baseline cortisol concentrations between young and old subjects.

We found no correlation between post DEX cortisol concentrations and age, neither after 1 mg DEX nor after 0.25 mg DEX administration. Furthermore, there was no difference between males and females concerning their post DEX cortisol concentrations using either one doses of DEX. Two previous studies of healthy individuals have also demonstrated no age-related differences in cortisol suppression after 1 mg DEX (16, 17). In two other studies, however, a decreased suppression was reported in older subjects after the 1 mg DST (14, 15).

The most important conclusions from our study are the observation of an individual stability of baseline cortisol levels, which is closely related to the feedback sensitivity of the HPA-axis in response to a low dose of DEX. These observations suggest a genetic influence on the setpoint of the HPA-axis.

List of references

1. Krieger DT, Allen W, Rizzo F et al. 1971 Characterization of the normal temporal pattern of plasma corticosteroid levels. J Clin Endocrinol Metab. 32:266-284.
2. Maxwell JD, Boyle JA, Greig WR et al. 1969 Plasma corticosteroids in healthy twin pairs. J Med Genet. 6:294-297.
3. Meikle AW, Stringham JD, Woodward MG et al. 1988 Heritability of variation of plasma cortisol levels. Metabolism. 37:514-517.
4. Colucci CF, D'Alessandro B, Bellastella A et al. 1975 Circadian rhythm of plasma cortisol in the aged (Cosinor method). Gerontol Clin. 17:89-95.
5. Touitou Y, Sulon J, Bogdan A et al. 1982 Adrenal circadian system in young and elderly human subjects: a comparative study. J Endocrinol. 93:201-210.
6. Blichert-Toft M. 1971 Assessment of serum corticotrophin concentration and its nyctohemeral rhythm in the aging. Gerontol Clin. 13:215-220.
7. Friedman M, Green MF, Sharland DE. 1969 Assessment of hypothalamic-pituitary-adrenal function in the geriatric age group. J Gerontol. 24:292-297.
8. Pavlov EP, Harman SM, Chrousos GP et al. 1986 Responses of plasma adrenocorticotropin, cortisol, and dehydroepiandrosterone to ovine corticotropin-releasing hormone in healthy aging men. J Clin Endocrinol Metab. 62:767-772.
9. Zumoff B, Fukushima DK, Weitzman ED et al. 1974 The sex difference in plasma cortisol concentration in man. J Clin Endocrinol Metab. 39:805-808.
10. Waltman C, Blackman MR, Chrousos GP et al. 1991 Spontaneous and glucocorticoid-inhibited adrenocorticotropic hormone and cortisol secretion are similar in healthy young and old men. J Clin Endocrinol Metab. 73:495-502.
11. Nugent CA, Nichols T, Tyler FH. 1965 Diagnosis of Cushing's syndrome. Single dose dexamethasone suppression test. Archives of Internal Medcine. 116:172-76.
12. Carroll BJ. 1984 Dexamethasone suppression test for depression. Adv Biochem Psychopharmacol. 39:179-188.
13. Guthrie S. 1991 The impact of dexamethasone pharmacokinetics on the DST: a review. Psychopharmacol Bull. 27:565-576.
14. Georgotas A, McCue RE, Kim OM et al. 1986 Dexamethasone suppression in dementia, depression, and normal aging. Am J Psychiatry. 143:452-456.
15. Weiner MF, Davis BM, Mohs RC et al. 1987 Influence of age and relative weight on cortisol suppression in normal subjects. Am J of Psychiatry. 144:646-649.
16. Ansseau M, von Frenckell R, Simon C et al. 1986 Prediction of cortisol response to dexamethasone from age and basal cortisol in normal volunteers: a negative study. Psychopharmacology. 90:276-277.

17. Tourigny-Rivard MF, Raskind M , Rivard D. 1981 The dexamethasone suppression test in an elderly population. Biol Psychiatry. 16:1177-1184.
18. Sapolsky RM, Krey LC , McEwen BS. 1986 The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. Endocr Rev. 7:284-301.
19. Hofman A, Grobbee DE, de Jong PT et al. 1991 Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. Eur J Epidemiol. 7:403-422.
20. Lamberts SWJ, De Jong FH, Birkenhager JC. 1977 Evaluation of diagnostic and differential diagnostic tests in Cushing's syndrome. Neth J Med. 20:267-274.
21. Koper JW, Stolk RP, de Lange P et al. 1997 Lack of association between five polymorphisms in the human glucocorticoid receptor gene and glucocorticoid resistance. Human Genetics. 99:663-668.
22. Kutemeyer S, Schurmeyer TH , von zur Muhlen A. 1994 Effect of liver damage on the pharmacokinetics of dexamethasone. Eur J Endocrinol. 131:594-597.
23. Jubiz W, Meikle AW, Levinson RA et al. 1970 Effect of diphenylhydantoin on the metabolism of dexamethasone. N Engl J Med. 283:11-14.
24. Crapo L. 1979 Cushing's syndrome: a review of diagnostic tests. Metabolism. 28:955-977.
25. Cronin C, Igoe D, Duffy MJ et al. 1990 The overnight dexamethasone test is a worthwhile screening procedure. Clin Endocrinol. 33:27-33.
26. Meikle AW. 1982 Dexamethasone suppression tests: usefulness of simultaneous measurement of plasma cortisol and dexamethasone. Clin Endocrinol. 16:401-408.
27. Weiner MF. 1989 Age and cortisol suppression by dexamethasone in normal subjects. J Psychiatr Res. 23:163-168.
28. Arana GW, Workman RJ , Baldessarini RJ. 1984 Association between low plasma levels of dexamethasone and elevated levels of cortisol in psychiatric patients given dexamethasone. Am J Psychiatry. 141:1619-1620.
29. Poland RE, Rubin RT, Lesser IM et al. 1987 Neuroendocrine aspects of primary endogenous depression. II. Serum dexamethasone concentrations and hypothalamic-pituitary-adrenal cortical activity as determinants of the dexamethasone suppression test response. Arch Gen Psychiatry. 44:790-795.
30. Kirschbaum C, Wust S, Faig HG et al. 1992 Heritability of cortisol responses to human corticotropin-releasing hormone, ergometry, and psychological stress in humans. J Clin Endocrinol Metab. 75:1526-1530.
31. Seeman TE, Robbins RJ. 1994 Aging and hypothalamic-pituitary-adrenal response to challenge in humans. Endocr Rev. 15:233-260.
32. Vermeulen A, Deslypere JP, Schelfhout W et al. 1982 Adrenocortical function in old age: response to acute adrenocorticotropin stimulation. J Clin Endocrinol Metab. 54:187-191.
33. West CD, Brown H, Simons EL et al. 1961 Adrenocortical function and cortisol metabolism in old age. J Clin Endocrinol Metab. 21:1197-1207.

CHAPTER 3

LACK OF ASSOCIATION BETWEEN FIVE POLYMORPHISMS IN THE HUMAN GLUCOCORTICOID RECEPTOR GENE AND GLUCOCORTICOID RESISTANCE.

Jan W. Koper, Ronald P. Stolk, Pieter de Lange, Nannette A.T.M. Huizenga, Gerd-Jan Molijn, Huibert A.P. Pols, Diederick E. Grobbee, Michael Karl, Frank H. de Jong, Albert O. Brinkmann, and Steven W.J. Lamberts.

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ABSTRACT

Glucocorticoid resistance due to mutations in the gene for the glucocorticoid receptor has been suggested to be more common than is known at present, due to the relative mildness of its symptoms and the difficulty of its diagnosis. To investigate the prevalence of mutations in the glucocorticoid receptor gene responsible for relative insensitivity to glucocorticoids, we carried out PCR/SSC analysis of the glucocorticoid receptor gene in a group of 20, otherwise healthy, persons with a reduced response in a dexamethasone suppression test and in 20 controls. We did not find mutations or polymorphisms associated with a reduced sensitivity for glucocorticoids. However we identified 5 novel polymorphisms in the gene for the human glucocorticoid receptor, which may be useful in analysing whether loss of (part of) the glucocorticoid receptor gene plays a role in glucocorticoid resistant malignancies.
Conclusion: although relative resistance to glucocorticoids seems to be rather frequent in otherwise healthy persons, it is not usually associated with mutations or polymorphisms in the glucocorticoid receptor gene.

INTRODUCTION

Glucocorticoid (GC) hormones are essential for postnatal life. GCs exert their function through the cellular glucocorticoid receptor (GR); absence of the GR in knock-out mice leads to death soon after birth (1). In humans, complete absence of functional GRs has not been observed. However, several families have been described with hereditary "partial" or "relative" GC resistance (2-5). In these four families, mutations in the part of the GR-gene coding for the hormone binding domain (HBD) of the protein were found to be the cause of a reduced affinity of the GR for GCs (2,5)or of a reduced number of receptors per cell due to a splice site deletion in one allele of the gene (4). In one case, a mutation in the HBD was shown to cause dominant negative inhibition of the wild-type GR (5).
The GR is a member of the intracellular- or steroid-hormone-binding superfamily of receptors. Other members of this family include the receptors for sex steroids, vitamins A and D and thyroid hormone. These intracellular (cytoplasmic and/or nuclear) receptors have a common structure which comprises an N-terminal transactivating domain, a central DNA-binding domain and a C-terminal hormone binding domain (6).
The symptoms of partial glucocorticoid resistance are the result of the role of the GR in the negative feedback mechanism that governs the hypothalamic-pituitary-adrenal(HPA)-axis (7). Primarily, the reduced GC sensitivity results in increased cortisol production by the adrenal glands, which compensates for the reduced feedback inhibition of the HPA. Increased adrenal activity also results in increased amounts of adrenal androgens and of mineralocorticoids. It is this overproduction of other adrenal steroids which causes the primary symptoms accompanying glucocorticoid resistance such as hirsutism, menstrual irregularities and fertility problems in women (8), while a case of isosexual precocious puberty has been described in a boy (3). Increased levels of mineralocorticoids lead to hypertension and hypokalemia (9). The symptoms, however, are usually relatively mild and very variable in nature (8). Thus, two of three siblings carrying the same mutation (4,8,10) were essentially without symptoms (8). The long-term effects of glucocorticoid resistance are unknown, but there are some indications that it may be involved in the development of hyperplasia of the pituitary corticotroph cells, and eventually the development of pituitary corticotroph tumors (5,11).

Apart from the four cases mentioned above, which have been fully elucidated at the genomic level, approximately 20 other patients have been described in whom glucocorticoid resistance has been clinically and biochemically diagnosed without further knowledge with regard to mutations of their GR genes (12-15). In two studies relatively large numbers of newly diagnosed cases of glucocorticoid resistance have been reported (8, 16).

In the present study we have investigated the prevalence of glucocorticoid resistance in a population based study. Subsequently, we have searched for mutations and polymorphisms in the GR-gene and investigated their relationship to the observed GC insensitivity.

METHODS

Study population

A random sample of participants from the Rotterdam Study was invited to participate in the present study. The Rotterdam Study is a population-based follow-up study of the determinants of chronic disabling diseases in the elderly. The study focuses on four areas of chronic disease: cardiovascular, neurogeriatric, locomotor, and ophthalmologic diseases; details have been published previously (17). The present study population included 216 persons aged 55 to 80 years without evident psychogeriatric or endocrine disease, including treated diabetes mellitus. From all subjects informed consent was obtained and the study has been approved by the medical ethics committee of the Erasmus University Medical School.

A reduced response in an overnight 1 mg dexamethasone suppression test (18) in 216 subjects (see below) was used as an indicator of relative glucocorticoid insensitivity. The 20 persons with the highest post-dexamethasone cortisol levels were selected. The post-dexamethasone cortisol concentrations in this group of persons was >50 nmol/L. From the remaining subjects (who all had post-dexamethasone cortisol levels <50 nmol/L) 20 age- and sex-matched controls were selected randomly. The GR gene of these 40 persons was analysed for the presence of mutations, using a polymerase chain reaction based analysis of single strand conformation, (PCR/SSC, 19) of the GR gene was carried out. In the case of two polymorphisms, their frequency was further estimated by additional PCR/SSC analysis of the relevant part of the gene in the remaining 176 individuals. The persons with reduced

suppression also underwent an extensive physical examination.

Dexamethasone suppression test and hormone determinations

The participants were seen at the Rotterdam Study research centre after an overnight fast and blood was drawn between 8.00 and 9.00 am. for measurement of the concentration of circulating cortisol. Subsequently, all participants were subjected to an overnight 1 mg dexamethasone suppression test (18). They were asked to take their dexamethasone tablets at 11.00 pm and fasting blood samples were again drawn the next morning between 8.00 and 9.00 am. The concentration of cortisol was determined using kits supplied by Diagnostics Products Corporation (Los Angeles CA) (8). To check for compliance and possible drug-induced abnormalities in the metabolism of dexamethasone, the dexamethasone concentration was also measured in a radioimmunoassay using antiserum obtained from IgG Corporation (Nashville, TN).

PCR/SSC analysis and nucleotide sequence determination

DNA was isolated from peripheral blood leukocytes using standard techniques. PCR amplification of the GR gene was carried out employing primer sequences and amplification conditions previously described (4) (Table 1 and Fig. 1). For the amplification of exons 3 through 8 intronic primer pairs complementary to sequences in the 5'- and 3'-exon-flanking regions were used. Exon 2, which encodes the entire N-terminal part of the glucocorticoid receptor, was amplified in five overlapping segments (see also Fig. 1). The non-coding exon 1 was not analyzed and of exon 9α only the 5' coding part was investigated. Exon 9β which can form part of the mature mRNA instead of exon 9α, after alternative splicing (20), resulting in a non-functional receptor protein, was not investigated. SSC analysis of the PCR products was carried out using 0.5 x MDE polyacrylamide gels (J.T. Baker Chemicals, Deventer, the Netherlands) under three conditions, as described (19), except that 5% glycerol was used instead of 10%. DNA-fragments displaying an abnormal migration pattern during SSC-analysis were subjected to direct sequencing using a modified Sanger-dideoxynucleotide chain termination method as previously described (4).

Table 1. Primers used for PCR/SSC and sequencing.

Exon 2/1	GAT	TCG	GAG	TTA	ACT	AAA	AG
	ATC	CCA	GGT	CAT	TTC	CCA	TC
Exon 2/2	CCA	AGC	AGC	GAA	GAC	TTT	TG
	TAC	CTG	GGG	ACC	CAG	AAG	AA
Exon 2/3	CCA	CAG	AGA	AGG	AGT	TTC	CA
	TTG	CCT	GAC	AGT	AAA	CTG	TG
Exon 2/4	CCA	GTA	ATG	TAA	CAC	TGC	CCC
	TTC	GAC	CAG	GGA	AGT	TCA	GA
Exon 2/5	AGT	ACC	TCT	GGA	GGA	CAG	AT
	GTC	CAT	TCT	TAA	GAA	ACA	GG
Exon 3	AGT	TCA	CTG	TGA	GCA	TTC	TG
	CGT	GAG	AAA	TAA	AAC	CAA	GT
Exon 4	GAC	AGA	AGG	CTG	TCC	TTA	TA
	CAT	TAT	GCG	TAT	CAA	GCA	TA
Exon 5	GAA	TAA	ACT	GTG	TAG	CGC	AG
	TAG	TCC	CCA	GAA	CTA	AGA	GA
Exon 6	GAT	CTT	CTG	AAG	AGT	GTT	GC
	GGG	AAA	ATG	ACA	CAC	ATA	CA
Exon 7	GAA	AGT	TCT	CCA	AAA	TTC	TG
	TTG	GTG	TCA	CTT	ACT	GTG	CC
Exon 8	GAC	ACA	GTG	AGA	CCC	TAT	CT
	CAC	CAA	CAT	CCA	CAA	ACT	GG
Exon 9α	GGA	ATT	CCA	GTG	AGA	TTG	GT
	TAT	AAA	CCA	CAT	GTA	GTG	CG

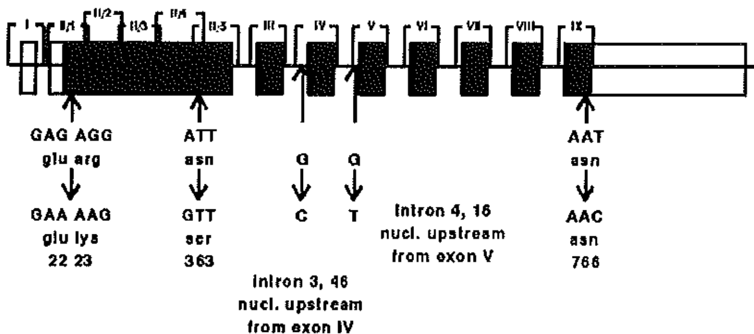

Figure 1. Genomic organization of the glucocorticoid receptor gene and the positions of the amplified fragments used for PCR/SSC analysis. The nature and position of the polymorphisms that were identified are shown below the figure. (Not drawn to scale.)

Data analysis

Gender-adjusted parameters were calculated for each participant by linear regression analysis. The clinical characteristics of the study population were compared between controls and the persons with reduced suppression using Student's t-test. The Wilcoxson rank-sum test was used to analyze the differences in characteristics between subjects with and without mutations in the glucocorticoid receptor gene. P-values smaller than 0.05 were considered statistically significant.

RESULTS

Clinical observations

In the group of 216 participants of The Rotterdam Study, 20 persons (prevalence: 9.3%, 95% C.I.: 5.4-13.2) were identified with a reduced response (cortisol > 50 nmol/L) in the 1 mg dexamethasone suppression test.

Table 2. Clinical characteristics of the study population.[1]

	response in the dexamethasone suppression test				
	post-dex [cortisol] <50 nmol/L		**post-dex [cortisol] >50 nmol/L**		**p-value**
Number	196		20		-
Women (%)	53.6		50.0		0.76
Age (years)	66.6	(0.4)	67.8	(1.4)	0.40
Body mass index (kg/m²)	26.5	(0.2)	25.6	(1.3)	0.35
Waist/hip ratio	0.92	(0.01)	0.91	(0.02)	0.68
Systolic blood pressure(mmHg)	139.3	(1.4)	137.6	(5.0)	0.70
Diastolic blood pressure (mmHg)	75.0	(0.7)	74.2	(2.3)	0.72
Hypertension[2] (%)	29.1		40.0		0.31
Ankle/arm blood pressure index	0.98	(0.02)	0.97	(0.08)	0.86
Potassium (mmol/L)	4.08	(0.02)	4.08	(0.08)	0.96
Fasting cortisol (nmol/l)	509.1	(9.6)	608.3	(55.4)	0.005
Cortisol after DST	22.8	(0.7)	149.1	(39.8)	-
DEX after DST	7.39	(0.24)	6.67	(1.10)	0.39

(1) Values are means with standard error in parentheses, or percentages.

In Table 2 the clinical characteristics are given for all subjects. The subjects with reduced response in the DST also had higher fasting cortisol levels. Dexamethasone concentrations were not different between the two groups. Physical examination of the 20 subjects with reduced suppression did not reveal signs or symptoms related to hypokalemia, hirsutism or infertility.

PCR/SSC- and DNA sequence-analysis

PCR/SSC-analysis revealed five SSC-variants (Figures 1 and 2, and Table 3). Four of these variants were observed in three or more participants in this study, while the fifth, observed in one person in this study, had previously been found in another person not included in the present study (J.W. Koper, unpublished observations). Sequence analysis showed that all SSC-variants were caused by point mutations.

In the case of exon 2/1 sequence analysis revealed two point mutations separated by one base-pair. The mutations were located at cDNA nucleotide positions (20) 198 and 200, respectively. The first mutation was silent, changing codon 22 from GA*G* to GA*A*, both coding for glutamic acid. The second mutation changed codon 23 from A*G*G to A*A*G resulting in an amino acid change from arginine to lysine. PCR/SSC analysis in 109 of the remaining 176 samples (i.e. in persons with a normal response in the DST) revealed a further 8 persons who were heterozygous for this polymorphism (Table 3).

In fragment 2/5 an *A*TT to *G*TT point mutation at cDNA position 1220 resulted, heterozygously, in an asparagine to serine change at codon 363 in four subjects. Subsequently, using PCR/SSC analysis of the remaining 176 DNA samples we discovered 9 additional subjects who were heterozygous for this polymorphism (Table 3).

The polymorphisms in the amplified segments containing exons 4 and 5 were located in the 5'-flanking intron sequences rather than in the exons themselves. In the fragment containing exon 4 a mutation was identified as a heterozygous G to C change 46 nucleotides upstream from the start of exon 4, which is at cDNA position 1549 (Table 3). The polymorphism in the fragment containing exon 5 was more frequent and was a G to T change 16 nucleotides upstream from the starting point of exon 5 which is located at cDNA position 1666. For this polymorphism 5 homozygous subjects were also identified (Fig. 2 and Table 3). The polymorphisms in the

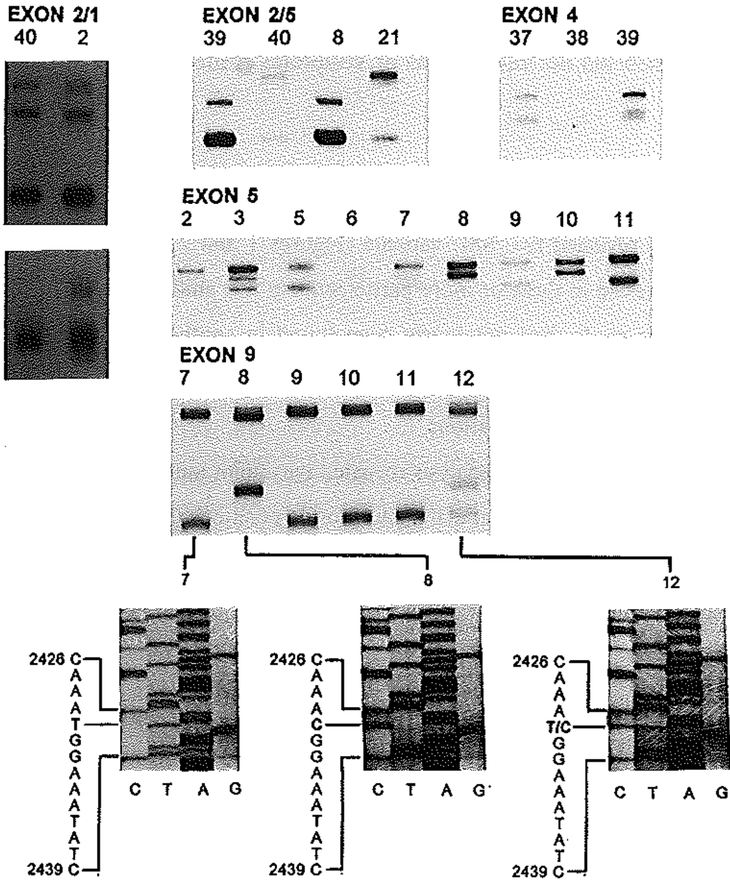

Figure 2. SSC patterns of all the polymorphisms: exon 2/1 wt (40) and heterozygote (2); exon 2/5 wt (39 and 8) and heterozygote (40 and 21); exon 4 wt (37 and 38) and heterozygote (40); exon 5 wt (5, 9 and 11), heterozygote (2, 3 and 7) and homozygote variant (6, 8 and 10); exon 9 wt (7, 9, 10 and 11), heterozygote (12) and homozygote variant (8). Sequence analysis for exon 9 of the samples 7, 8 and 9.

Table 3. Frequencies of the polymorphisms in the glucocorticoid receptor gene

	subjects with post-dex cortisol <50 nmol/L (%)	subjects with post-dex cortisol >50 nmol/L (%)	all subjects	frequency of the variant alleles (%)(95% C.I.)
codons 22+23: wild type GAGAGG (GluArg), variant GAAAAG (*GluLys*)				
GAGAGG/GAGAGG	121/129	17/20	138/149	
GluArg/GluArg	(93.8)	(85.0)	(92.6)	
				GAAAAG
GAGAGG/GAAAAG	8/129	3/20	11/149	11/298
GluArg/*GluLys*	(6.2)	(15.0)	(7.4)	(3.7%)
				(0.6-6.8)
GAAAAG/GAAAAG	0/129	0/20	0/149	
GluLys/GluLys	(0.0)	(0.0)	(0.0)	
codon 363: wild type ATT (Asn), variant GTT (*Ser*)				
ATT/ATT	183/196	20/20	203/216	
Asn/Asn	(93.4)	(100.0)	(94.0)	
				GTT
ATT/GTT	13/196	0/20	13/216	13/432
Asn/*Ser*	(6.6)	(0.0)	(6.0)	(3.0%)
				(0.7-5.3)
GTT/GTT	0/196	0/20	0/216	
Ser/Ser	(0.0)	(0.0)	(0.0)	
intron 3, 46 nucleotides upstream from exon 4: wild type G, variant *C*				
G/G	20/20	19/20	39/40	
	(100.0)	(95.0)	(97.5)	
				C
G/*C*	0/20	1/20	1/40	1/80
	(0.0)	(5.0)	(2.5)	(1.3%)
				(0-4.8)
C/C	0/20	0/20	0/40	
	(0.0)	(0.0)	(0.0)	
intron 4, 16 nucleotides upsteam from exon 5: wild type G, variant *T*				
G/G	9/20	10/20	19/40	
	(45.0)	(50.0)	(47.5)	
				T
G/*T*	10/20	7/20	17/40	25/80
	(50.0)	(35.0)	(42.5)	(31.3%)
				(16.6-45.9)
T/T	1/20	3/20	4/40	
	(5.0)	(15.0)	(10.0)	
codon 766: wild type AAT (Asn), variant AAC (*Asn*)				
AAT/AAT	16/20	14/20	30/40	
Asn/Asn	(80.0)	(70.0)	(75.0)	
				AAC
AAT/AAC	4/20	5/20	9/40	11/80
Asn/*Asn*	(20.0)	(25.0)	(22.5)	(13.7%)
				(2.9-24.6)
AAC/AAC	0/20	1/20	1/40	
Asn/Asn	(0.0)	(5.0)	(2.5)	

fragments containing exons 4 and 5 might be associated with sequences important in the splicing process, however we did not observe differences in the glucocorticoid binding capacity of peripheral blood leukocytes of persons with these polymorphisms (results not shown). Finally, the polymorphism in exon 9α, which was also relatively frequent, was caused by a silent T to C point mutation in codon 766 (AA*T* to AA*C*, both coding for asparagine). One person homozygous for this mutation was also identified (Fig 2). A single case of this polymorphism has been reported previously (21).

The polymorphisms reported here were neither associated with clinical (hirsutism, hypokalemia, hypertension) glucocorticoid resistance, nor with a reduced response in the dexamethasone suppression test (for all analyses $p>0.4$). None of the physiological parameters investigated differred in a significant way between the various genotypes. Table 3 shows the distribution of genotypes over the two subgroups from the dexamethasone suppression test, all genotypes, with the exception of the codon 363 polymorphism, are evenly divided over the two subgroups.

DISCUSSION

Glucocorticoid resistance in the cases that have been described until now was found to be caused by mutations in the hormone-binding domain of the GR (2-4). Thus in "classical" glucocorticoid resistance the glucocorticoid receptor is the key-modulator of glucocorticoid sensitivity. Two reports show relatively high numbers of new patients with a reduced glucocorticoid sensitivity, based on clinical and biochemical observations in the endocrine out-patients clinic (8,16). Both these reports focussed on persons with an otherwise unexplained reduced response in the 1 mg DST. We set out to determine whether mutations in the GR gene also played a role in the general differences in glucocorticoid sensitivity, such as they are observed in the general population.

Our present results indicate that biochemical glucocorticoid resistance is more common than expected: 20 out of 216 subjects (9.3%, 95% C.I.: 5.4-13.2) in an elderly non-hospitalized population were shown to have a reduced response in the 1 mg DST. However, no "classical" clinical and biochemical glucocorticoid resistance, accompanied by mutations in the hormone-

binding domain of the GR, was identified in any of these subjects.

We found five polymorphisms in the GR gene. However, none of these polymorphisms were associated with parameters indicative of glucocorticoid resistance. The double mutation in codons 22 and 23 has also been tested in in vitro expression experiments (22). However, these mutations do not seem to alter the efficacy of the GR in such systems. Similarly the polymorphism in codon 363 did not alter the function of the GR (4, 22, 23).

The possible effects of the intronic mutation in the 5'-flanking region to exon 5 are more difficult to assess. It seems unlikely that this mutation affects the splicing-process in some way, as no significant changes in the number of receptors, nor in their affinity were observed in persons homozygous for this polymorphism. Another possibility is that this polymorphism is linked to another mutation in or near the GR gene that was not detected using the PCR/SSC approach of this study.

Recently, a number of papers (24-26), reported that the ß-form of the human glucocorticoid receptor, a natural splice variant, is expressed in many tissues and might play a role in the response to glucocorticoids, as it was shown to have a dominant negative effect on the function of the αGR (24-26). In future research it will be interesting to investigate if there is a relation between the relative levels of expression of these two GR forms, both at the mRNA and at the protein levels, and the observed differences in sensitivity to glucocorticoids.

From the present investigation we conclude that biochemical glucocorticoid resistance is quite common and that a number of polymorphisms exist in the gene for the GR, but that these polymorphisms do not cause the observed glucocorticoid resistance.

These polymorphisms, however, may be useful in determining whether loss of (part of) one of the copies of the GR gene has occurred in malignancies known to be glucocorticoid resistant (c.f. 23, 27). We are currently investigating the role of the GR in corticotroph pituitary tumors and we have indications that there is loss of heterozygosity for the GR in a number of these tumors, as observed by analysing these intragenic polymorphisms (N.A.T.M.H., unpublished observations).

List of references

1. Cole T, Blendy J, Monaghan A et al. 1995 Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev 9:1606-1621.
2. Hurley DM, Accili D, Stratakis CA et al. 1991 Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucocorticoid resistance. J Clin Invest 87:680-686.
3. Malchoff DM, Brufsky A, Reardon G, McDermott P et al. 1993 A mutation of the glucocorticoid receptor in primary cortisol resistance. J Clin Invest 91:1918-1925.
4. Karl M, Lamberts SW, Detera-Wadleigh SD et al. 1993 Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. J Clin Endocrinol Metab 76:683-689.
5. Karl M, Lamberts SWJ, Koper JW et al. 1996 Cushing's disease preceded by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proc Assn Am Phys 108:296-307.
6. Beato M, Herrlich P, Schütz G. 1995 Steroid hormone receptors: many actors in search of a plot. Cell 83:851-857.
7. Chrousos GP, Detera-Wadleigh SD, Karl M. 1993 Syndromes of glucocorticoid resistance. Ann Intern Med 119:1113-1124.
8. Lamberts SW, Koper JW, Biemond P et al. 1992 Cortisol receptor resistance: the variability of its clinical presentation and response to treatment. J Clin Endocrinol Metab 74:313-321.
9. Vingerhoeds AC, Thijssen JH, Schwarz F. 1976 Spontaneous hypercortisolism without Cushing's syndrome. J Clin Endocrinol Metab 43:1128-1133.
10. Lamberts SW, Poldermans D, Zweens M et al. 1986 Familial cortisol resistance: differential diagnostic and therapeutic aspects. J Clin Endocrinol Metab 63:1328-1333.
11. Lamberts SWJ, van der Lely AJ, de Herder WW. 1995 Transsphenoidal selective adenectomy is the treatment of choice in patients with Cushing's disease. Considerations concerning preoperative medical treatment and the long-term follow-up. J Clin Endocrinol Metab 80:3111-3113.
12. Iida S, Gomi M, Moriwaki K et al. 1985 Primary cortisol resistance accompanied by a reduction in glucocorticoid receptors in two members of the same family. J Clin Endocrinol Metab 60:967-971.
13. Brönnegård M, Werner S, Gustafsson JA. 1986 Primary cortisol resistance associated with a thermolabile glucocorticoid receptor in a patient with fatigue as the only symptom. J Clin Invest 78:1270-1278.
14. Gomi M, Iida S, Tsugawa M et al. 1987 In vitro studies of skin fibroblasts from a patient with a new type of primary cortisol resistance: glucocorticoid receptor status and glucocorticoid effect on DNA synthesis. Horm Metab Res 19:328-330.
15. Nawata H, Sekiya K, Higuchi K et al. 1987 Decreased deoxyribonucleic acid binding of glucocorticoid-receptor complex in cultured skin fibroblasts from a patient with the glucocorticoid resistance syndrome. J Clin Endocrinol Metab 65:219-226.

16. Werner S, Thoren M, Gustafsson JA et al. 1992 Glucocorticoid receptor abnormalities in fibroblasts from patients with idiopathic resistance to dexamethasone diagnosed when evaluated for adrenocortical disorders. J Clin Endocrinol Metab 75:1005-1009.
17. Hofman A, Grobbee DE, de Jong PT et al. 1991 Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. Eur J Epidemiol 7:403-422.
18. Nugent C, Nichols T, Tyler F. 1965 Diagnosis of Cushing's syndrome. Single dose dexamethasone suppression test. Arch Int Med 116:172-176.
19. Orita M, Iwahana H, Kanazawa H et al. 1989 Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci U S A 86:2766-2770.
20. Hollenberg SM, Weinberger C, Ong ES et al. 1985 Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 318:635-641.
21. Soufi M, Kaiser U, Schneider A et al. 1995 The DNA and steroid binding domains of the glucocorticoid receptor are not altered in mononuclear cells of treated CLL patients. Exp Clin Endocrinol 103:175-183.
22. De Lange L, Koper JW, Huizenga NATM et al. 1997 Differential hormone-dependent transcriptional activation and -repression by naturally occurring human glucocorticoid receptor variants. Mol Endocrinol 11:1156-1164.
23. Gaitan D, DeBold RC, Turney MK et al. 1995 Glucocorticoid receptor structure and function in an adrenocorticotropin-secreting small cell lung cancer. Mol Endocrinol 9:1193-1201.
24. Bamberger CM, Bamberger AM, De Castro M et al. 1995 Glucocorticoid receptor ß, a potential endogenous inhibitor of glucocorticoid action in humans. J Clin Invest 95:2435-2441.
25. Oakley RH, Sar M, Cidlowski JA. 1996 The human glucocorticoid receptor ß isoform. J Biol Chem 271:9550-9559.
26. De Castro M, Elliot S, Kino T et al. 1996 The non-ligand binding ß-isoform of the human glucocorticoid receptor (hGRß): tissue levels, mechanism of action, and potential physiologic role. Mol Med 2:597-607.
27. Ray DW. 1996 Molecular mechanisms of glucocorticoid resistance. J Endocrinol 149:1-5.

CHAPTER 4

A POLYMORPHISM IN THE GLUCOCORTICOID RECEPTOR GENE MAY BE ASSOCIATED WITH AN INCREASED SENSITIVITY TO GLUCOCORTICOIDS *IN VIVO.*

Nannette A.T.M. Huizenga, Jan W. Koper, Pieter de Lange, Huibert A.P. Pols, Ronald P. Stolk, Huibert Burger, Diederick E. Grobbee, Albert O. Brinkmann, Frank H. de Jong and Steven W.J. Lamberts

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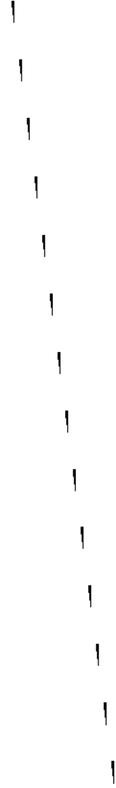

ABSTRACT

We investigated whether a polymorphism at nucleotide position 1220, resulting in an asparagine to serine change at codon 363 in the glucocorticoid receptor (GR) gene is associated with an altered sensitivity to glucocorticoids.

In a group of 216 elderly persons, 13 heterozygotes for the N363S polymorphism were identified by PCR/SSCP analysis. In two DEX suppression tests (DSTs), using 1 and 0.25 mg DEX, the circulating cortisol and insulin concentrations were compared between N363S carriers and controls. In the 1 mg DST there were no differences between N363S carriers and controls with respect to adrenal suppression, but there was a significantly higher insulin response in N363S carriers. In the 0.25 mg DST, a significantly larger cortisol suppression and higher insulin response were seen in N363S carriers. Comparison of blood pressure, body mass index (BMI) and bone mineral density (BMD) between the N363S carriers and controls showed that N363S carriers had a higher BMI, but normal blood pressure. There was an obvious trend towards lower age-, BMI- and sex-adjusted BMD in the lumbar spine in N363S carriers. GR characteristics measured in 41 controls and 9 N363S carriers in periferal mononuclear leucocytes showed no differences between N363S carriers and controls with respect to GR number and ligand binding affinity.However, there was a trend towards greater sensitivity to DEX in the carriers' lymfocytes, in a mitogen induced cell proliferation assay. In transfection assays the capacity of the codon 363 variant to activate MMTV promotor mediated transcription in COS-1 cells was unaltered when compared to the wild type GR.

We conclude that in 6.0 % of our study population, a polymorphism in codon 363 of the GR gene was found. Individuals carrying this polymorphism appeared healthy at clinical examination, but had a higher sensitivity to exogenously administered glucocorticoids, both with respect to cortisol suppression and insulin response. Life-long exposure to the mutated allele may be accompanied by an increased BMI and a lowered BMD in the lumbar spine, but does not affect blood pressure.

INTRODUCTION

There is a considerable variability in the sensitivity to glucocorticoids (Gcs) across individuals. Early morning serum cortisol concentrations show a great diversity between individuals. On the other hand, there is a marked individual stability of baseline cortisol concentrations within persons which is closely related to the feedback sensitivity of the hypothalamo-pituitary-adrenal (HPA)-axis to a low dose of dexamethasone (DEX) (Huizenga et al 1997, submitted). Extreme examples of variability in sensitivity to GCs are the cortisol hyperreactive syndrome and cortisol resistance (CR). In 1990, Iida et al (1) described a patient who presented with signs and symptoms of Cushing's syndrome in spite of persistent hypocortisolemia. So far, this is the only case reported with cortisol hyperreactivity. CR is a rare condition as well. The first patient was described in 1976 (2) and since then, only about ten symptomatic and asymptomatic patients and family members have been identified (3-5). Already15 years ago it was suggested that CR might be the result of a defect in the functional characteristics of the glucocorticoid receptor (GR). The GR has been cloned and sequenced (6) and found to be organized into a series of discrete domains wich mediate the receptor functions of hormone-binding, DNA-binding and transcriptional modulation (7). The binding of GCs to the GR induces a series of cellular events wich results in the activation or repression of a network of GC responsive genes and produces a specific cellular response (8). Thus far, the molecular basis of CR has been elucidated in four kindreds (9-12). In all reported cases, mutations in the hormone binding domain of the GR-gene appeared to be the cause of CR. The molecular basis of cortisol hyperreactivity (1) has not been elucidated yet.

Variability in the sensitivity to GCs is also observed in patients treated with GCs. GCs are important therapeutic agents, used for the treatment of various inflammatory and autoimmune diseases. Although plasma concentrations of GCs can be ascertained, their functional effects on target tissues are very difficult to predict (13). In clinical observations, a considerable variability between subjects is seen in their sensitivity to GC therapy both with regard to efficacy as well as to the prevalence and severity of side effects.

In a previous study, two dexamethasone (DEX) suppression tests (DSTs) were performed in 216 elderly individuals in order gain insight in the sensitivity to GCs in the normal population

(Chapter 2). Furthermore, a general screening of the GR-gene in 40 persons was performed using PCR/SSCP-analysis (14). Based on indications that a previously reported polymorphism in the 3'-region of exon 2 which encodes for the transactivation domain of the GR of the GR gene (Asn363Ser, N363S, Figure 1C) might be associated with an increased sensitivity to GCs (14), we reexamined our study population for this polymorphism and analysed whether its presence was associated with increased GC-sensitivity.

SUBJECTS, METHODS AND MATERIALS.

For the present study, a sample of participants from the Rotterdam Study was invited for an additional examination. The Rotterdam Study is a population-based cohort study of the determinants of chronic disabling diseases in the elderly. All approximately 10,000 inhibitants of a suburb of Rotterdam, aged 55 years and over were invited to participate as described elsewere (15). The population for the present study included 216 persons aged 55 to 80 years (102 men and 114 women with mean ages of 67.7 ± 5.6 (SE) and 65.8 ± 6.1 years, respectively) who had completed the baseline visit for the Rotterdam Study not more than 6 months earlier. Subjects with acute, psychiatric or endocrine diseases, including diabetes mellitus treated with medication, were not invited. Compared to other participants of the Rotterdam Study of the same age without diabetes mellitus, there were no differences in age and gender distribution and cardiovascular risk factors. From all subjects written informed consent was obtained and the study was approved by the Medical Ethics Committee of the Erasmus University Medical School.

Two DSTs were carried out as described previously (Chapter 2). In brief, participants were seen at the research centre after an overnight fast. Blood was drawn by venapuncture in order to determine serum cortisol and insulin concentrations. Participants were given a tablet of 1.00 mg DEX and instructed to ingest it at 11.00 pm. Next morning, fasting blood samples were obtained at the same time as the previous morning. In these samples, cortisol, insulin and DEX concentrations were measured. Circulating cortisol DEX concentrations were determined as described previously (Chapter 2). Serum insulin concentrations were determined using a commercially available radioimmunoassay (Medgenix Diagnostics, Brussels, Belgium). Intra-

and inter-assay variation were 8.0 and 13.7% respectively.
Two and a half years later, all 216 participants were invited for a 0.25 mg DST.164 subjects agreed to participate in this second test (76 men and 88 women with mean ages of 69.1± 5.9 and 67.6 ± 5.6 years, respectively). The same procedures were used as described for the 1 mg DST. In the fasting and post 0.25 mg DEX samples, glucose concentrations were determined using routine standard laboratory methods.
Both as part of the baseline examinations of the Rotterdam Study, and at the second determination, body weight and height were measured in order to calculate body mass index (BMI, kg/m^2), and sitting blood pressure was measured at the right upper arm with a random-zero sphygmomanometer. Bone mineral density (BMD) measurements were performed by dual-energy X-ray absorptiometry (DXA), using a DPX-L densitometer (Lunar Radiation Corporation, Madison, WI, USA). Standard positioning was used with anterior-posterior scans of the lumbar spine and the right proximal femur. In cases of a history of hip fracture or prothesis implantation, the left femur was scanned. Using standard software the vertebrae L2 to L4 and the femoral neck were analysed. Quality assurance included calibration with the standard of the machine, and was performed routinely every morning. The in vivo coefficient of variation for the BMD measurements was 0.9% in the lumbar spine and 3.2% in the femoral neck (16)

PCR/SSCP analysis of the glucocorticoid receptor gene.
DNA was isolated from periferal blood leucocytes of all subjects using standard techniques. PCR amplification and SSCP analysis of the 3'-region of exon 2 of the glucocorticoid receptor gene were carried out using primer sequences and amplification and electrophoresis conditions previously described by Koper et al (14).

Whole cell DEX binding assay and mitogen induced proliferation assay
From 41 randomly selected controls and nine carriers of the N363S mutation (see results), 40 ml heparinized blood was drawn for a whole cell DEX binding assay and a mitogen induced proliferation assay. Glucocorticoid receptor characteristics in mononuclear leucocytes and the sensitivity of mononuclear leucocytes to the inhibition of phytohaemagglutinin (PHA)

stimulated incorporation of [^{3}H]-thymidine by DEX were determined as described previously (3).

Cell culture and transfections

Monkey kidney (COS-1) cells were maintained in Dulbecco's Modified Essential Medium-Ham's F-12 tissue culture medium (Life Technologies, Gaithersburg, MD) supplemented with 5% dextran coated chroal treated Foetal Calf Serum (Life Technologies). For transcription regulation studies, cells were plated at 1.0 x 10^5 cells/well (10 cm^2), grown for 24 hr, and transfected overnight by calcium phosphate precipitation, as described previously (17). Cells were transfected with 250 ng of GR expression plasmid and 250 ng reporter plasmid per well. After transfection, experimental media were added. After an incubation period of 24h, cells were harvested for the luciferase (LUC) assay, as described previously (18).

Statistical analysis

Results are reported as mean ± SE unless otherwise stated. Serum cortisol, insulin and glucose concentrations, bloodpressure, BMI, BMD and receptor characteristics were compared between N363S carriers and controls, using Student's t-test.To control for possible confounders, the analyses were adjusted for BMI if appropriate using multiple linear regression analysis. Values for BMD were expressed as Z-scores (number of standard deviations below or above the age-, BMI-, and sex-standardized average value obtained from 2446 males and 3368 females who participated in the baseline visit for the Rotterdam study) (16).

RESULTS

After the 1 mg DST, 5 individuals were identified with a post DEX cortisol concentration >140 nmol/l, which is the cut-off point for a normal test result in our clinic when the DST is used for the screening for Cushing's Syndrome. These five subjects were further investigated for cortisol resistance (14), and were left out of the calculations described here. Screening for the codon 363 polymorphism was performed using PCR/SSCP-analysis. The abnormal pattern in the 3'-fragment of exon 2 was found in 13 individuals out of the whole study population of

216 subjects (6.0%, Figure 1A). Sequence analysis of the fragment showed an A to G point-mutation at cDNA position 1220, resulting in an asparagine to serine change at codon 363 (Figures 1B and 1C) .The mutation was found to be heterozygous in all cases.

The subjects without the mutation in codon 363 (controls) and the 13 persons heterozygous for the polymorphism (N363S carriers) have been investigated further with respect to their HPA-axis and sensitivity to GCs.

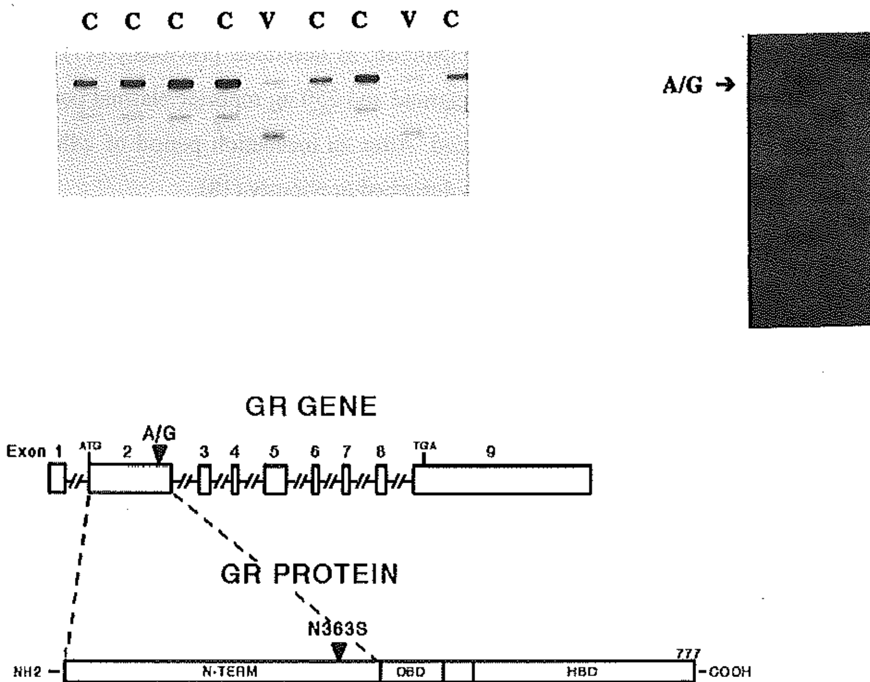

Figure 1. (A) PCR/SSCP analysis of the 3'-part of exon II of the glucocorticoid receptor gene. The analysis revealed a polymorphism in 13 out of 216 elderly individuals (C: control, V: variant) **(B)** Sequence analysis of the polymorphism in the 3'-part of exon II of the glucocorticoid receptor gene, showing a heterozygous A to G point mutation at cDNA position 1220. **(C)** Structure of the human glucocorticoid receptor gene and protein and its functional domains (DBD:DNA-binding domain; HBD:hormone-binding domain), indicating the position of an asparagine to serine change at codon 363 as a result of the A to G point mutation at cDNA position 1220 of the glucocorticoid receptor gene.

In vivo results

The group of N363S carriers consisted of 4 men and 9 women, while in the control-group, the sexes were equally divided. There was one female N363S carrier who showed an additional mutation in the 5'-prime part of exon 2 of the glucocorticoid receptor gene. She was further investigated for cortisol resistance (14), and left out of all comparisons.

At the first examination no significant difference in age between the groups was present as shown in Table 1a. The N363S carriers had a higher mean BMI compared to controls, which did not reach statistical significance (P=0.07). Systolic and diastolic blood pressures were not different between the groups (Table 1a).

At the second examination 164 of the initial 216 individuals participated, 9 of whom had the codon 363 polymorphism. The female N363S carrier with the additional point mutation in exon 2 was excluded again from the comparison, and so were two subjects who had a post DEX cortisol concentration >140 nmol at the first determination (Table 1b). In the control group the sexes were equally divided, the group of N363S carriers now consisted of 5 women and 3 men. At the second examination, the N363S carriers had a significantly higher BMI as compared to the control group (P<0.04). In the first determination, there was no statistically significant difference in BMI between controls and N363S carriers in this subgroup (26.1 ± 0.28 in controls versus 28.1 ± 1.30 in N363S carriers, P=0.09). As in the first examination, there were no differences between the groups with respect to systolic or diastolic blood pressure.

Physical examination of the N363S carriers revealed no evident abnormalities. One male N363S carrier had hypertension, and two females had hyperinsulinemia (fasting insulin>25 mU/l).

Cortisol concentrations.

Table 2a shows the concentrations of fasting serum cortisol before and after administration of 1 mg DEX, the DEX concentration and the cortisol suppression in reaction to DEX (Δ cortisol). There were no differences between the controls and the N363S carriers, neither in basal levels nor in response to the administration of 1 mg DEX.

Table 1a. Age, BMI and blood pressure at first examination in controls (n=198) and N363S carriers (n=12)

	Controls		**N363S carriers**		
	mean	**SE**	**mean**	**SE**	**P-value***
Age (years)	66.6	0.44	68.5	1.64	0.28
BMI (kg / m²)	26.6	0.26	28.1	1.09	0.07
SBP (mmHg)	139	1.35	137	7.00	0.77
DBP (mmHg)	74.9	0.71	73.1	2.84	0.54

* Test for the difference between controls and N363S carriers.

SBP: systolic blood pressure, DBP: diastolic blood pressure

Table 1b. Age, BMI and blood pressure at second examination in controls (n=153) and N363S carriers (n=8)

	Controls		**N363S carriers**		
	mean	**SE**	**mean**	**SE**	**P-value***
Age (years)	68.3	0.49	69.3	2.36	0.63
BMI (kg / m²)	25.5	0.30	28.3	1.52	0.04
SBP (mmHg)	143	1.50	140	10.7	0.57
DBP (mmHg)	88.1	0.86	83.7	6.37	0.26

* Test for the difference between controls and N363S carriers.

SBP: systolic blood pressure, DBP: diastolic blood pressure

Table 2b provides the same parameters before and after the administration of 0.25 mg DEX. Again, there were no differences in baseline cortisol, but there was a larger decrease in cortisol concentration (Δ cortisol) in response to 0.25 mg DEX in the N363S carriers than in controls (280.5 nmol/l in controls versus 373.9 nmol/l in N363S carriers, $P<0.09$, unadjusted data).

This difference in Δ cortisol was statistically significant when the data were adjusted for BMI (443.8 in controls versus 553.6 in N363S carriers, $P<0.05$). Adjusting for BMI was necessary because there was a statistically significant difference in BMI between controls and N363S carriers, and BMI is known to influence the cortisol concentration.The actual DEX concentrations were equal in both groups ($P=0.50$), so the higher response in the N363S carriers was not due to higher DEX concentrations.

Table 2a. Cortisol and DEX concentrations in controls (n=198) and N363S carriers (n=12) before and after 1 mg DEX

	Controls		N363S carriers		
	mean	**SE**	**mean**	**SE**	**P-value***
Fasting cortisol (nmol/l)	513.5	11.1	532.2	32.4	0.49
Post DEX cortisol (nmol/l)	26.26	1.22	25.80	3.45	0.97
Δ cortisol (nmol/l)	488.1	10.9	506.3	33.5	0.50
DEX (nmol/l)	7.300	0.25	8.500	1.44	0.30

* Test for the difference between controls and N363S carriers, adjusted for BMI.

Table 2b. Cortisol and DEX concentrations in controls (n=153) and N363S carriers (n=8) before and after 0.25 mg DEX

	Controls		N363S carriers		
	mean	**SE**	**mean**	**SE**	**P-value***
Fasting cortisol (nmol/l)	541.7	12.0	566.5	44.9	0.53
Post DEX cortisol (nmol/l)	261.2	11.4	192.6	38.1	0.13
Δ cortisol (nmol/l)	280.5	12.6	373.9	37.2	<0.05
DEX (nmol/l)	2.820	0.13	3.300	0.55	0.48

* Test for the difference between controls and N363S carriers, adjusted for BMI.

Insulin and glucose concentrations

Tables 3a and 3b show the fasting insulin concentrations before and after the administration of 1 and 0.25 mg DEX, respectively, and the increase in insulin levels in response to DEX administration. In order to be certain that only the data from subjects with a normal carbohydrate tolerance were analysed, subjects with hyperinsulinaemia or diabetes mellitus (fasting insulin or glucose concentrations above values of 25 mU/l or 7.8 mmol/l, respectively), were excluded from this analysis (20 controls excluded, n=178 and 2 N363S carriers excluded, n=10). There was a significant increase in insulin concentrations in response to the administration of 1 mg DEX among the remaining 188 subjects (12.9 ± 0.53 mU/L before, and 19.1± 0.80 mU/L after DEX administration, respectively, P<0.001). There were no significant differences between the groups in fasting or post DEX insulin concentrations, but there was a significantly larger increase in insulin levels in the N363S carriers in response to 1 mg DEX (p<0.01) compared to controls. As mentioned before, these differences in insulin response were not due to differences in DEX concentrations between the two groups (Table 2a).

Table 3a. Insulin concentrations in controls (n=178) and N363S carriers (n=10) before and after 1 mg of DEX

	Controls		**N363S carriers**		
	mean	**SE**	**mean**	**SE**	**P-value***
Fasting insulin (mU/l)	11.8	0.39	11.2	1.69	0.34
Post DEX insulin (mU/l)	17.6	0.67	23.5	5.19	0.08
Δ insulin (mU/l)	5.84	0.57	12.3	4.09	<0.01

* Test for the difference between controls and N363S carriers, adjusted for BMI.
Subjects with fasting insulin>25 mU/l were left out of the calculation; 2 N363S carriers and 20 controls were excluded.

After the administration of 0.25 mg DEX, there was a drop in insulin concentrations in the controls. In the N363S carriers there was a slight increase in insulin concentrations. After comparison of the insulin responses to 0.25 mg DEX between the two groups, again a

significantly larger response in the N363S carriers became apparent (p< 0.03). Glucose levels did not increase in response to DEX administration, nor were there differences in glucose concentrations between the two groups.

Table 3b. Insulin concentrations in controls (n=127) and N363S carriers (n=7) before and after 0.25 mg of DEX

	Controls		N363S carriers		
	mean	**SE**	**mean**	**SE**	**P-value***
Fasting insulin (mU/l)	14.8	0.46	13.7	2.29	0.16
Post DEX insulin (mU/l)	13.7	0.51	15.5	2.83	0.69
Δ insulin (mU/l)	-1.10	0.36	1.80	1.98	0.03
Fasting glucose (mmol/l)	5.60	0.06	5.40	0.29	0.26
Post DEX glucose (mmol/l)	5.48	0.05	5.50	0.25	0.75
Δ glucose (mmol/l)	- 0.12	0.04	0.07	0.09	0.19

* Test for the difference between controls and N363S carriers, adjusted for BMI.
Subjects with fasting insulin>25 mU/l or fasting glucose >7.8 nmol/l were left out of the calculation; 1 N363S carrier and 26 controls were excluded.

Bone mineral density

In Figure 2, the mean values and standard deviation for BMD in the lumbar spine and the femoral neck in controls and N363S carriers expressed as Z-scores are shown. The figure shows that in the lumbar spine there was a trend towards lower BMD in the N363S carriers compared to controls (z-scores -0.48 versus 0.02 respectively, P=0.08). In the femoral neck, there were no differences in BMD between the two groups (P=0.67).

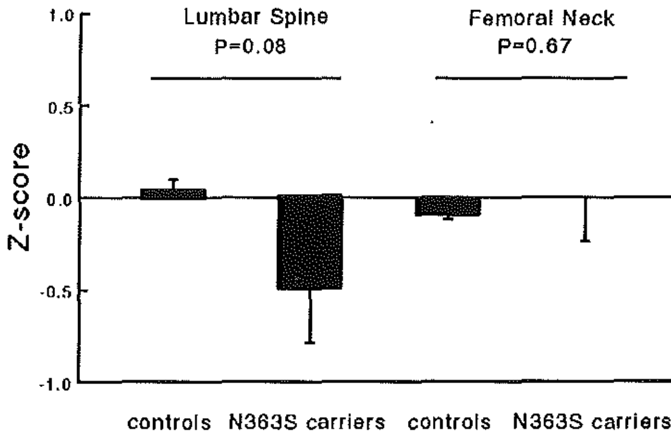

Figure 2. Mean values and SD for bone mineral density in the lumbar spine and the femoral neck in controls and N363S carriers, expressed as Z-scores. There is a trend towards lower BMD in the lumbar spine in N363S carriers compared to controls (Z-scores -0.48 in N363S carriers versus 0.02 in controls, P=0.08). There are no differences in feroral neck BMD between both groups (P=0.67).

In vitro results

Whole cell DEX binding assay and mitogen induced proliferation assay

Figure 3 shows the results from 50 whole cell DEX binding assays and mitogen induced proliferation assays (41 randomly selected controls and nine N363S carriers). The number of receptors (n,Figure 3A) and the dissociation constant (K_d, figure 3B) were not different in both groups (n=7056 ± 200 in controls, n=7242 ± 645 in N363S carriers; K_d=10.6 ± 0.63 in controls, K_d=8.5 ± 1.14 in N363S carriers respectively). Also, no statistically significant difference in IC_{50} values between the N363S carriers and controls was found; as shown in figure 3C, there was a trend towards a lower IC_{50} in the N363S carriers (IC_{50} =47.7± 9.6 in controls, IC_{50}=21.8 ± 6.4 in N363S carriers, respectively) .

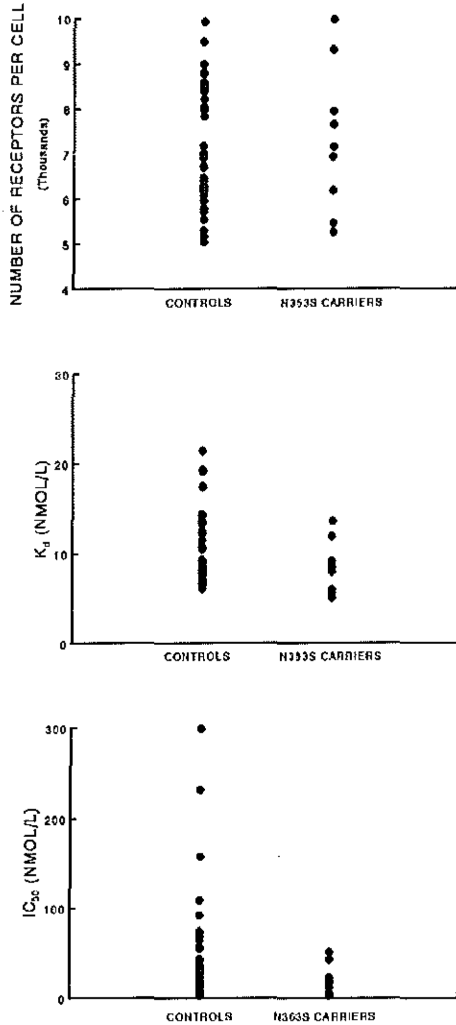

Figure 3. Glucocorticoid receptor characteristics in peripheral blood mononuclear leucocytes in 41 randomly selected controls and 9 N363S carriers. **(A)** Number of receptors in controls and N363S carriers obtained from whole cell DEX binding assays. There is no difference between controls and N363S carriers in number of receptors per cell. **(B)** Ligand affinity of the receptors (K_d) in controls and N363S carriers obtained from whole cell DEX binding assays. There is no difference in K_d between controls and N363S carriers. **(C)** Concentrations of DEX necessary to achieve 50 % of the maximal inhibition (IC_{50}) in mitogen induced proliferation assays in controls and N363S carriers. There are no differences in IC_{50} values between controls and N363S carriers.

Transcriptional activation studies

To investigate whether the observed codon change at position 363 resulted in an altered capacity of the GR protein to activate transcription, we co-transfected a plasmid containing this variant receptor and a Mouse Mammary Tumor Virus (MMTV)-driven luciferase (LUC) reporter plasmid in COS-1 cells. Cells were incubated with increasing concentrations of DEX. At DEX concentrations between zero and 100 nmol/l, the capacity of the N363S variant to activate transcription from the MMTV promotor of the N363S variant was unaltered compared to the wild type GR (Figure 4).

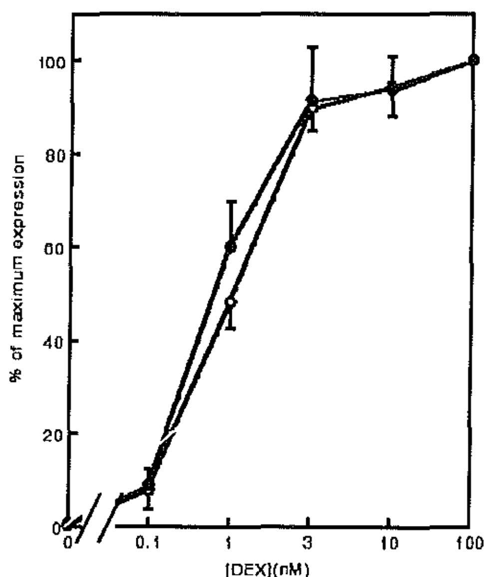

Figure 4. Transcriptional activation of an MMTV-LUC reporter gene by the wild-type receptor (closed circles) and the codon 363 mutant (open circles). Data are the average of three separate transfection assays, each performed in duplicate. At concentrations of DEX between zero and 100 nmol/L, the capacity of the N363S variant to activate the transcription from the MMTV promotor was unaltered compared to the wild type GR.

DISCUSSION

In this study, we examined differences in the sensitivity to GCs within a normal elderly population, using DSTs with two different doses of DEX. In a previous study (14), looking for mutations in the GR gene which cause the clinical syndrome of CR (9-12), we found a polymorphism which appeared to be associated with increased sensitivity to GCs. This polymorphism located at nucleotide position 1220 (AAT to AGT), results in a asparagine to serine change in codon 363 of the GR protein. This polymorphism was heterozygously present in 13 out of the 216 subjects. One of these subjects had hypertension and two had elevated insulin concentrations. The other N363S carriers were healthy elderly individuals, and showed no signs or symptoms of CR.

A 1 mg DST carried out to asses differences in GC sensitivity showed no differences in early morning post-DEX cortisol concentrations. Because 1 mg may be a relatively high dose (93% of the subjects suppressed to a level ≤ 50 nmol/l) we also carried out a 0.25 mg DST. In this experiment, post DEX cortisol concentrations showed a much broader range than after 1 mg DEX(Chapter 2).The decease in early morning cortisol levels (Δ cortisol) was significantly greater in N363S carriers than in controls. The absolute post-DEX cortisol levels were also lower in N363S carriers, but this difference was not statistically significant.

Another parameter for the sensitivity to exogenously administered GCs is the fasting insulin concentration before and after DEX administration. It has been recognized for a long time that GCs impair insulin mediated glucose metabolism and induce insulin resistance (19). In this study, fasting insulin levels before DEX were similar in the two groups, but in response to the administration of 1 mg DEX there was a significantly larger increase in the serum insulin levels in N363S carriers than in controls. After the administration of 0.25 mg DEX, there was a slight drop in insulin concentrations in the control group, whereas in the N363S carriers, mean insulin concentrations rose. It appears that in controls 0.25 mg DEX is not sufficient to induce insulin resistance, while in the N363S carriers this low dose of DEX forces the insulin concentrations to increase in order to maintain a normal plasma glucose concentration. These data support the assumption that N363S carriers have an increased sensitivity to GCs.

With respect to the potential long term effects of the codon 363 mutation, three parameters

were investigated: BMI, blood pressure and BMD. GCs are involved in the syndrome of abdominal obesity due to stimulatory effects on lipid accumulation in adipose tissue (20). Patients with Cushing's syndrome have a centripetal fat distribution, resulting in a higher BMI. This seems to be due to an increased lipoprotein lipase activity in combination with a lowered capacity for fat mobilization in the central regions (21). The net effect of hypercortisolemia is an increase at triglyceride storage in visceral adipocytes (22). At the second examination, the N363S carriers had a significantly higher BMI than controls, while at the first examination there was a trend towards a higher BMI in the N363S carriers. The higher BMI observed in in these persons supports the view that they are more sensitive to effects of CGs. There were, however, no differences in blood pressure between the two groups, neither at the first, nor at the second examination.

It is well known that patients under treatment with GCs show a reduced bone mass and an increased fracture risk. GCs have been found to influence bone and calcium metabolism at several levels (23, 24). The effects of GCs on BMD are more profound on trabecular bone than on cortical bone (24). The data from the present investigation indeed demonstrate a lower BMD in the lumbar spine of the N363S carriers compared to control subjects. However, this difference in BMD did not reach statistical significance. In the femoral neck, there were no differences in BMD between the groups.

Other investigations have previously found the N363S polymorphism in a CR-patient, who also showed a splice-site deletion (10) and in the small cell long cancer (SCLC) cell lines DMS-79 (25) and COR L24 (26). These authors found no significant difference in the capacity of this variant GR relative to the wild type to activate transcription of the CAT gene in an MMTV driven reporter construct, after transfection into COS-1 cells. Our own results using the more sensitive MMTV-LUC system also do not indicate a difference between N363S and wild type GR in this respect. Apart from transcriptional activation, GR is also involved in transcriptional repression of target genes. It does so either by binding directly to so-called negative glucocorticoid responsive elements (nGREs) (27, 28), or via interactions with factors such as activating protein-1 (AP-1) (29, 30) or nuclear factor (NF)-κB (21-23), thereby preventing these factors from activating their target genes. Co-transfection experiments have shown that the codon 363 variant has an unaltered capacity to repress target genes via nGREs

or via the above mentioned transcription factors (31).

Our results of the whole cell DEX binding assays demonstrate no differences between the variant- and the wild type GR in this respect, but as the mutation is located in the N-terminal part of the GR, no alterations in the number of receptors or the ligand binding capacity in peripheral mononuclear leucocytes from N363S carriers were expected. The mitogen induced proliferation assay on the other hand, reflects the cellular response to GCs (32). One would expect to find alterations in the suppressibility of mitogen-stimulated thymidine incorporation in cultured leucocytes from N363S carriers. Indeed, a trend towards lower IC_{50} values in the N363S carriers compared to controls was observed, but these differences did not reach statistical significance, probably as a result of the wide range of values obtained in controls .

In summary, we have showed significant effects of the N363S mutation on changes in insulin and cortisol levels after the administration of DEX, indicating increased sensitivity to acutely increased GC levels. Long term effects of increased sensitivity to endogenous Gcs - which might be partly compensated by counterregulatory mechanisms - could be reflected in the trends to increased BMI and decreased BMD in trabecular bone. In this connection, it is of interest that about 5% of the patients treated with Gcs develop severe side effects soon after starting the therapy; N363S carriers might be included in this subpopulation. The apparent discrepancy between these *in vivo* effects and our inability to confirm this increased sensitivity to Gcs *in vitro* is likely to be due to the earlier mentioned broad spectrum of regulatory mechanisms in which Gcs and their receptors are involved. We showed earlier that GR mutations can effect transcriptional activation and -repression differently (31). It could well be that COS-1 cells lack the transcription factors determining the effect of the N363S mutation *in vivo* and that the presence of endogenous GR in these cells is an impediment for the study of subtle effects of this mutation *in vitro*.

Finally, our results may indicate that this polymorphism is linked to an additional genetic alteration in a gene of which the product is involved in the observed phenotypic differences. This yet unknown factor might interact with the GR protein to exert its effect. However, for diagnostic purposes, the recognition of a point mutation in the GR gene linked to a phenotypic difference may prove very useful, whether or not this may solely, in part or not at all be a consequence of altered GR function. A next step will be to investigate whether the codon 363

polymorphism might predict an increased sensitivity to the development of early and/or serious side effects during therapy with GCs.

In conclusion, our findings show that *in vivo* the codon 363 variant of the GR is associated with an increased sensitivity to GCs in the direct response to exogenously administered DEX. BMI and BMD might also be effected by this variant GR. The molecular basis for this increased sensitivity remains to be elucidated.

List of references

1. Lida S, Nakamura Y, Fujii H et al. 1990 A patient with hypocortisolism and Cushing's syndrome-like manifestations: cortisol hyperreactive syndrome. J Clin Endocrinol Metab. 70:729-737.
2. Vingerhoeds AC, Thijssen JH, Schwarz F. 1976 Spontaneous hypercortisolism without Cushing's syndrome. J Clin Endocrinol Metab. 43:1128-1133.
3. Lamberts SWJ, Koper JW, Biemond P et al. 1992 Cortisol receptor resistance: the variability of its clinical presentation and response to treatment. J Clin Endocrinol Metab. 74:313-321.
4. Bronnegard M, Werner S, Gustafsson JA. 1986 Primary cortisol resistance associated with a thermolabile glucocorticoid receptor in a patient with fatigue as the only symptom. J Clin Invest. 78:1270-1278.
5. Arai K, Chrousos GP. 1995 Syndromes of glucocorticoid and mineralocorticoid resistance. Steroids. 60:173-179.
6. Hollenberg SM, Weinberger C, Ong ES et al. 1985 Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature. 318:635-641.
7. Giguere V, Hollenberg SM, Rosenfeld MG et al. 1986 Functional domains of the human glucocorticoid receptor. Cell. 46:645-652.
8. Wright AP, Zilliacus J, McEwan IJ et al. 1993 Structure and function of the glucocorticoid receptor. J Steroid Biochem Mol Biol. 47:11-19.
9. Hurley DM, Accili D, Stratakis CA et al. 1991 Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucocorticoid resistance. J Clin Invest. 87:680-686.
10. Karl M, Lamberts SWJ, Detera-Wadleigh SD et al. 1993 Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. J Clin Endocrinol Metab. 76:683-689.
11. Malchoff DM, Brufsky A, Reardon G et al. 1993 A mutation of the glucocorticoid receptor in primary cortisol resistance. J Clin Invest. 91:1918-1925.
12. Karl M, Lamberts SWJ, Koper JW et al. 1996 Cushing's disease preceded by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proc Ass Am Phys. 108:296-307.
13. DeRijk RH, Petrides J, Deuster P et al. 1996 Changes in corticosteroid sensitivity of peripheral blood lymphocytes after strenuous exercise in humans. J Clin Endocrinol Metab. 81:228-235.
14. Koper JW, Stolk RP, de Lange P et al. 1997 Lack of association between five polymorphisms in the human glucocorticoid receptor gene and glucocorticoid resistance. Human Genetics. 99:663-668.
15. Hofman A, Grobbee DE, de Jong PT et al. 1991 Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. Eur J Epidemiol. 7:403-422.
16. Burger H, v Daele PLA, Algra D et al. 1994 The association between age and bone mineral density in men and women aged 55 years and over: The Rotterdam Study. Bone Miner. 25:1-13.

17. Jenster G, Trapman J, Brinkmann AO. 1993 Nuclear import of the androgen receptor. Biochem J. 293:761-768.
18. Kuil CW, Berrevoets CA, Mulder E. 1995 Ligand-induced conformational alterations of the androgen receptor analyzed by limited trypsinization. J Biol Chem. 270:27569-27577.
19. Tappy L, Randin D, Vollenweider P et al. 1994 Mechanisms of dexamethasone-induced insulin resistance in healthy humans. J Clin Endocrinol Metab. 79:1063-1069.
20. Bouchard C, Bray GA, Hubbard VS. 1990 Basic and clinical aspects of regional fat distribution. Am J Clin Nutr. 52:946-950.
21. Rebuffe-Scrive M, Krotkiewski M, Elfverson J et al. 1988 Muscle and adipose tissue morphology and metabolism in Cushing's syndrome. J Clin Endocrinol Metab. 67:1122-1128.
22. Rebuffe-Scrive M, Bronnegard M, Nilsson A et al. 1990 Steroid hormone receptors in human adipose tissues. J Clin Endocrinol Metab. 71:1215-1219.
23. Reid IR, Gluckman PD, Ibbertson HK. 1989 Insulin-like growth factor I and bone turnover in glucocorticoid-treated and control subjects. Clin Endocrinol. 30:347-353.
24. Lukert BP , Raisz LG. 1990 Glucocorticoid-induced osteoporosis: pathogenesis and management. Ann Intern Med. 112:352-364.
25. Gaitan D, DeBold CR, Turney MK et al. 1995 Glucocorticoid receptor structure and function in an adrenocorticotropin-secreting small cell lung cancer. Mol Endocrinol. 9:1193-1201.
26. Ray DW, Littlewood AC, Clark AJ et al. 1994 Human small cell lung cancer cell lines expressing the proopiomelanocortin gene have aberrant glucocorticoid receptor function. J Clin Invest. 93:1625-1630.
27. Sakai DD, Helms S, Carlstedt-Duke J et al. 1988 Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Genes Dev. 2:1144-1154.
28. Drouin J, Sun YL, Nemer M. 1989 Glucocorticoid repression of pro-opiomelanocortin gene transcription. J Steroid Biochem. 34:63-69.
29. Heck S, Kullmann M, Gast A et al. 1994 A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. Embo J. 13:4087-4095.
30. Liu W, Hillmann AG, Harmon JM. 1995 Hormone-independent repression of AP-1-inducible collagenase promoter activity by glucocorticoid receptors. Mol Cell Biol. 15:1005-1013.
31. de Lange P, Koper JW, Huizenga NATM et al. 1997 Differential hormone-dependent transcriptional activation and -repression by naturally occurring human glucocorticoid receptor variants. Mol Endocrinol.11: 1156-1164.
32. Kraft N, Hodgson AJ, Funder JW. 1979 Glucocorticoid receptor and effector mechanisms: a comparison of the corticosensitive mouse with the corticoresistant guinea pig. Endocrinology. 104:344-349.

CHAPTER 5

FIVE PATIENTS WITH BIOCHEMICAL AND/OR CLINICAL GENERALIZED GLUCOCORTICOID RESISTANCE WITHOUT ALTERATIONS IN THE GLUCOCORTICOID RECEPTOR GENE.

Nannette A.T.M. Huizenga, Pieter de Lange, Jan W. Koper, Wouter W. de Herder, Roger Abs, Jan H.L.M. v Kasteren, Albert O. Brinkmann, Frank H. de Jong and Steven W.J. Lamberts.

(Submitted for publication).

ABSTRACT

Cortisol resistance (CR) is a rare disease characterized by a generalized reduced sensitivity of end-organs to the actions of glucocorticoids (GCs). GC effects are mediated by the glucocorticoid receptor (GR). The molecular alterations in CR described thus far were located in the hormone binding domain of the GR gene. Recent reports of a considerable prevalence of abnormalities in the GR in patients attending the endocrine clinic prompted us to carry out further investigations with respect to GR protein and GR gene in patients attending the endocrine clinic for a broad spectrum of complaints and biochemical evidence suggesting a CR. In the present study, we describe 5 patients with biochemical and clinical CR. All patients showed a diurnal rhythm of serum cortisol concentrations, albeit at a high level, an insufficient suppression of serum cortisol concentration in reaction to 1 mg of dexamethasone (DEX), variable degrees of androgen overproduction, in the absence of clinical signs and symptoms of Cushing's syndrome. Three of the four female patients presented with complaints of androgen overproduction, two of them in combination with fatigue. The other female patient had severe steroid resistant asthma. The only male patient and his son were asymptomatic.

In 4 patients, we investigated receptor protein characteristics on mononuclear leukocytes in a whole cell DEX binding assay, and studied the ability of DEX to inhibit mitogen induced cell proliferation in mononuclear leukocytes *in vitro*. In all patients investigated, we found alterations in receptor number or ligand affinity and/or the ability of DEX to inhibit mitogen induced cell proliferation.

In order to investigate the molecular defects leading to the clinical and biochemical pictures in these patients, we screened the GR gene using PCR/SSCP/sequence analysis. No GR gene alterations were found in these patients.

In conclusion, the five patients described had clinical and biochemical evidence of CR but no abnormalities were demonstrated in the GR gene.

Probably, as yet undefined alterations somewhere in the cascade of events starting with ligand binding to the GR protein, and finally resulting in the regulation of the expression of GC responsive genes, or post-receptor defects or interactions with other nuclear factors form the pathophysiologic basis of CR in these patients.

INTRODUCTION

Cortisol resistance (CR) is a rare condition characterized by a generalized reduced sensitivity of end-organs to glucocorticoids (GCs). The disease is typified by increased serum cortisol levels, a normal circadian rhythm of cortisol concentrations albeit at an elevated level, relative resistance of adrenal cortisol suppression to dexamethasone (DEX) and the absence of the clinical stigmata of Cushing's syndrome (1). After the original description of a father and son with CR by Vingerhoeds (2), about 30 symptomatic patients and (a)symptomatic family members have been identified (1-13). In generalized CR, the decreased sensitivity to cortisol results in activation of the HPA-axis with increased ACTH and cortisol secretion. In most cases, the elevated cortisol levels sufficiently compensate to overcome peripheral cortisol resistance. The elevated cortisol concentrations are beneficial in these patients and no signs or symptoms of Cushing's syndrome are present. The clinical presentation of CR is in most instances related to the secondary ACTH-driven overproduction of adrenal androgens and mineralocorticoids. Hypertension and hypokalemic acidosis may develop secondary to overproduction of mineralocorticoids (1, 2). In females, the secondary overproduction of adrenal androgens may result in acne, hirsutism, menstrual irregularity and infertility (1, 6). The overproduction of adrenal androgens gives rise to signs of hyperandrogenism in females only, because of the much higher gonadal androgen production in males compared to females. In some patients with CR chronic fatigue is the only symptom (5), probably due to an insufficient overproduction of cortisol. Finally, several patients with steroid resistant asthma have been described, which in two cases appeared to be caused by generalized CR (13).

The biological effects of cortisol are mediated by the GC receptor (GR), a member of the family of intracellular hormone receptors (14), to which the vitamin D and retinoic acid receptors belong as well. These receptors are characterized by a three-domain structure: a carboxy terminal ligand-binding domain, a central DNA-binding domain and an N-terminal domain responsible for the interactions with other transcription factors (14). Hormone-bound GRs translocate to the nucleus and regulate the expression of GC responsive genes.

It has become clear from previous studies that the molecular basis of CR may be located in the GR gene. Up to the present, this has been elucidated in four different kindreds (15-18), in

which mutations in the hormone binding domain of the GR-gene appeared to be the cause of CR.

Because of the large variation in clinical manifestation of CR, the disease might be less rare than previously thought. In six patients described by Lamberts et al (1), at least 3 different clinical forms of CR could be identified.

In the present study, we report five additional patients with very diverse clinical presentations. Eventually, in all patients clinical and/or biochemical cortisol resistance was diagnosed. Using PCR/SSCP and sequence analysis, we screened the GR gene in these patients for alterations, in order to characterize the cortisol resistance on the molecular level as well. No evidence for mutations in the GR gene was found in any of the patients, however. Suggestions for other possible mechanisms of CR in these individuals are made.

MATERIALS AND METHODS

Biochemical determinations

The diagnostic procedures used in measuring the activity and responsiveness of the HPA axis in the patients tested in our own clinic have been described previously (19, 20) Circulating cortisol, ACTH, desoxycortisol, androstenedione, dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS) and testosterone levels were determined by RIA kits. The inter- and intra-assay variations of these determinations have been reported previously (1). Urinary cortisol was measured with the same RIA kit as that used for circulating cortisol. Patient 3 (see results) was tested in Antwerp (Belgium). Differences in normal values compared to the normal values in our clinic are indicated in the results section.

Peripheral blood mononuclear cells

40 ml of blood was drawn into heparinized tubes between 8.00h and 9.00h by vena puncture. Peripheral blood mononuclear leukocytes (PBMLs) were isolated as described previously (21). The blood was diluted twofold with saline and layered over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). The PBML enriched interphase was isolated and washed twice with saline. The final cell pellet was resuspended in 15 ml RPMI-1640 medium (Gibco Europe, Breda, the

Netherlands), containing 15 mM Hepes, 10% charcoal adsorbed fetal calf serum (Amstelstad/Flow, Zwanenburg, the Netherlands), 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 1.5 mg/ml fungizone. The cells were incubated for 30 minutes at 37°C in a shaking waterbath in order to remove endogenous cortisol. The cell suspension was centrifuged and resuspended in 15 ml medium. This procedure was repeated twice more. Finally, the cells were resuspended at a density of 2.5-10 x 10^6 cells per ml in the medium.

Whole cell dexamethasone binding assay

The whole cell dexamethasone binding assay was performed as described previously (21). Briefly: incubation was started in a volume of 240 μl (0.5-2 x10^6 cells) containing ^{3}H-dexamethasone at concentrations of 1.3 to 40 nM without (total binding) and with (non-specific binding) a 400-fold excess of unlabelled dexamethasone. Two tubes without labelled dexamethasone were incubated under the same conditions for determination of cell number and viability at the end of the procedure. The tubes were incubated during 1 hour at 30 °C in a shaking water bath. The incubation was stopped by the addition of 2 ml cold saline, followed by centrifugation and two washing steps. Finally, the cells were resuspended in 250 μl medium. Radioactivity in 200 μl of this suspension was counted in a liquid scintillation counter. Specific binding was calculated by subtracting non-specific binding from total binding. Receptor number and ligand affinity ($1/K_d$) were calculated from the data using the method of Scatchard (22). Normal values for the number of receptors per cell (6352 ± 310 per cell) and K_d (7.7 ± 0.4 nmol/l) were described previously (1, 23)

Inhibition of mitogen-stimulated incorporation of [^{3}H]-*thymidine by DEX*

The sensitivity of mononuclear leukocytes to the inhibition of phytohaemagglutinin (PHA) stimulated incorporation of [^{3}H]-thymidine by DEX was determined as described previously (1). After isolation, mononuclear leukocytes were seeded, PHA (5 μg/mL) and DEX in 9 concentrations between 0 and 1000 nmol/L were added. The cells were incubated at 37°C during 96 hour. The last four hours of incubation was in the presence of 0.5 μCi of [^{3}H]-thymidine. At the end of the incubation the cells were harvested, washed and incorporated [^{3}H]-thymidine was measured by liquid scintillation counting. The concentration of DEX

necessary to achieve 50% of the maximal inhibition (IC_{50}) was calculated. Normal values for IC_{50} (<15 nmol/l) were described previously (1, 23)

PCR/SSCP analysis of the glucocorticoid receptor gene.

DNA was isolated from peripheral blood leukocytes using standard techniques. PCR amplification of the glucocorticoid receptor gene was carried out using primer sequences and amplification conditions previously described by Karl et al (16). SSCP analysis of the PCR-products was carried out using 0.5 x MDE polyacrylamide gels (from J.T. Baker Chemicals, Deventer, the Netherlands) at 4 °C in the presence and absence of 5% glycerol, generally as described by Orita et al (24). DNA-fragments displaying an abnormal migration pattern during SSCP-analysis were subject to direct sequencing using a modified Sanger-dideoxynucleotide chain termination method (16, 25). In all patients, a second PCR amplification of the exons 2 and 4 to 9 of the GR gene was carried out using other primer sequences (data not shown, sequences available on request), in order to obtain PCR fragments with a length of about 200 nucleotides. SSCP analysis of the PCR fragments was carried out as described above. Finally, PCR fragments of exons 4 to 9 (coding for the hormone binding domain of the GR) in all patients and of the whole coding part of the GR gene in patients 1 and 2, were subjected to direct sequencing as described by Karl et al (16).

RESULTS

Patient 1 was first investigated at the age of 18 years because of hirsutism, menstrual irregularities and weight gain of 15 kg over the past two years. Physical examination revealed an obese young woman (length 1.60 m, weight 85 kg), with a normal fat distribution. Blood pressure was 160/85 mm Hg. She had profound hirsutism on arms, legs, chest and especially face. She had a round face and acne. There was no skin atrophy.

Endocrine testing showed a diurnal rhythm of plasma cortisol concentrations at an elevated level (Table 1). 24 hour urinary cortisol secretion was elevated (Table 1). ACTH secretion was in the higher normal range. A 1 mg overnight dexamethasone suppression test (DST) showed insufficient cortisol suppression (323 nmol/l, Table 1), but intravenous administration of a

high-dose DEX adequately suppressed cortisol secretion. Further studies (Table 2) showed increased androstenedione and testosterone levels and a slightly increased desoxycortisol concentration. At that time, the diagnosis of pituitary dependent Cushing's syndrome was made and a left sided adrenalectomy was performed followed by external pituitary radiation.

A year and a half later, the patient was admitted with a fever, vomiting and diarrhoea. Although the cortisol concentration at the time of admission was normal (528 nmol/l), there was a dramatic improvement of the clinical condition following intravenous administration of hydrocortisone. In retrospect, relative adrenocortical insufficiency during this intercurrent disease was suspected.

One year later, the patient presented with increasing fatigue, further weight gain, dizziness and increasing hirsutism. Physical examination showed an obese woman with a normal fat distribution. She had a round face and profound hirsutism on the chest. Endocrine screening revealed an insufficient cortisol suppression in the 1 mg overnight DST. Because of the pituitary radiation, no further endocrine testing of the HPA-axis was possible. She was treated with oral contraceptives containing cyproterone acetate.

Twelve years after the first admission, the patient was screened with respect to cortisol receptor characteristics. It appeared that she had a normal number of receptors per cell in mononuclear leukocytes, and also the ligand binding affinity of the receptors was normal (Table 4). From the mitogen induced proliferation assay, however, it became clear that the PBMLs were extremely insensitive to DEX (Table 4, Figure 1). PCR/SSCP analysis of the GR gene showed an abnormal pattern in the N-terminal part of exon 2. Sequence analysis of the fragment revealed the presence of a polymorphism, leading to a GAG to GAA (both Glu) in codon 22 and a AGG to AAG (Arg to Lys) change in codon 23 as described previously by Koper et al (25). Further sequence analysis of the whole coding part of the GR gene revealed no additional alterations.

Based on clinical and biochemical findings, the diagnosis of generalized cortisol resistance was made, and a treatment with low doses DEX at night was started. Cortisol-, androstenedione- and testosterone levels were significantly suppressed during DEX treatment (Table 2), and there was a slight improvement of the hirsutism.

Table 1. Patient data concerning the HPA-axis in 4 patients with partial cortisol resistance.

			Diurnal rhythm of cortisol (nmol/l)					
Pt	**sex**	**age** (yr)	8.00 hr	17.00 hr	22.00 hr	**1 mg DST** (nmol/l)	**ACTH** (ng/l)	**UCS** (↑,↓,=)
1	F	18	653	508	403	323	46	↑
3	M	65	524	364	107	471	25	↑
4	F	33	1355	1040	870	857	28	↑
5	F	33	321	252	89	503	17	=

Diurnal rhythm of cortisol: Normal values: 8.00 hr: 200-800 nmol/l;
17.00 hr:< 75% of 8.00 hr value;
22.00 hr:< 75% of 8.00 hr value.

1 mg DST 1 mg dexamethasone suppression test,
cortisol at 8.00 hr, normal value <140 nmol/l

UCS: As the methodology used differed between patients, only an indication of the result in comparison with normal levels is given.

ACTH: Normal value <60 pmol/l

Patient 2, a girl born in 1976, presented at the age of 7 years with very severe asthma. Because of the severity of the asthma, she spent several times in a center for asthmatic diseases in the Swiss Alps. Initially, she was treated with inhalation corticosteroids, β-sympaticomimetics and anti cholinergic drugs in combination with short periods of high doses of orally administered corticosteroids (prednisone). When she was referred to our clinic at the age of 17 years, she had been treated with prednisone 25 mg daily continuously for two years, which regularly had to be elevated to a dose of 60 mg per day over a period of 10-12 days. Despite this therapeutic regimen, she still had severe complaints of asthma, not allowing her to live a normal life. Because this very poor clinical response to therapy in combination with the fact that she had no signs or symptoms of Cushing's syndrome, she was referred to our clinic for investigation

whether this steroid resistant asthma was based on a defect in the GR gene.
Physical examination revealed a tall, slender young woman, without signs or symptoms of Cushing's syndrome. She had a normal blood pressure. There were no signs of skin atrophy, hirsutism, hematomas or muscle weakness. Using oral contraceptives, she had regular menses. Despite the continuous high dose prednisone therapy during puberty, her final height was over 10 cm above the parents' height (1.70 m). Because of the high dose of prednisone the patient was taking, we were not able to perform regular testing of the HPA-axis. Furthermore, as became clear from a previous study (21), prednisone is an agent that interferes with the whole cell DEX binding assay and the mitogen induced thymidine incorporation assay, making it impossible to obtain meaningful results from these tests.
Using PCR/SSCP analysis, we found the same polymorphism as described for patient 1 in exon 2 of the GR gene. No other mutations were found despite sequencing of the whole coding part of the GR gene.
The patient,s grandparents, parents and her two younger brothers were tested with respect to CR as well.They were all healthy without clinical signs of GR. They all showed a normal cortisol suppression in the 1 mg overnight DST. Nevertheless, it appeared that the grandmother (from mothers side), the mother and the oldest brother showed the same polymorphism in exon 2 of the GR gene.

Patient 3 is a 65 years old Belgian man. Hypertension was diagnosed at the age of 50. Fifteen years before presentation, a CT scan revealed a tumor in the right kidney and a tumorous enlarged right adrenal gland. He underwent a partial right sided nefrectomy in combination with an adrenalectomy. Histopathological evaluation revealed an infiltrating clear-cell adenocarcinoma of the kidney and nodular adrenal hyperplasia.
Two years later, he presented with obesity and insulin resistance. He was treated with a diet and biguanides. Serum cortisol concentrations and 24 hr urinary cortisol excretion were tested because of the adrenal hyperplasia that was previously found. Basal cortisol concentration at that time was 432 nmol/l at 8.00 am, which is within the normal range. Nevertheless, 24 hr urinary cortisol excretion was increased considerably to twofold over the normal range.
Five years later, a CT scan revealed a nodule of 2 cm in a nodular enlarged left adrenal gland.

There were no clinical symptoms of Cushing's syndrome.

Endocrine testing at that time (Table1) showed a normal diurnal rhythm of cortisol concentrations with high-normal ACTH concentrations. There was an insufficient suppression of cortisol in the overnight 1 mg DST (471 nmol/l). In response to the iv administration of 0.1 U/kg insulin, he developed hypoglycemia followed by a just subnormal increase in cortisol levels (Table 3).

Three yaers later, a CT scan confirmed the macronodular hyperplasia of the left adrenal gland. A CT scan of the pituitary revealed no abnormalities. At present, he is still asymptomatic without signs or symptoms of Cushing's syndrome.

In the whole cell DEX binding assay, a normal number of GC receptors per cell in PBMLs became apparent (Table 4). On the other hand, the receptors showed a diminished affinity for DEX, with a K_d of 22.3 nmol/l (Figure 2). In the mitogen induced proliferation assay, a maximal inhibition of 77% could be achieved with an IC_{50} of 33.1 nmol/l, which is in the higher normal range.

Using PCR/SSCP analysis of the whole coding part of the GR gene in combination with sequence analysis of the whole hormone binding domain (exons 4-9), no alterations in the GR gene could be identified.

The patient's three sons and only daughter were tested with respect to CR as well. One of the sons had an increased 24 hr urinary cortisol excretion. He was a healthy young man, and had no hypertension. In a 1 mg overnight DST, he showed an insufficient cortisol suppression (cortisol after 1 mg of DEX was 364 nmol/l). Also in the son, no alterations in the GR gene could be identified.

As the increased 24 hr urinary cortisol excretion and the diminished cortisol suppression in the overnight 1 mg DST were found by coincidence, and the patient was, apart from the increased blood pressure, asymptomatic, he was not treated with DEX.

(exons 4 to 9) revealed no alterations in this part of the gene.
Her parents and only sister were healthy and showed a normal cortisol suppression in an overnight 1 mg DST.
Based on the clinical and biochemical data, the diagnosis of generalized cortisol resistance was made. The patient has been treated with low doses of DEX for the past three months. Within a couple of weeks a dramatic improvement of the clinical course was observed: Fatigue and mental instability greatly improved. The patient started working again and jogs 10 km every other day. Nevertheless, Table 3 shows that the HPA axis was not sufficiently suppressed by 0.5 mg of DEX administration. Cortisol and desoxycortisol concentrations were still elevated while adrenal androgen production was not suppressed. Probably, this low dose of DEX was a sufficient dose to induce the clinical improvement in the patient, but was not high enough to adequately suppress the HPA axis.

Patient 5 is a 33 year old woman who was admitted for analysis of severe tiredness, hirsutism and oligomenorrhea. Furthermore, there were complaints of diminished concentration and muscle weakness. She was not able to perform her job as a schoolteacher anymore. In the past, she had been analysed several times for fatigue and pain in the throat which was interpreted as a viral infection. Furthermore, she had hirsutism and irregular menstruations.
Endocrine testing showed a normal circadian rhythm of cortisol, ACTH and a normal urinary cortisol excretion (Table 1). There was an insufficient cortisol suppression in reaction to the administration of 1 mg of DEX (Table 1). A normal GH response in reaction to an insulin induced hypoglycemia was observed, but cortisol concentrations did not increase sufficiently (Table 3). Table 2 shows that the desoxycortisol concentration was slightly elevated and the androgen concentrations were increased considerably. The patient had no brothers or sisters, her parents were both healthy and showed normal cortisol concentrations and a normal adrenal cortisol suppression in an overnight 1 mg DST.
Analysis of cortisol receptor characteristics in mononuclear leukocytes showed a normal K_d, in combination with a significantly lowered number of receptors per cell (Figure 3). The bioassay showed a normal capacity to inhibit mitogen induced proliferation (Table 4). PCR/SSCP analysis of the whole coding part of the GR gene showed no abnormal migration patterns.

Furthermore, sequence analysis of the hormone binding domain of the GR gene (exons 4-9) revealed no alterations in this part of the gene.

Based on the clinical and biochemical determinations, the diagnosis of partial cortisol resistance was made. The patient has been treated with low doses of DEX for the past 6 months. Table 3 shows that the androgen concentrations are still slightly elevated, but suppressed as compared to pre-treatment values. Hirsutism has improved and the menstrual pattern has normalized. Furthermore, the tiredness has dramatically improved, the patient feels extremely well for the first time in years, and started to work again on a regular basis.

Table 4. Cortisol receptor characteristics and inhibition of PHA-stimulated [^{3}H]-thymidine incorporation in mononuclear leukocytes by dexamethasone.

	mononuclear leukocyte cortisol receptor characteristics		**inhibition of PHA-stimulated [^{3}H]-thymidine incorporation by DEX**	
Patient	**no. of receptors / cell**	**K_d (nmol/l)**	**maximal inhibition (%)**	**IC_{50} (nmol/l)**
1	7429	8.6	0	∞
3	6659	22.3	77	33.1
4	5938	17.4	35	∞
5	3500	8.1	95	27.7

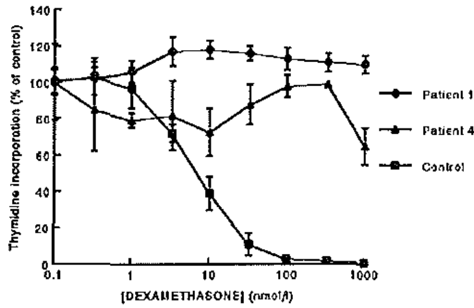

Figure 1. Mitogen induced proliferation assay in patient 1 (circles), patient 4 (triangles) and a healthy control (squares). In both patients DEX showed a dramatically reduced capacity to inhibit mitogen induced cell proliferation

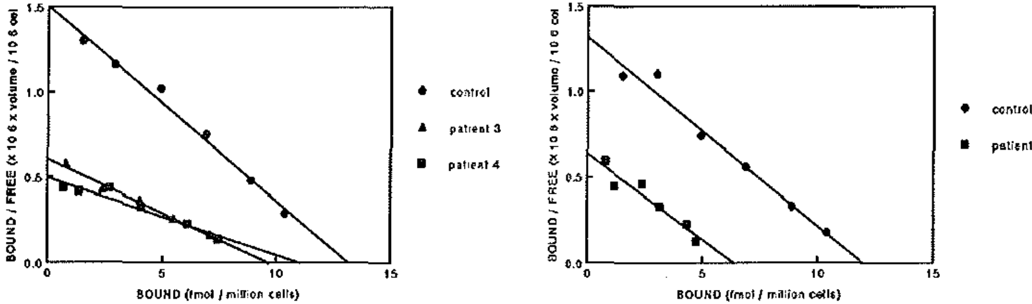

Figure 2. (right) Whole cell DEX binding assay in patients 3 (triangles) and 4 (squares) and a healthy control (circles). Both patients showed a decreased affinity of the GR to DEX.

Figure 3. (left) Whole cell DEX binding assay in patient 5 (squares) and a healthy control (circles). The patients showed only half of the normal receptor number per cell.

DISCUSSION

Ever since the first description 1976 by Vingerhoeds et al (2), only about 30 patients and (asymptomatic) family members with CR have been described. Up till now, the molecular basis for CR has been described in four patients and affected (asymptomatic) family members (15-18). In all four cases, mutations in the hormone binding domain of the GR gene were responsible for the clinical manifestation of cortisol resistance. Recent reports of a considerable prevalence of possible abnormalities in the GR in patients attending the endocrine clinic for hypokalemia, hypertension, acne, hirsutism and menstrual disorders (1, 11), prompted us to carry out a thorough investigation with respect to the GR protein and the GR gene in patients attending the endocrine clinic for a broad spectrum of complaints suggesting a possible CR. In the present study, we describe 5 patients with variable clinical presentation which all seem clarified by the syndrome of generalized CR.

In patient 1, extreme hirsutism was the most profound complaint after she was treated for Cushing's disease (at that time the treatment of choice was adrenalectomy followed by pituitary radiation). Two years later she was readmitted with clinical signs and symptoms which were probably related to relative adrenocortical insufficiency. About 20 years later at retesting , it appeared that her PBMLs were extremely insensitive to the inhibitory effect of DEX on mitogen induced proliferation. Treatment with low doses of DEX decreased the serum androgen concentrations, and there was a slight improvement of the hirsutism after several months of treatment. Patient 4 had complaints of hyperandrogenism, mood unstability, increasing fatigue and hypertension. Endocrine testing elsewhere pointed towards Cushing's disease. She underwent transsphenoidal pituitary surgery, during which normal pituitary tissue was removed. Further investigations showed that the GR ligand affinity on PBMLs was markedly diminished. From the clinical history of these two patients, it can be concluded that it is important to distinguish Cushing's syndrome from CR in cases of hypercortisolemia. The patients showed an increased (patient 4) or high normal (patient 1) early morning cortisol concentration, but, in both patients, there still was a virtually normal diurnal rhythm of cortisol, albeit at an elevated level. In Cushing's syndrome, the diurnal rhythm of cortisol is

absent. Another important observation in patient 4 was that she had a completely normal bone mineral density despite the elevated cortisol concentrations, while also both cortisol and GH levels adequately increased in response to an insulin induced hypoglycemia. Both observations would not have been observed in an individual with Cushing's syndrome. On the other hand, it cannot be excluded that patient 4, who showed very high serum cortisol concentrations will eventually develop Cushing's syndrome. Karl et al (18) described a patient who developed pituitary Cushing's syndrome preceded by generalized CR. A previous study by our group, however, indicated that in patients with Cushing's disease due to ACTH secreting pituitary adenomas, no GR gene alterations in DNA extracted from PBMLs could be identified (26). In patient 4, we observed clinical improvement during treatment with low doses of DEX. Nevertheless, cortisol and desoxycortisol concentrations remained elevated, while adrenal androgen production was not suppressed. Probably, this low dose of DEX was sufficient to induce the clinical improvement in the patient, but was not high enough to adequately suppress the HPA axis.

In patients 4 and 5, fatigue was, besides signs of hyperandrogenism, an additional complaint. In a previous report by Bronnegard (5), a family with CR was described, in whom chronic fatigue was the only symptom. It was hypothesized that there was an insufficient compensation by increased GC secretion in certain target tissues, like for example the central nervous system. In patient 5, a normal diurnal rhythm of cortisol concentrations was seen, but she showed insufficient adrenal cortisol suppression in an overnight 1 mg DST. Furthermore, an insufficient cortisol response in reaction to an insulin induced hypoglycemia was seen. After treatment with low doses of DEX, the patient felt much better and the fatigue disappeared. In this patient, an insufficient increment of GC secretion to overcome the CR might be an explanation for the clinical picture.

Patient 2 was a young girl with severe asthma, who was using prednisone therapy for several years, without beneficial effects with respect to her asthma, but also without adverse effects with respect to signs and symptoms of Cushing's syndrome. It was striking that there was no growth impairment during puberty, and her final length was well above her parents' length. Because of the GC therapy, we could not perform endocrinological testing, nor determine

receptor protein characteristics. Nevertheless, the diagnosis CR was based on the complete absence of signs and symptoms of Cushing's syndrome. The results of a number of studies (27, 28) suggest that in cases of rheumatoid arthritis, asthma and sepsis, high local concentrations of cytokines induce a localized CR at the site of inflammation, which can insufficiently be overcome by excess exogenous GCs. This reversible cytokine-induced form of CR should be differentiated from the situation in our patient in whom steroid resistant asthma was probably due to an irreversible (generalized) CR, which was previously described in two patients by Sher et al (13). This differentiation can be made on base of the appearance of side effects: In patients with cytokine-induced CR, GCs do have deleterious systemic effects, while in patients with generalized CR, no side effects of treatment with high doses of GCs are seen.

Patient 3 was an asymptomatic man, in whom a greatly increased urinary cortisol excretion was identified as he was endocrinologically tested because of an incidentally discovered adrenal enlargement. It appeared that there was an insufficient adrenal suppression in the overnight 1 mg DST, a normal diurnal rhythm of serum cortisol concentrations with ACTH concentrations around the upper limit of the normal values. Also his asymptomatic son showed an increased urinary cortisol excretion and insufficient cortisol suppression in the 1 mg DST. It appeared that the GR in PBMLs from the patient showed diminished affinity for DEX.

To search for possible alterations in the GR gene explaining the clinical pictures in the patients described in this study, PCR/SSCP analysis of the whole coding part of the GR gene was performed. Furthermore, the hormone binding domain of the GR gene (exons 4-9) was screened using sequence analysis. Two of the patients described carried a polymorphism characterized by a double point mutation at codon 22 and 23. This polymorphism has previously been described in a general population screening by Koper et al (25). In that study, a 1 mg overnight DST was performed in 216 healthy elderly individuals. The same polymorphism was found in this population study in eleven individuals, including a person with the highest post DEX cortisol concentration (256 nmol/l). Moreover, two other subjects with diminished cortisol suppression (post DEX cortisol concentration were 110 and 241 nmol/l, respectively) were found to carry the same mutation. However, eight other subjects out of this group of 216 volunteers, showed this polymorphism in combination with a normal cortisol

suppression in reaction to 1 mg of DEX. From patient 2, we screened her family with respect to this polymorphism as well. It appeared that her healthy grandmother, mother and brother carried the mutation. They all showed a normal cortisol suppression in the 1 mg DST. In *in vitro* expression experiments, this polymorphism showed no alterations in the efficacy of the GR to activate transcription in such systems compared to the wild-type receptor (29). Possibly, in the patients with CR carrying this polymorphism, the mutation is linked to another mutation / polymorphism in or near the GR gene that was not detected in our study using the PCR/SSCP/sequence approach.

In patients 3-5, who all showed clear clinical and biochemical pictures of CR, no GR gene alterations were found however. It is clear that there is a discrepancy between the clinical and biochemical picture in these patients on the one side, and the molecular characterization on the other side. This is especially true for the patients who showed a lowered K_d. In these patients one would expect to find alterations in the hormone binding domain of the GR gene. Patient 5 showed only half of the normal numbers of GR per cell in PBMLs. In a family with only half of the number of receptors previously described by Karl et al (16), it appeared that this was due to a splice site mutation in one of the alleles of the GR gene, resulting in an unstable mRNA. Another possibility might be that there is a mutation/deletion in one of the alleles which alters the structure of the hormone binding domain in such a way, that no ligand can be bound. On the other hand, one would expect to find such alterations using PCR/SSCP/sequence analysis.

Not only GR expression level and ligand binding affinity, but also ligand induced GR conformation changes, dissociation from heat shock proteins, phosphorylation and nuclear transformation are important determinants of GC sensitivity (30). Another possibility could be that post-receptor defects or interactions with other nuclear factors form the pathophysiologic basis of CR in these patients. In order to investigate the GR gene more extensively, a next step will be to look at the transcriptional level by studying mRNA expression.

It can be concluded that in all five patients described, there was clinical and/or biochemical evidence for generalized CR. On the other hand, we could not identify GR gene alterations which give a molecular explanation for the clinical and biochemical CR.

List of references

1. Lamberts SWJ, Koper JW, Biemond P et al. 1992 Cortisol receptor resistance: the variability of its clinical presentation and response to treatment. J Clin Endocrinol Metab 74:313-321
2. Vingerhoeds AC, Thijssen JH, Schwarz F. 1976 Spontaneous hypercortisolism without Cushing's syndrome. J Clin Endocrinol Metab 43:1128-1133
3. Chrousos GP, Vingerhoeds AC, Brandon D et al. 1982 Primary cortisol resistance in man: a glucocorticoid receptor mediated disease. J Clin Invest 69:1261-1268.
4. Lida S, Moriwaki K, Itoh Y et al. 1985 Primary cortisol resistance accompanied by a reduction in glucocorticoid receptors in two members of the same family. J Clin Endocrinol Metab 60:967-971.
5. Bronnegard M, Werner S, Gustafsson JA. 1989 Primary cortisol resistance associated with a thermolabile glucocorticoid receptor in a patient with fatigue as the only symptom. 78:1270-1278.
6. Lamberts SWJ, Poldermans D, Zweens M et al. 1986 Familial cortisol resistance: differential diagnostic and therapeutic aspects. J Clin Endocrinol Metab 63:1328-1333
7. Nawata H, Sekiya K, Higuchi K et al. 1987 Decresed deoxyribonucleic acid binding of glucocorticoid receptor complex in cultured skin fibroblasts from a patient with the glucocorticoid resistance syndrome. J Clin Endocrinol Metab 65:219-226.
8. Vercei P, Frank K, Haack D et al. 1989 Primary glucocorticoid receptor defect with likely familial involvement. Cancer Res 49(supp) 2220s-2221s.
9. Jaursch-Hancke C, Allolio B, Reincke M et al. 1989 Primary cortisol resistance as a differential diagnosis of Cushing's syndrome. Acta Endocrinol Copenh 120(supp) 113.
10. Malchoff CD, Javier EC, Malchoff DM et al. 1990 Primary cortisol resistance presnting as isosexual precocity. J Clin Endocrinol Metab 70:503-507.
11. Werner S, Thoren M, Gustafsson JA et al. 1992 Glucocorticoid recptor abnormalities in fibroblasts from patients with idiopathic resistance to dexamethasone diagnosed when evaluated for adrenocortical disorders. J Clin Endocrinol Metab 75:1005-1009.
12. Norbiato G, Bevilaqua M, Vago T et al. 1992 Cortisol resistance in acquired immunodeficiency syndrome. J Clin Endocrinol Metab 74:608-613.
13. Sher ER, Leung DYM, Surs W et al. 1994 Steroid resistant asthma. Cellular mechanisms contributing to inadequate response to glucocorticoid therapy. J Clin Invest 93:33-39.
14. Giguere V, Hollenberg SM, Rosenfeld MG et al. 1986 Functional domains of the human glucocorticoid receptor. Cell. 46:645-652
15. Hurley DM, Accili D, Stratakis CA, et al. 1991 Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucocorticoid resistance. J Clin

Invest. 87:680-686.

16. Karl M, Lamberts SWJ, Detera-Wadleigh SD et al. 1993 Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. J Clin Endocrinol Metab. 76:683-689.

17. Malchoff DM, Brufsky A, Reardon G et al. 1993 A mutation of the glucocorticoid receptor in primary cortisol resistance. J Clin Invest. 91:1918-1925.

18 Karl M, Lamberts SWJ, Koper JW et al. 1996 Cushing's disease preceded by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proc Ass Am Phys. 108:296-307.

19. Lamberts SWJ, Klijn JGM, de Jong FH. 1987 The definition of true recurrence of pituitary dependent Cushing's syndrome after transsphenoidal operation. Clin Endocrinol 26:707-712.

20. Biemond P, de Jong FH, Lamberts SWJ. 1990 Continuous dexamethasone infusion for seven hours in patients with the Cushing Syndrome. Ann Intern Med 112:738-742.

21. Molijn GJ, Spek JJ, van Uffelen JCJ et al. 1995 Differential adaptation of glucocorticoid sensitivity of peripheral blood mononuclear leukocytes in patients with sepsis or septic shock J Clin Endocrinol Metab 80:1799-1803.

22. Scatchard G. 1949. The attractions of proteins for small molecules and ions. Ann NY Ac Science 51:660-672.

23. Huizenga NATM, Koper FJW, de Lange P et al. 1998 A polymorphism in the glucocorticoid receptor gene is associated with increased sensitivity to glucocorticoids in vivo. J Clin Endocrinol Metab 83:144-151

24. Orita M, Iwahana H, Kanazawa H et al. 1989 Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci U S A 86:2766-2770.

25. Koper FJW, Stolk RP, de Lange P et al. 1997 Lack of association betwee five polymorphisms in the glucocorticoid receptor gene and glucocorticoid resistance. Hum Genet 99:663-668.

26. Huizenga NATM, de Lange P, Koper JW et al. 1998 Human ACTH secreting pituitary adenomas show frequent loss of heterozygosity at the GR gene locus. J Clin Endocrinol Metab

27. Corrigan CJ, Brown JPH, Barnes NC et al. 1991 Glucocorticoid resitance in chronic asthma: glucocorticoid pharmacokinetics, glucocorticoid receptor characteristics and inhibition of peripheral blood T cell proliferation by glucocorticoid in vitro. Am Rev Respir Dis 144:1016-1025.

28. Corrigan CJ, Brown JPH, Barnes NC et al. 1991 Glucocorticoid resistance in chronic asthma: peripheral blood T lymphocyte activation and comparison of the T lymphocyte inhibitory effects of glucocorticoids and cyclosporin A. Am Rev Respir Dis 144:1026-1032.

29. de Lange P, Koper FJW, Huizenga NATM et al. 1997 Differential hormone-dependent transcriptional activation and repression by naturally occuring human glucocorticoid receptor variants. Mol Endocrinol 11:1156-1164.

30. Bamberger CM, Schulte HM, Chrousos GP. 1996 Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocrine Rev 17:245-261.

CHAPTER 6

CUSHING'S DISEASE PRECEDED BY GENERALIZED GLUCOCORTICOID RESISTANCE: CLINICAL CONSEQUENCES OF A NOVEL, DOMINANT-NEGATIVE GLUCOCORTICOID RECEPTOR MUTATION.

Michael Karl, Steven W.J. Lamberts, Jan W. Koper, David A. Katz, Nannette A.T.M. Huizenga, Tomoshige Kino, Bassem R. Haddad, Mark R. Hughes, and George P. Chrousos.

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ABSTRACT

Generalized glucocorticoid resistance is associated with chronic hyperactivation of the hypothalamic-pituitary-adrenal-axis, compensating for impaired glucocorticoid receptor function. We report a unique patient with sporadic generalized glucocorticoid resistance who, at age 33, presented with infertility and hypertension and, at 38, developed pituitary Cushing's disease. Leukocyte-binding studies revealed normal affinity of the glucocorticoid receptor but a reduction of binding sites by 50%. [^{3}H]-thymidine incorporation by this patient's lymphocytes was not suppressible by dexamethasone. He bad a novel heterozygous missense mutation in the glucocorticoid receptor gene (isoleucine 559 to asparagine 559). The mutant receptor exhibited a strong dominant-negative effect on the ability of the wild-type receptor to induce gene transcription in vitro. The mutation was present in all of the patient's cultured lymphoblasts and fibroblasts as well as in 50% of his sperm, as demonstrated by single-cell polymerase chain reaction; it was not present in his parents and seven siblings. This novel mutation was thus both de novo and present in the germ line. Immunohistochemical staining of this patient's pituitary corticotropinoma revealed accumulation of p53 protein, indicating the presence of a putative somatic oncogenic mutation in the p53 gene in the tumor cells. Investigation of the lymphoblast and skin fibroblast cultures for p53 abnormalities did not show any aberration. Thus, a novel de novo germ line mutation of the glucocorticoid receptor with strong dominant-negative activity caused severe sporadic generalized glucocorticoid resistance, which preceded corticotroph adenoma formation. The latter probably was due to the combined effects of chronic corticotroph hyperstimulation, decreased glucocorticoid negative feedback, and at least one subsequent somatic defect in the control of the cell cycle.

INTRODUCTION

Increased secretion of cortisol is present in both generalized glucocorticoid resistance (GGR) (1,2) and Cushing's disease (3). Yet in the former, circadian and stress induced secretion of cortisol remain intact, whereas in the latter, they are disrupted. Patients with GGR develop neither Cushing's syndrome stigmata nor glucocorticoid deficiency. Their excess adrenocorticotropic hormone (ACTH) secretion, however, results in increased production of adrenal steroids with salt-retaining or androgenic activity. Salt retaining steroids cause hypertension or hypokalemic alkalosis, whereas androgenic adrenal steroids lead to symptoms and signs of androgen excess, such as acne, hirsutism, and disturbances of reproductive functions in women and virilization in children. GGR has been associated with quantitative or qualitative defects the glucocorticoid receptor (GR) (2). To date, the molecular basis of GGR has been elucidated in three families (4-6). In Cushing's disease one sees the deleterious effects of chronic hypercortisolism in humans (3). This condition usually is caused by ACTH hypersecretion from an ACTH-secreting pituitary adenoma. The hypercortisolism produces the characteristic features of Cushing's syndrome, including central obesity, moon facies, muscle wasting, easy bruisability, depression, glucose intolerance or diabetes, and osteoporosis. In Cushing's disease, the negative-feedback loop of the hypothalamic-pituitary-adrenal (HPA) axis is disrupted at the level of the ACTH-secreting adenoma, and the elevated ACTH levels usually are accompanied by bilateral adrenal hyperplasia or unilateral or bilateral adrenocortical macronodules (7,8). The chronic exposure of corticotrophs to elevated levels of hypothalamic releasing factors has been suggested as a possible mechanism leading to pituitary tumorigenesis (8). However, recent reports of putative somatic mutations of tumor suppressor genes, such as p53 (9), or oncogenes, such as Gs (10), in corticotropinomas suggested that inherent adenomatous cell-specific changes might play a primary role in the pathogenesis of these neoplasms.

In this study, we present the unique case of a Dutch man with GGR who developed aggressive Cushing's disease at the age of 38 years. We examined the molecular pathophysiology of GGR of this patient, with findings that provided new insights on both the mechanisms of glucocorticoid resistance and the pathogenesis of Cushing's disease.

METHODS

Subjects and family history

A moderately obese man was evaluated for infertility at the age of 33 years. He first was found to be oligospermic and mildly (stage 1) hypertensive (145/90 mm Hg). He was followed regularly and when his arterial blood pressure increased to 150/100 mm Hg (stage 2) at the age of 36 years, he was started on standard doses of hydrochlorothiazide. On this regimen over several weeks, he developed severe life-threatening, symptomatic hypokalemia (plasma K 1.0 mmol/l). Reevaluation at this time revealed hypercortisolism, without any clinical evidence of Cushing's syndrome (11). His bone density was normal. He was started on dexamethasone, 3 mglday PO and felt well over the next 2 years. His blood pressure normalized. His morning serum cortisol levels and ACTH levels,. however, remained elevated at 3000 nmol/l (normal, 200-580 nmol/l) and 25.2 pmol/l (normal 4.4-14.3 pmol/l), respectively, as was his urinary free cortisol excretion at 87,500 nmol/day (normal, < 450 nmol/day). Two years later, he became floridly cushingoid over a period of 6 weeks. His blood pressure was elevated (165/105 mm Hg), his serum cortisol levels increased to 10700 nmol/l, and his urinary free cortisol excretion nearly doubled to 160000 nmol/d. Despite discontinuation of the dexamethasone substitution, the patient continued to gain centripedal weight, his hypertension worsened and he complained of severe back pain. Bone densitometry performed at that time was within the normal range but significantly increased in the lumbar spine, hips, and lower arm compared with the mean of age- and sex-matched controls. Serum cortisol concentrations increased further to levels between 20000 and 24000 nmol/l, without diurnal variation. The diagnosis of pituitary Cushing's disease was made, but pituitary magnetic resonance imaging was negative. The patient elected to have bilateral adrenalectomy. After an uneventful surgery, the patient was placed on 1.5 mg dexamethasone and 50 µg fludrocortisone by mouth daily, and his condition improved. Blood pressure normalized, and he needed no further antihypertensive medication. However, his morning plasma ACTH levels increased in the subsequent months to a maximum of 130 pmol/l. Repeat magnetic resonance imaging 8 months after adrenalectomy revealed a pituitary tumor with a diameter of 9 mm, which was removed by transsphenoidal

adenomectomy. Histological examination of the pituitary tissue confirmed the diagnosis of a corticotropinoma and excluded corticotroph hyperplasia. After the transsphenoidal surgery (TSS), the patient has remained on 1.5 mg dexamethasone and 50 μg fludrocortisone daily by mouth, without any stigmata of Cushing's syndrome. [^{3}H]dexamethasone binding and [^{3}H]thymidine incorporation studies were performed with the patient's leukocytes after the TSS. Three years later, circulating ACTH levels vary between 33 and 40 pmol/l. The patient has been symptom free and normotensive. The patient's parents, five brothers, and two sisters had no clinical or biochemical evidence of GGR (Figure 1).

Whole cell dexamethasone binding assay

GR concentrations and affinity for [^{3}H]dexamethasone were determined in Ficoll-Isopaque extracted mononuclear leukocytes as previously described (11) Ligand incubation was carried out for 1 hr at 30° C.

Inhibition of mitogen-stimulated incorporation of [^{3}H]thymidine by dexamethasone

Mitogen-stimulated incorporation of [^{3}H]thymidine was inhibited by dexamethasone as previously described (11). Mononuclear leukocytes isolated with the Ficoll-Isopaque method were seeded at 10^5 cells/well, and 5 μg, phytohemagglutinin (PHA) was added to each well. Subsequently cells were exposed to dexamethasone at six different concentrations ranging from 3-1000 nM for 72 hr. [^{3} H]thymidine was added to each well, and incubation was allowed for 4 hr. After centrifugation, precipitation, and washing of the cell pellet, radioactivity was determined by liquid scintillation counting. The concentration of dexamethasone necessary to achieve 50% of the maximal inhibition of thymidine incorporation (IC_{50}) then was estimated graphically by graph fitting.

Establishment of a permanent lymphoblast cell line

Epstein-Barr virus transformed (EBV-transformed) lymphoblast lines were established from the patient peripheral blood lymphocytes, as previously described (12). The cell lines were grown in RPMI- 640 medium supplemented with 10% fetal bovine serum (FBS), and 2 mmol/l

glutamine in the presence of 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL, Gaithersburg, MD) in a 5% carbon dioxide atmosphere.

Establishment of a fibroblast line

Fibroblast cultures were derived from skin and prepared as previously described (13). Cells were maintained in culture as described earlier.

COS-7 cell lines

COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% charcoal-treated FBS (HyClone, Logan, UT), 2 mmol/l glutamine in the presence of 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL) in a 5% carbon dioxide atmosphere.

Extraction of DNA

DNA was extracted either directly from isolated peripheral white blood cells of the patient, his relatives, and control subjects or from the EBV-transformed lymphoblast cell lines and cultured skin fibroblasts using the QIAamp Blood Kit (Qiagen Inc., Chatsworth, CA).

Extraction of RNA

Total RNA was isolated from the EBV cell line and skin fibroblasts using a monophase solution of phenol-guanidinium thiocyanate (RNA STAT-60) in a single-step method according to the manufacturer's instructions (TEL-TEST "B", Inc., Friendswood, TX).

Amplification of genomic DNA by Polymerase Chain Reaction (PCR)

PCR was performed as previously described, with minor modifications (5). Briefly, amplifications were carried out in a total volume of 50 μg containing 50-100 ng of genomic DNA, 15 pmol of each nucleotide primer, 0.2 mM dNTP, 50 mM KCl, 10 mM Tris (pH 8.3), 1.5-3 mM $MgCl_2$ and 2.5 units of Taq-DNA-Polymerase (Boehringer Mannheim, Indianapolis, IN) or Ampli-Taq (Perkin-Elmer, Emeryville, CA). PCR was performed for 30 cycles each

cycle consisting of incubations for 30 sec at 94°C for denaturation, for 30 sec at 55°C or 60°C for annealing, and for 45 sec at 72°C for primer extension in a GeneAmp PCR System 9600 (Perkin-Elmer, Emeryville, CA). In the first cycle, the denaturation period was lengthened to 4 min, and temperature was increased to 95°C; at the end of the last cycle, extension was lengthened to 4 min. Primer pairs were complementary to sequences in the 5'- or 3'-exon-flanking regions of the GR -gene and of exons 5-8 of the p53 gene, respectively (5).

PCR of single cell extracts

Extracts of single cells from the lymphoblast or fibroblast cultures and sperm were amplified using a specific primer pair located in the 5'- and 3'-intron-flanking region of exon 5 using the same conditions as described previously. An aliquot (2 µl) of the first PCR amplification was reamplified using nested primer pairs.

Synthesis of cDNA

Total RNA was reverse transcribed using a first-strand cDNA synthesis kit (Boehringer Mannheim). Briefly, RNA (1 µg) was denatured at 65°C for 15 min and chilled on ice for 5 min. Reverse transcription was carried out in a total volume of 20 µl containing 10 mM Tris, 50 mM KCl (pH 8.3), 5 mM $MgCl_2$,1 mM each of dATP, dCTP, dTTP, and dGTP, 50 units of RNase inhibitor, 20 units of avian myeloblastosis virus (AMV) reverse transcriptase and 50 pmol of a specific antisense primer of the GR cDNA. The reaction mixture was incubated first at room temperature for 10 min and then at 42°C for 1 hr. Thereafter, the AMV reverse transcriptase was denatured by incubating the reaction at 99°C for 5 min and cooling on ice for 5 min. PCR amplification was performed in a total volume of 100 µl containing an aliquot of the first-strand cDNA reaction (5-10 µl) in the presence of 10 mM Tris, 50 mM KCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dTTP, and dGTP, 50 pmol of each, a specific sense and antisense cDNA primer, and 2.5 units of Taq DNA Polymerase (Boehringer Mannheim) or Ampli-Taq (Perkin-Elmer). PCR was carried out for 40 cycles in a GeneAmp PCR System 9600 (Perkin-Elmer) using the cycling conditions previously described.

Direct sequencing of asymmetric PCR-amplified products

Single-stranded DNA was synthesized using as template the product of the symmetrical PCR. For the amplification of one DNA strand selectively, only a sense or an antisense primer (50 pmol) was added, and the asymmetric PCR was carried out for 20 cycles. Single-stranded PCR products then were purified using a QIAquick-spin PCR purification kit (Qiagen Inc., Chatsworth, CA), according to the manufacturers instructions. Direct sequencing of the purified asymmetric PCR products was performed by the Sanger-dideoxynucleotide chain termination method, using modified T7-DNA polymerase in the presence of Redivue [α-^{35}S] dATP (Amersham Life Science Inc, Arlington Heights, IL), as previously described (5).

Sau-3A Digestion of the Exon 5-Amplicon of the GR

The amplicon of exon 5, including intron-flanking regions (470 or 421 bp, respectively) harbored a single recognition site for the restriction enzyme Sau-3A (GATC). Digestion of the wild-type exon 5 amplicon with Sau-3A, under standard conditions, resulted in two fragments of 273 and 197 bp or 254 and 167 bp, respectively. A mutation within this region abolished the ability of Sau-3A to digest the fragment. Digested or undigested fragments were visualized by agarose gel electrophoresis.

Construction of Plasmid pRShGR-Asn559

An expression vector for the mutant GR asparagine 559 (Asn559) was constructed by replacing a ClaI/SauI fragment, including human glucocorticoid receptor (hGR) nuelcotides 1524-2159 of plasmid pRSh-GRα (kindly provided by Dr. R.M. Evans, Salk Institute), with the corresponding cassette harboring the mutant adenosine (A) substitution. Site-directed mutagenesis by overlap extension PCR was employed to replace the wild-type thymine (T) at nucleotide position 1808, with the mutant A using the expression vector pRShGRα as a template. Each of two pairs of oligonucleotides were used in separate PCR reactions to amplify overlapping hGR cDNA fragments that incorporated the base substitution. An aliquot of each of the two initial reactions then was added to a third PCR reaction in order to amplify the cDNA region including nucleotides 1524-2159. The product of the third PCR was digested with ClaI and SauI. The ClaI-XhoI fragment of the pRShGRα (including nucleotides 1524-

2463) was inserted into the multiple cloning site of the cloning vector pBluescript II phagemid (Stratagene, La Jolla, CA). After digestion of the recombinant plasmid with ClaI and SauI, the digested PCR fragment was ligated into the plasmid to replace the wild-type sequence. Subsequently, the recombinant ClaI-XhoI fragment of the hGRα cDNA was ligated back into pRShGRα. The entire ClaI/SauI cDNA insert then was sequenced to confirm the A1808 substitution without any other base changes. The recombinant plasmid was designated pRShGR-Asn559.

Transfection Studies

COS-7 cells (ATCC) cultured in DMEM supplemented with 10% charcoal-treated FBS, 1% glutamine and penicillin-streptomycin were tranfected using lipofectine or lipofectamine (Gibco-BRL) as previously described with minor modifications (5,14). Briefly, 12 to 14 hr prior to transfection, COS-7 cells were harvested and seeded in six well plates (Costar, Cambridge, MA) at a density of 4.2 x 10^5 cells/35-mm well. At 70-80% confluency, cells were incubated for 5 h in 1 ml of Opti-MEM (Gibco-BRL) containing 6 μl of lipofectamine or 10 μl of lipofectine and the plasmids to be transfected. For reporter gene expression, 0.4 μg of pRShGRα or pRShGR-Asn559 was incubated with 1.6 μg of the reporter plasmid pLTR-Luc (gift of Dr. G. Hager, National Cancer Institute, NIH. Bethesda, MD). Twenty hours after transfection, cells were incubated with graded concentrations of dexamethasone (10^{-11}-10^{-5} M). For coexpression of wildtype and mutant GR, increasing quantities of pRShGR-Asn559 (0.02, 0.04, 0.06, and 0.10 μg) were added to constant amounts of pRShGRα (0.02 μg) and pLTR-Luc (1.0 μg). The plasmid pSV-β-gal served as a control for transfection efficiency in these experiments (0. 1 μg). The total amount of DNA transfected remained constant by the addition of appropriate amounts of plasmid pRSv-erbA. Cells were stimulated with medium containing 10^{-7} M dexamethasone. For Western blot and hormone-binding analyses, cells were transfected with 0.4 μg of pRShGR-Asn559 or pRShGRα with or without reporter genes.

Luciferase Assay

COS-7 cells cotransfected with the wild-type GR expression vector pRShGRα, the mutant GR expression vector pRShGR-Asn559, or a combination of pRShGRα and pRShGR-Asn559

together with the reporter plasmid pLTR-Luc were harvested 48 hr after transfection. Briefly, media were removed from the cell culture plates, and the cells were rinsed twice with phosphate buffered saline without Ca^{2+} or Mg^{2+}. Cells were covered with 350 μl of cell lysis buffer (0.1 M potassium phosphate buffer, pH 7.8, 1% Triton X-100, 1 mM DTT, and 2 mM EDTA) and incubated at 4°C for 20 min. Cells were collected by scraping, transferred to an Eppendorf-centrifuge tube and spun for 10 sec to remove cellular debris. For the luciferase assay 100 μl of assay buffer solution consisting of 30 mM Tricine, 3 mM ATP, 15 mM $MgS0_4$, and 10 mM DTT (pH 7.8) were added to 5 μl of cell extract. Luciferase activity was measured on a Monolight 2010 Luminometer after the injection of 100 μl of 1 mM D-Luciferin (pH 6.1-6.5) (Analytical Luminescence Laboratory, San Diego, CA). Protein concentrations of the extracts were determined using a Coomasie Protein Assay Reagent (Pierce Chemical Co., Rockford, IL) to normalize for cell lysate. In the coexpression studies of wt-hGRα and mutant GRAsn559, galactosidase activity was determined in the samples using, a galactosidase assay system (Promega, Madison, WI).

Protein-Electrophoresis and Western Blot Analysis

Cells transfected with 0.4 μg of pRShGR-Asn559 or pRShGRα, with or without 1.6 μg of pLTRLuc, were harvested by trypsinization with 250 μl TrysinVersene (0.05% Trysin and 0.02% Versene in EBSS without calcium and magnesium, Biofluids, Inc., Rockville, MD) for 3-5 min, collected in microcentrifuge tubes, and washed three times with phosphate suffered saline. Cell pellets were stored at -70°C. Samples were resuspended in TAPS-Buffer (pH 8.8). Protein, 10 μg/lane, was applied to 8% precast polyacrylamide gels, run according to Laemmli, and transferred onto nitrocellulose by electroelution. The blot was preincubated in milk block buffer. A polyclonal rabbit anti-human GR antibody (Clone 57, Affinity Bioreagents, Neshanic Station, NJ) was applied overnight at 4°C (1:5000 dilution).The blot was washed and incubated with peroxidase-conjugated antirabbit immunoglobulin antibody (1:2000, Dako, Copenhagen, Denmark) at 22°C for 1 hr. Bands were detected by chemiluminescence (15).

Receptor binding assays in vitro and determination of relative binding affinities

COS-7 cells were transfected with 0.4 μg of plasmids pRShGRα, pRShGRβ or pRShGR-Asn559 as described earlier. Thirty-six hours after transfection, cells were washed three times with Opti-MEM (Gibco BRL) and incubated with 10 nM of [1, 2, 4-^{3}H]-labeled dexamethasone (Amersham Life Science Inc., Arlington Heights, IL) in the absence or presence of increasing concentrations of radioinert competitor (10^{-12} -10^{-5} M) dexamethasone (Sigma Chemical Company, St. Louis, MO). After a 1hr incubation at 37°C, cells were washed three times with ice-cold phosphate-buffered saline, trypsinized by addition of 600 μl of Trysin-Versene (0.05% Trysin and 0.02% Versene in EBSS without calcium or magnesium, Biofluids) at 37°C for 3-5 minutes, and collected in microcentrifuge tubes. Aliquots of 300 ml of the suspension were transferred into scintillation vials containing 15 ml of scintillation fluid (Aquassure, Dupont NEN, Boston, MA). Radioactivity was determined by scintillation counting (Beckmann 6000IC counter) of duplicate samples for each concentration. Relative binding was expressed as a percentage of the control incubates in the absence of radioinert competitor.

Immunohistochemical Procedures

Tissue removed at surgery was fixed in 10% neutral buffered formalin and processed for paraffin embedding in standard fashion. Sections were cut at 5 μm and stained with hematoxylin and eosin, Wilder's reticulin, and periodic acid Schiff. Sections for immuno-histochemistry of the pituitary gland were stained using an avidin-biotin complex method with XantiACTH (polyclonal, 1:4000, kindly provided by the National Hormone and Pituitary Agency, NIDDK), and with anti-p53-antibodies (Polyclonal,1:50, Dako), on a Ventana 320 ES automated stainer (16).

RESULTS

In Vitro [^{3}H]dexamethasone Binding and [^{3}H]thymidine Incorporation Studies

Binding studies were performed on mononuclear leukocytes isolated from peripheral blood of the patient after he had had the adrenalectomy and TSS and while he was receiving

dexamethasone. These studies demonstrated a normal GR affinity (K_d = 7.5 nM, controls = 7.7 ± 0.2; n = 18) and a markedly reduced GR number (2929 sites/cell, controls 7191 ± 216; n = 18) (Figure 2). These results were consistent either with expression of only one of the two GR alleles, as previously reported in another family with GGR (5), or with the expression of one normal allele coding for a ligand-binding receptor and one mutated allele coding for a protein unable to bind ligand. Interestingly, this patient's cells showed no suppression of [^{3}H]thymidine incorporation by concentrations of dexamethasone as high as 10^{-6} M. suggesting marked impairment of the function of the expressed ligand binding GR (Figure 2).

Analysis of the GR gene and cDNA

The exons of the entire coding region of the GR-gene (2- 8 and 5'-region of 9α) including the intronic flanking regions, were amplified by PCR and analyzed by direct sequencing (17). A single missense mutation was disclosed in exon 5, in the coding region of the GR gene of the patient (Figure 3, top). In one of the two alleles, the nucleotide thymine (T) of the wild-type sequence was substituted by adenine (A) at cDNA position 1808 (18) (Figure 3, middle, left). Sense and antisense strands of the independently amplified amplicons of exon 5 were sequenced several times to verify the base change, thereby excluding a PCR-introduced artefact.

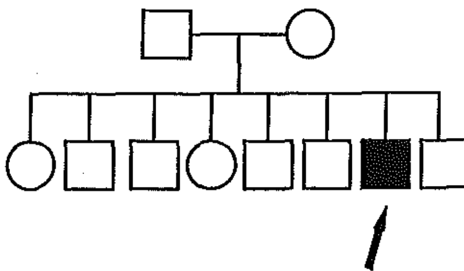


Figure 1. Family pedigree. The propositus, the second youngest of a nonconsanguineous marriage, is indicated by the arrow. He had severe generalized glucocorticoid resistance and developed a pituitary corticotropinoma at age 38. Neither of his parents and none of his siblings had the clinical or biochemical signs of glucocorticoid resistance or Cushing's disease. (Males are represented by squares, females by circles).

The expression of both mutant and wild-type alleles in the patient was confirmed by sequencing cDNA transcripts derived from reverse transcriptase PCR-amplified RNA extracted from the lymphoblast and fibroblast culture (Figure 3, middle, right). The location of the heterozygous mutation corresponded to the second base of codon 559 (ATC to AAC) in the GR (Figure 3, bottom). The nucleotide substitution was responsible for an amino acid change from the neutral and hydrophobic isoleucine (Ile) to the neutral but polar asparagine (Asn). Investigation of the corresponding amplicon in the patient's relatives, in contrast, excluded the presence of the mutation in both of his parents and in each of his siblings (see Figure 1). Therefore, the alteration identified in the patient originated either as a de novo germ-line mutation or as a somatic mutation that occurred postzygotically during early embryogenesis.

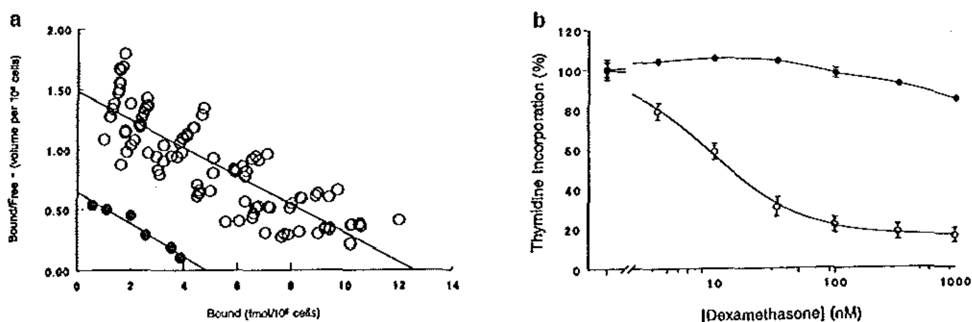


Figure 2. Whole-cell [^{3}H]dexamethasone binding assay and dexamethasone-inhibition of PHA-stimulated [^{3}H]thymidine incorporation. (a) Scatchard analysis of whole-cell ligand-binding assays of mononuclear leukocytes with [^{3}Hldexamethasone. Open circles represent the results from 18 control subjects, closed circles the results from the patient. Glucocorticoid binding sites were reduced by 50% in the patient (2929 sites/cell, controls 7191 ± 216), while the K_d remained in the normal range (7.5 nM; controls 7.7 ± 0,2). (b) Inhibition by dexamethasone of mitogen-stimulated incorporation of [^{3}H]thymidine in mononuclear leukocytes. The open symbols represent dexamethasone-induced inhibition of [^{3}H]thymidine incorporation in nine control subjects. The closed circles represent the result of the measurement in the patient. Maximal inhibition was defined as the inhibition observed with 1 μM dexamethasone. The concentration of dexamethasone needed to achieve 50% of the maximal inhibition (IC_{50}) was estimated by curve fitting. The maximal inhibition in nine control subjects was 85 ± 4%, the IC_{50} was 10.1 ± 1.1 nM. No significant inhibition of [^{3}H]thymidine incorporation by dexamethasone was observed in the propositus.

We employed the technique of single-cell PCR amplification to search for the presence and prevalence of the alteration in cells of different tissues. Single cells of the fibroblast and lymphoblast cell cultures, as well as single sperm, were separated by a micromanipulation process and lysed. The genomic DNA obtained from a single cell then was subjected to two PCR reactions consisting of 30 cycles each. An aliquot of the first specifically primed PCR was reamplified in a second reaction, using a pair of nested primers. The amplification yielded a single fragment of 421 bp. The wild-type sequence of the amplicon harbored a single GATC recognition site for the restriction enzyme Sau-3A. This restriction enzyme recognition site was eliminated by the mutation at cDNA position 1808 (GAAC). Digestion of the wild-type amplicon by Sau-3A yielded two fragments of 167 and 254 bp, respectively, whereas the mutant fragment remained uncut. Screening of the products of the single-cell PCR by Sau-3A digestion demonstrated the presence of both the mutant and wild-type alleles in each of the fibroblasts and lymphoblasts investigated. In addition, the mutation was detected in 50% of the haploid sperm.

Following a similar strategy, we screened for the presence of the mutation in normal subjects. DNA extracted from peripheral white blood cells of more than 30 unrelated controls were specifically amplified by PCR and digested by Sau-3A. The mutation was not detected in any of these subjects, excluding the base change as a common polymorphism. In addition, in more than 100 normal Dutch subjects, no such polymorphism was detected in the ligand-binding domain of the receptor by single-strand conformational polymorphism analysis.

Analysis of the GR mutation

The impact of the amino acid substitution Asn559 on the function of the GR was studied by cloning the T-to-A nucleotide substitution into a wild-type GR expression vector and transfecting COS-7 cells with the mutant vector. In transfected COS-7 cells, expression of mutant hGR-Asn559 and wild-type hGRα were demonstrated by Western blot analysis, with both species migrating at the expected molecular size (Figure 4a). The mutant hGR-Asn559 receptor showed a basal binding capacity and relative binding affinity similar to that observed

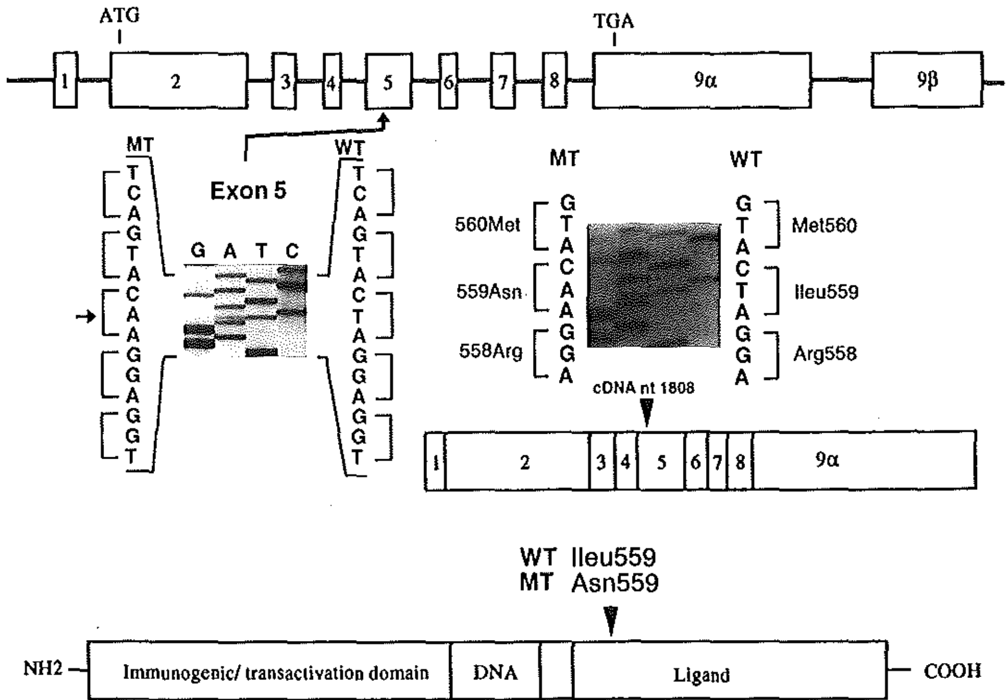


Figure 3. Analysis of the glucocorticoid receptor gene and its expression. (Top) Exons are represented by open boxes; lines between indicate intervening sequences (not to scale). The amino-terminal of the receptor is encoded by exon 2; the two zinc-fingers of the DNA binding domain are encoded separately by exons 3 and 4. The cortisol-binding domain is assembled from exons 5-8, including the 5'-end of exon 9α. (Middle, left) The autoradiogram allows visualization of both alleles of the patient obtained by direct sequencing of amplified DNA. In exon 5, a heterozygous base change substitutes the wild-type T by A, as indicated by the small arrow. The location of the mutation within the gene is shown by the long arrow. The presence of the heterozygous GR gene mutation was confirmed in the patient's peripheral white blood cells, EBV-transformed lymphoblast cells, a fibroblast cell line and in 50% of sperm cells. (Middle, right) Sequence analysis of the cDNA revealed the presence of the wild-type and mutant nucleotide at position 1808, confirming the expression of both alleles in the patient's cultured lymphoblasts and fibroblasts. The mutation at the second letter of codon 559, CTA to CAA, predicts the substitution of the wild-type amino acid isoleucine (Ileu) by asparagine (Asn). (Bottom) The amino acid change is located within the 5'-region of the ligand binding domain of the GR protein.

in cells transfected with the non-ligandbinding GRβ-isoform (Figure 4b). The mutant hGR-Asn559 thus had lost any ability to bind hormone. Consequently, the mutant Asn-559 receptor did not induce expression of a glucocorticoid-responsive luciferase reporter gene (Figure 4c). Importantly, as shown in cotransfection studies, the mutant hGR-Asn559 impaired the ability of the wild-type GR to activate reporter gene expression, exhibiting a dominant negative effect on GR function (Figure 4d).

Analysis of the p53 tumor suppressor gene and protein

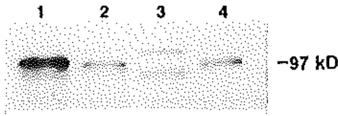
Immunohistochemical staining using anti-p53 antibodies revealed nuclear accumulation of p53 protein in a small number of the ACTH-producing pituitary adenoma cells, indicating the presence of an oncogenic somatic mutation in the p53 gene of these cells. The attempt to amplify DNA extracted from micro-dissected pituitary tumor tissue for further analysis was unsuccessful, despite different primer combinations and short amplicons. Analysis of exons 5-8 of the p53 gene of DNA from peripheral white blood cells was in accordance with the wild-type sequence and did not reveal any aberration (19).

DISCUSSION

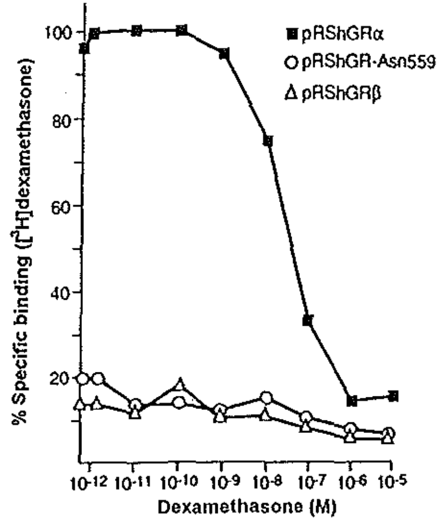
In this study, we report the development of a corticotroph adenoma in a patient with GGR. The patient presented initially with hypokalemia and hypertension. During treatment for GGR with pharmacological doses of dexamethasone, but years after the onset of this therapy, the patient developed signs and symptoms of Cushing's disease. Despite discontinuation of the glucocorticoid substitution, symptoms of Cushing's disease worsened. Eventually, the patient underwent bilateral adrenalectomy and subsequently, when a pituitary magnetic resonance imaging scan became positive for a tumor, he had TSS to remove a histologically verified corticotropinoma.

Pituitary tumors have not been described in syndromes of complete androgen or thyroid hormone resistance. Pituitary gonadotropinomas or thyrotropinomas are rare. Enlargement of the sella turcica has been noted in a few patients with longstanding Klinefelter's syndrome, and even if this represented gonadotropinoma formation, it would still be exceedingly rare (20).

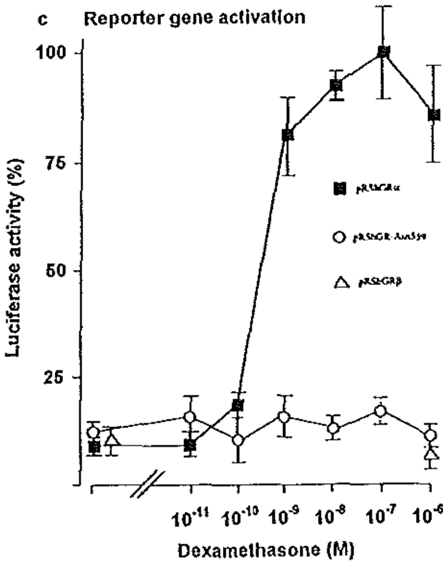
a Western blot



b Dexamethasone-binding studies



c Reporter gene activation



d Inhibition of hGRα-mediated gene transcription by hGR-Asn559

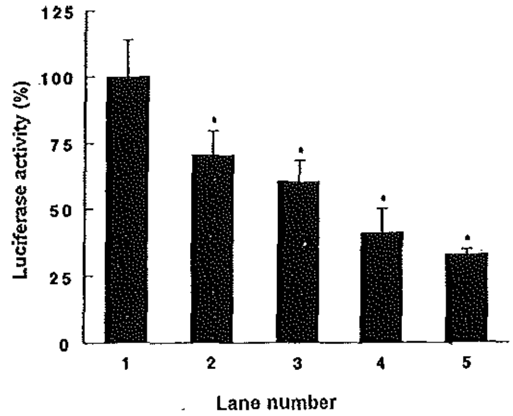


Figure 4. In vitro transfection studies. (a) Western blot analysis. Protein extracts of COS-7 cells transfected with the vectors encoding for the cDNA of the mutant GR-Asn559 (lane 1) or the wild-type GR (lane 2) were analyzed for their expression. Extracts of untransfected COS-7 cells (lane 3) and HeLa (lane 4) served as negative or positive controls, respectively. The 97-kD line indicates the expected size of the human GR. Both mutant GR-Asn559 (lane 1) and wild-type GR (lane 2) were expressed in the transfected COS-7 cells and migrated at the same distance as the naturally expressed GR of HeLa Cells (lane 4). Interestingly, two bands with different migration patterns were detected in the untransfected COS-7 cells which might represent nonfunctioning GR (lane 3). These bands were not detectable HeLa cells (lane 3). (b) Determination of relative binding affinities of wild-type hGRα, and mutant hGR-Asn559. COS-7 cells transfected with the wild type hGRα, mutant hGR-Asn559 or hGRβ expression vector, were incubated with 10 nM [^{3}H]dexamethasone in the presence of increasing concentrations of radioinert (10 - 10) dexamethasone. Each point represents the average of duplicate samples and is expressed as a percentage of the control incubates in the absence of a radioinert competitor. The mutant hGR-Asn559 was unable to bind ligand. Expression of wild-type GRα, and mutant hGR-Asn559 was confirmed by Western blot analysis, as shown in Figure 4. (c) Transcription activation of LTR-Luc by wild-type hGRα or mutant hGR-Asn559. COS-7 cells were transfected with the expression vector for the wild-type GR or the mutant GR-Asn559 and the reporter plasmid pLTRLuc. Control transfections were performed by co-transfecting the cells with the truncated GR isoform GRβ and the reporter plasmid. Transfected cells were exposed to increasing concentrations of dexamethasone. The ability of expressed wild-type GRα, mutant GR-Asn559, and the isoform GRβ to stimulate the luciferase (Luc) gene expression was assessed by measuring luciferase activity. Expression of wild-type GRα and mutant hGR-Asn559 was confirmed by Western blot analysis, as shown in Figure 4a. (d) Inhibition of hGRα-mediated gene transcription by hGR-Asn559. COS-7 cells were co-transfected with a constant quantity of wild-type hGRα (0.05 μg; lanes 1-5), and increasing amounts of the mutant hGR-Asn559 with 0.05 μg (lane 2), 0.1μg (lane 3), 0.15 μg (lane 4), and 0.25 μg (lane5). The amount of the reporter plasmid pLTR-Luc, as well as the amount of pSV-β-gal as a control of transfection efficacy, remained constant at 1.0 μg and 0.1 μg, respectively (lanes 1-5). The total amount of DNA transfected was kept constant by appropriate addition of plasmid pRSV-erbA, as previously described (33). Luciferase activity was assessed after stimulation by 10^{-7} M dexamethasone. Variations in transfection efficiency were normalized by galactosidase activity. A strong dexamesthasone-dependent dominant-negative effect of the mutant hGR on the wild-type GR was seen. Each column represents the mean ± SEM of one out of three experiments performed in triplicate. (*$p < 0.05$, ANOVA followed by Student t test with Bonferroni correction.)

One should note that in androgen-resistant patients, increased levels of estrogens, produced by peripheral aromatization of androgens, might play a role in suppressing adenoma formation. Similarly, patients with primary hypothyroidism rarely develop thyrotropinomas; this is true also for patients with untreated primary Addison's disease who rarely develop

corticotropinomas (21).

We have previously reported a somatic frame shift mutation of the GR gene in one of four patients with Nelson's syndrome (22). The mutation was restricted to pituitary tumor tissue, presumably leading to a decrease to half the number of receptors in the aggressively growing adenoma. Although we could not directly show a cause and effect between that mutation and corticotroph tumorigenesis, those data together with the data from this patient suggest that exposure of the corticotroph to enhanced stimulation by increased production of hypothalamic corticotropin releasing hormone and arginine-vasopressin or decreased primary sensitivity of the corticotroph to negative glucocorticoid feedback may lead to adenoma formation.

Analysis of the GR gene of our patient disclosed a novel heterozygous missense mutation (T to A) in exon 5 at cDNA position 1808 (17,18). This base change predicted the substitution of the neutral and hydrophobic amino acid Ileu by the neutral and polar Asn at codon 559, in the proximal region of the ligand-binding domain. The expression of both wildtype and mutant receptors in the patient's tissues was confirmed by sequencing the cDNA transcripts of the patient's lymphoblasts and fibroblasts, which were of both the wild and mutant type. Comparison with the corresponding positions in the rat (23), mouse (24), and guinea pig (25) GR revealed that Ileu was conserved in all of them, whereas the related amino acid Leu was present in the human mineralocorticoid (26), progesterone (27), and androgen receptor (28). These findings suggest that the presence of Ileu or Leu at this location has major structural and functional significance. Indeed, alignment of the three-dimensional structures of the ligand binding domains of the retinoid X receptor (RXR), human retinoic acid receptor (RAR), and thyroid hormone receptor (TR) with the unknown arrangement of the hormone binding domain of the GR demonstrated that Ileu 559 is at the beginning of α-helix 3, which is involved in forming the entrance of the ligand binding pocket (29-31).

Ligand-binding studies in peripheral lymphocytes of the patient, performed after TSS, and while he was on substitution therapy with 1.5 mg dexamethasone, revealed a normal affinity but only half of the expected binding sites. In addition, thymidine incorporation was not inhibited by dexamethasone in this patient. When GR function was tested in an *in vitro* cell system, the mutated GR-Asn559 was unable to bind ligand and, in addition, failed to activate gene transcription. The location of the mutation at the entrance of the putative ligand-binding

pocket suggests that the polar Asn prevents entry of the ligand or, alternatively, abolishes the entire cavity formation (31). Importantly, as shown in cotransfection studies, the mutated GR-Asn559 impaired the ability of the wildtype GR to induce gene transcription. This mutation, therefore, exhibited a strong dominant-negative effect on the function of the wild-type GR. This is the first mutation identified in the GR of a patient with GGR that impairs the action of the wild-type receptor. In addition to preventing ligand binding, this mutation might exert its dominant-negative effects by potentiating the effects of co-repressors or inhibititing the effects of co-activators of the physiological GR encoded by the normal allele (32,33).

Dominant-negative effects of mutated receptors were reported previously in the syndrome of thyroid hormone resistance (34). In addition, dominant inhibitory actions of physiologically expressed receptor isoforms were demonstrated for the human progesterone receptor-A (35) and, recently, for the GR isoform β (36). Actually, our patient's dominant-negative mutant receptor appears to be two- to three-fold more potent in its action than the natural GR-β isoform. Previously identified mutations of the GR in patients with GGR had no apparent dominant-negative effect on the wild-type receptor (4,6). Thus, heterozygotic patients were asymptomatic with only mild hypercortisolism (37,38).The degree of the biochemical abnormality in these heterozygotes was less than that of members of a family with a 50% reduction of GRs (11,39) owing to nonexpression of one of the GR alleles (5).

The GR mutation of the patient was not detected in either of his parents or in any of his five brothers and two sisters, which agreed with the lack of a clinical or biochemical picture of GGR in these subjects. Therefore, this mutation appeared to be a sporadic de novo germ-line mutation or a somatic mutation that occurred postzygotically during embryogenesis. That it was a de novo germ-line mutation was supported by the lack of mosaicism in the patient's tissues and by its presence in 50% of his sperm. Indeed, single-cell PCR amplification employing single fibroblasts, lymphoblasts, or sperm separated by micromanipulation revealed that the diploid cells had both the wild-type and the mutant alleles, whereas approximately half of the haploid sperm carried the genetic defect. The appearance of a de novo germ-line mutation is in accordance with the fact that the patient is the second youngest of eight children, whose conception took place when his father was 41 and his mother 38 years old.

Chronic activation of the HPA axis caused adrenal hyperplasia in the patient and, although not

proven by image analysis or pathological examination, might also have resulted in corticotroph hyperplasia, similar to that previously observed in patients with untreated Addison's disease (21). However, the patient also developed an ACTH-secreting pituitary adenoma, which was accompanied by further enlargement of the adrenal glands and development of frank Cushing's disease. Chronic exposure of the pituitary to increased concentrations of hypothalamic releasing factors might have rendered the corticotroph cells of this patient more susceptible to tumor-related genetic changes. Recently, accumulation of the tumor suppressor p53 -gene was shown in as many as 60% of spontaneously occurring corticotropinomas, suggesting that a somatic mutation of this gene had taken place within these tumors (9). Indeed, immunohistochemical staining of the chromophobe pituitary adenoma of our patient also revealed accumulation of p53 protein, indicating the presence of a putative oncogenic mutation in the p53 gene. Our attempt to amplify DNA extracted from microdissected pituitary tumor tissue to examine the potential presence of an oncogenic mutation in the p53 gene was noncontributory. This probably was due to the overnight formaldehyde fixation process of the pituitary tissue, a procedure often used by pathologists. Therefore, alteration responsible for p53 accumulation of the corticotropinoma remained unknown in the patient. Sequencing of exons 5-8 of the p53 gene using DNA from peripheral white blood cells revealed identity to the wild-type sequence. Consequently, an inherited germ-line p53 mutation, as in the Li-Fraumeni syndrome (40), was unlikely in this patient. This was in accordance with the medical history and clinical presentation of the family members, who were generally healthy.

Hence, our investigation provided further pathophysiological insights on glucocorticoid action in humans and on the potential role that the GR may play in pituitary corticotroph tumorigenesis. It appears that chronic exposure of the pituitary to increased concentrations of hypothalamic releasing factors and insufficient suppression by glucocorticoids might render the corticotroph cells susceptible to tumor-related genetic changes.

List of references

1. Chrousos GP, Vingerhoeds A, Brandon D et al. 1982 Primary cortisol resistance in man: A glucocorticoid receptor-mediated disease. J Clin Invest 69: 1 261-1269
2. Chrousos GP, Detera-Wadleigh SD, Karl M. 1993 Syndromes of glucocorticoid resistance. Ann Intern Med 119: 1113-1124
3. Orth D, Kovacs WJ, DeBold CR. 1992 The adrenal cortex. In: Wilson J.D. and Foster D.W. (eds.) Williams Textbook of Endocrinology. 8th ed. Philadelphia: Saunders, 489-619.
4. Hurley DM, Accili D, Stratakis CA. 1991 Point mutation causing a single amino acid substitution in the hormoe binding domain of the glucocorticoid receptor in familial glucocorticoid resistance. J Clin Invest 87: 680-686.
5. Karl M, Lambert S.W.J, Detera SD et al. 1993 Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. J Clin Endocrinol Metab. 78: 683-689.
6. Malchoff DM, Brufsky A, Reardon G et al. 1993 A mutation of the glucocorticoid receptor in primary cortisol resistance. J Clin Invest 91: 1918-1925.
7. Tsigos C, Papanicolaou D, Chrousos GP 1995 Advances in the diagnosis and treatment of Cushing's syndrome. Balliere's Clin Endocrinol Metab 9: 315-336.
8. Karl M, Chrousos GP. Experimental and clinical models of CRH-induced pituitary tumors. In: Melmed, S. (ed.) Oncogenesis and Moleculcar Biology of Pituitany Tumors. Front. Horm. Res. 20: 54-57 1, Basel: Karger.
9. Buckley N, Bates AS, Broome JC et al. 1994 P53 protein accumulates in Cushing's adenomas and invasive nonfunctional adenomas. J Clin Endocrinol Metab 79:1513-1516.
10. Williamson EA, Ince PG, Harrison D et al. 1995 G-protein mutations in human pituitary adrenocorticotrophic hormone-secreting adenomas. Eur J Clin Invest 25:128-131.
11. Lamberts SWJ, Koper JW, Biemond P et al. Cortisol receptor resistance: The variability of its clinical presentation and response to treatment. 1992 J Clin Endocrinol Metab 74:313-321.
12. Tomita M, Brandon DD, Chrousos GP et al. 1986 Glucocorticoid receptors in Epstein-Barr virus-transformed lymphocytes from patients with glucocorticoid resistance and glucocorticoid-resistant New World primate species. J Clin Endocrinol Metab. 62:1145-1154.
13. Eil C, Lippman ME, Loriaux DL 1980 A dispersed whole cell method for the determination of androgen receptors in human skin fibroblasts. Steroids 35: 289-304.
14. Felgner PL, Gader TR, Holm M et al. 1987 Lipofection: A highly efficient, lipid-mediaited DNA-transfection procedure. Proc Natl Acad Sci U.S.A. 84: 7413-7417.
15. Durrant I, Benge LCA, Sturrack C et al. 1990 The application of enhanced chemiluminescence to membrane-based nucleic acids detection. Biotechniques 8: 564-570.

16. Reincke M, Karl M, Travis W et al. 1994 P53 mutations in human adrenocortical neoplasms: Immunohistochemical and molecular studies. J. Clin. Endocrinol Metab. 78:790-794.

17. Encio IJ, and Detera-Wadleigh SD. 1991 The genomic structure of the human glucocorticoid receptor. J Biol Chem 266:7182-7188.

18. Hollenberg SM, Weinberger C, Ong ES et al. 1985 Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 318:635-641.

19. Levine AJ, Momand J, Finaly CA. 1991 The p53 inmour suppressor gene. Nature 351:453-456.

20. Thorner MO, Vance ML, Horvath E et al. 1992 The anterior pituitary. In: Wilson J.D. and Fostor D.W. (eds). Williains Textbook of Endocrinology,. 8th ed. Philadelphia: Saunders, 221-310.

21. Scheithauer BW, Kovacs K, Randall RV 1983 The pituitary gland in untreated Addison's disease. Arch Pathol Lab Med 107:484-487.

22. Karl M, von Wichert G, Kempter E et al. 1996 Nelson's syndrome associated with a somatic frame shift mutation in the glucocorticoid receptor gene. J Clin Endocrinol Metab 81:124-129.

23. Miesfeld R, Rusconi S, Godowski PJ et al. 1986 Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor-cDNA. Cell 46:389-399.

24. Danielsen M, Northrop JP, Ringold GM 1986 The mouse glucocorticoid receptor: Mapping of functional domains by cloning, sequencing and expression of wildtype and mutant receptor proteins. EMBO J 5:2513-2522.

25. Keightly MC, Fuller PJ. 1994 Unique sequences in the guinea pig glucocorticoid receptor induce constitutive transactivation and decrease steroid sensitivity. Mol Endocrinol 8:431-439.

26. Arriza JL, Weinberger C, Cerelli G et al. 1987 Cloning of the human mineralocorticold receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science 237:268-275.

27. Misrahi M, Atger M, d'Auriol L et al. 1987 Complete amino acid sequence of the human progesterone receptor deduced from cloned cDNA. Biochem Biophys Res Commun 143:740-748.

28. Lubahn DB, Joseph DR, Sar M t al. 1988 The human androgen receptor: Complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate. Mol Enodocrinol 2:1265-1275.

29. Renaud J-P, Rochel N, Ruff M et al. 1995 Crystal structure of the RAR-γ ligand-binding domain bound to all-trans retinoic acid. Nature 378:681-689.

30. Wagner RL, Apriletti JW, McGrath ME et al. 1995 A structural role for hormone in the thyroid hormone receptor. Nature 378:690-697.

31. Wurtz J-M, Bourguet W, Renaud J-P et al. 1996 A canonical structure for the ligand-binding domain of nuclear receptors. Nature Structural Biol 3:87-94.

32. Chen JD, Evans RM. 1995 A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377:454-457.

33. Onate SA, Tsai SY, Tsai M-J et al. 1995 Sequence and characterization of a cofactor for the steroid hormone receptor superfamily. Science 270:13541357.
34. Refetoff S, Weiss RE, Usala SJ 1993 The syndromes of resistance to thyroid hormone. Endocrine Rev 14: 348-399.
35. Vetego E, Shahbaz MM, Wen DX et al. 1993 Human progesterone receptor A form is a cell- and promoter specific repressor of human progesterone receptor B function. Mol Endocrinol 7: 244-1255.
36. Bamberger CM, Bamberger AM, Castro M et al 1995 Glucocorticoid receptor β, a potential endogenous inhibitor of glucocorticoid receptor action in man. J Clin Invest 95:2435-2441.
37. Chrousos GP, Loriaux DL, Brandon D et al. 1983 Primary cortisol resistance: A familial syndrome and an animal model. J Steroid Biochem 19:567 575.
38. Malchoff CD, Javier EC, Malchoff DM et al. 1990 Primary cortisol resistance presenting as isosexual precocity. J Clin Endocrinol Metab 70:503 507.
39. Lamberts SWJ, Poldermans D, Zweens M et al. 1986 Familial cortisol resistance: Differential diagnostic and therapeutic aspects. J Clin Endocrinol Metab 63:1328-1333.
40. Srivastava S, Zou Z, Pirollo K et al. 1990 Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature 348: 47-749.

CHAPTER 7

HUMAN ACTH SECRETING PITUITARY ADENOMAS SHOW FREQUENT LOSS OF HETEROZYGOSITY AT THE GLUCOCORTICOID RECEPTOR GENE LOCUS.

Nannette A.T.M. Huizenga, Pieter de Lange, Jan W. Koper, Richard N. Clayton, William E. Farrell, Aart Jan van der Lely, Albert O. Brinkmann, Frank H. de Jong and Steven W.J. Lamberts

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ABSTRACT

Objective: Corticotrophinomas are characterized by a relative resistance to the negative feedback action of cortisol on ACTH secretion. In this respect there is a similarity with the clinical syndrome of cortisol resistance (CR). Since CR can be caused by genetic abnormalities in the glucocorticoid receptor (GR) gene, we investigated whether the insensitivity of corticotrophinomas to cortisol is also caused by (de novo) mutations in the GR gene.

Methods: We screened the GR gene in leucocyte- and tumor DNA from 22 patients with Cushing's disease for mutations using PCR/SSCP-analysis. In a previous study, we have identified 5 polymorphisms in the GR gene in a normal population. These polymorphisms were used as markers for the possible occurrence of loss of heterozygosity (LOH) at the GR gene locus.

Results: Except for one silent point-mutation, we did not identify novel mutations in the GR gene in leucocytes or corticotrophinomas from these patients. Of the 22 patients, 18 were heterozygous for at least one of the polymorphisms. In six of these patients LOH had occurred in the tumor DNA. Of 21 patients examined for LOH on chr 11q13 only one, a corticotroph carcinoma, showed allelic deletion. As controls we studied 28 pituitary tumor of other subtypes (11 clinically non-functioning, 8 prolactinomas and 9 GH-producing adenomas) and found evidence for LOH in only one prolactinoma.

Conclusions: In 6 patients LOH was found at the GR gene locus (chr 5) in DNA derived from adenoma cells. Our observations indicate for the first time that LOH at the GR gene locus is a relatively frequent phenomenon in pituitary adenomas of patients with Cushing's disease. This might explain the relative resistance of the adenoma cells to the inhibitory feedback action of cortisol on ACTH secretion. The specificity of the GR LOH to corticotrophinomas supports this concept. Somatic mutations of the GR are not a frequent cause for relative cortisol resistance in these cells.

INTRODUCTION

Cushing's disease is caused by an ACTH-secreting pituitary adenoma arising from corticotrophic cells. The adenomas are characterized by a relative resistance of the tumor cells to the feedback inhibition by glucocorticoids (GCs) on ACTH secretion (1,2). The biological effects of cortisol (and other GCs) are mediated by the glucocorticoid receptor (GR), a member of the family of intracellular hormone receptors (3). These receptors are characterized by a three-domain structure: a carboxy- terminal ligand-binding domain with also transcriptional activity, a central DNA-binding domain and an N-terminal domain essential for transcription activation (3). GC effects can either be the result of DNA binding of the ligand-bound receptor, or interactions of the receptor with other proteins within the nucleus (4). Mutations in the hormone binding domain of the GR gene have been described as the cause of familial glucocorticoid resistance (CR) (5-8). In a recent study, Karl and co-workers (9) screened the GR gene in patients who developed Nelson's syndrome after bilateral adrenalectomy for Cushing's disease, and found a heterozygous somatic frame shift mutation in one out of 4 macroadenomas investigated.

In the present study, we analysed the GR gene in leucocyte and tumor DNA samples from 22 patients with Cushing's disease, using PCR/SSCP analysis, to see whether somatic mutations in the GR gene in tumor DNA were responsible for the relative cortisol resistance of the corticotrophic tumor cells. In a previous study (10), PCR/SSCP screening revealed 5 GR polymorphisms in a normal population. Sequence analysis led to the disclosure of point mutations in the PCR fragments containing the exons II, IV, V and IX, respectively. In two of these polymorphisms (summarised in table 1) the variant sequences occur with relatively high frequencies (exon V in 31.3% and exon IX in 13.7% of the investigated population). This allowed us to use these polymorphisms as markers for the possible occurrence of loss of heterozygosity (LOH) at the GR gene locus. The patients investigated were a subgroup of the patients studied by Buckley et al (11), who were tested for accumulation of p53. Since there are reports that the MEN-1 locus on chromosome 11q13 is potentially involved in the pathogenesis of all types of pituitary adenoma (12), we also examined this region of the genome for LOH in this unselected cohort of corticotrophinomas (13).

PATIENTS AND METHODS

All 22 patients included in the study had clinical features of Cushing's syndrome and biochemical evidence of excessive cortisol production. All failed to suppresss with low dose (4 x 0.5 mg) dexamethasone. With regard to the differential diagnosis different definitions of suppression with high dose (4 x 2 mg) dexamethasone suppression tests were used (e.g. 17-OHCS, urinary free cortisol and sometimes plasma cortisol values). The high dose dexamethasone test does not allow us to distinguish precisely the sensitivity to dexamethasone amongst this group of 22 patients with histologically proven ACTH-secreting pituitary adenomas.

Using PCR/SSCP we analysed the GR gene in tumor and leucocyte DNA samples from 22 patients with pituitary dependent Cushing's syndrome, all due to surgically and histologically defined adenomas. All patients were operated on by transsphenoidal selective adenomectomy. Tumor samples were fixed in 10% formalin and embedded in paraffin. Tumor DNA was extracted from slide material using standard methods (13), with the benefit that the DNA originates from histologically defined adenoma tissue, therefore being as homogenous as possible. Leucocyte DNA was extracted from peripheral leucocytes using standard methods. Tumors were divided into categories according to invasiveness, based on the patients' CT and MRI reports as described by Bates et al (13, 14). Grades 1 and 2 include microadenomas and macroadenomas without radiological evidence of invasion, respectively. Grade 3 are macroadenomas with local invasion. One patient (no 5) was previously described (15). She had a corticotrophic carcinoma, with intracerebral metastases demonstrated on MRI scan and at autopsy (Grade 4).

In order to investigate the specificity of the changes in the GR gene in corticotropinomas, we also studied 28 other pituitary adenomas (11 clinically non-functioning, 8 prolactinomas and 9 GH-secreting adenomas).

PCR/SSCP analysis of the glucocorticoid receptor gene.

Polymerase chain reaction (PCR) amplification of the GR gene was carried out using primers previously described by Koper et al (10). Because of the very limited quantity of the DNA

samples, PCR amplification was performed in two steps. The first amplification was performed in a total volume of 20 μL containing leucocyte or tumor DNA solution, 6 pmol of each oligonucleotide from 4 different primer pairs, 200 μM of each dNTP, 3mM $MgCl_2$, 0.5 U Ampli Taq (Perkin Elmer), 50 mM KCl and 10 mM Tris (pH 8.3). A 1:300 dilution of the primary PCR product was used as a template for a second PCR amplification under the same conditions, using one primer pair in each reaction. Single strand conformation polymorphism (SSCP) analysis of the PCR products was carried out using 0.5 x MDE polyacrylamide gels (J.T. Baker Chemicals, Deventer, the Netherlands) in the presence or absence of 5% glycerol at 4°C as described by Orita et al (16). Each SSCP gel contained samples from controls with the normal sequence and from controls with each of the five GR gene polymorphisms previously described by Koper et al (10). DNA fragments displaying an abnormal migration pattern during SSCP-analysis were amplified with the same primers for direct sequencing using a modified Sanger-dideoxynucleotide chain termination method, described in detail by Karl et al (7).

Loss of heterozygosity on chromosome 11

PCR amplification was performed using primers spanning the microsatellite repeat at the PYGM locus (11q13). Primer sequences for the amplification were 5'-GGAATGCTGATTTCCAGGTT-3', and 5'-CTGTCAGGTAGCAACTGACATC - 3' respectively. PCR reaction conditions were: Primer annealing at 50-54°C for 2 min, primer extension at 72°C for 2 min and denaturation at 94°C for 1 min and final extension at 72°C for 5 mins. Leucocyte and tumor PCR products were run adjacently on 8 - 10% non-denaturing polyacrylamide gels, fixed in 10% methanol/10% acetic acid and then incubated in 0.1% aqueous silver nitrate for 15mins. Following two brief washes in distilled water, products were visualised by development in an aqueous solution of 1.5% sodium hydroxide and 0.1% formaldehyde. Allelic loss is identified by a reduction in intensity of >80% or absence of one of the expected PCR products (13). Results were reviewed independently by two observers and allelic deletion only recorded if agreed by both. All samples in which LOH was identified underwent repeat PCR amplification and gel electrophoresis with identical results.

RESULTS

Age, sex and tumor grading of the 22 patients investigated are shown in Table 1. There were 20 female and two male patients, ages ranged from 13 to 58 years. There were three invasive tumors, four macroadenomas without invasion and 15 microadenomas. Patient 18 showed a novel SSCP pattern in exon VI of both tumor and leucocyte DNA. Direct sequence analysis showed that the abnormal pattern was the result of a silent heterozygous point mutation (GCA to GCG at codon 618, both coding for an alanine in the receptor protein, data not shown). None of the other DNA samples analysed revealed novel SSCP variants in the GR gene. The GR PCR fragments containing the polymorphisms as described earlier (10) served as markers for possible loss of heterozygosity (LOH) in the present study. The polymorphism in the PCR-fragment containing exon IV was not present in these 22 patients. In one patient (number 12), the polymorphism in PCR fragment exon II/1 was present in leucocyte DNA. In the tumor DNA of this patient, however, the polymorphism was absent. This suggests that in the tumor the variant allele was lost (Table 1). The polymorphism in the 3'-part of exon II (PCR fragment exon II/5) was present in 2 subjects (2 and 8). The polymorphism was seen in both leucocyte and tumor DNA, indicating that there was no LOH in this part of the GR gene in these two subjects (Table 1).

Figure 1 shows the SSCP patterns of the polymorphism in the PCR fragment containing exon V in controls (AA: homozygous for the normal allele; AB: heterozygous; BB: homozygous for the variant allele), together with the leucocyte and tumor SSCP patterns from several patients.

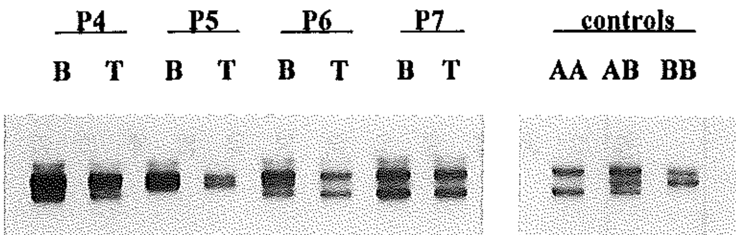

Figure 1. SSCP patterns of the polymorfhisms in the PCR fragment containing exon V in controls (AA, homozygous for the normal allele; AB, heterozygous; BB, homozygous for the variant allele), together with the leukocyte and tumor SSCP patterns from several patients. P6 revealed pattern AA in the tumor DNA, which in this case represents the presence of one A allele.

Table 1. Sex, age, tumor grading, possible loss of heterozygosity at the GR gene locus or chromosome11, and P53 staining in corticotropinomas from 22 patients withpituitary dependent Cushing's disease.

				Glucocorticoid receptor gene PCR fragment					
patient no	**sex**	**age**	**grade**	**Exon II/1**	**Exon II/5**	**Exon V**	**Exon IX**	**chrom 11**	**P53**
1	F	15	1	NI	NI	retained	retained	retained	nuc C
2	F	50	2	NI	retained	retained	retained	retained	nuc D
3	F	44	1	NI	NI	retained	retained	retained	nuc B
4	F	60	1	NI	NI	retained	retained	retained	neg
5	M	39	3 /4	NI	NI	NI	retained	**LOH**	cyt D
6	F	50	2	NI	NI	**LOH**	retained	retained	neg
7	F	52	1	NI	NI	NI	NI	retained	neg
8	F	40	3	NI	retained	NI	NI	retained	neg
9	F	28	2	NI	NI	**LOH**	**LOH**	retained	nucB
10	F	48	1	NI	NI	**LOH**	retained	retained	nuc D
11	F	13	1	NI	NI	retained	NI	retained	ND
12	F	25	1	**LOH**	NI	**LOH**	NI	retained	nuc C
13	F	57	3	NI	NI	NI	NI	retained	neg

14	F	33	1	NI	NI	retained	NI	retained	neg
15	F	58	1	NI	NI	NI	NI	retained	neg
16	F	37	2	NI	NI	retained	NI	retained	nuc A
17	F	22	1	NI	NI	retained	NI	retained	nuc A
18	M	28	1	NI	NI	**LOH**	NI	retained	neg
19	F	26	1	NI	NI	NI	NI	ND	ND
20	F	32	1	NI	NI	retained	retained	retained	neg
21	F		1	NI	NI	retained	NI	retained	neg
22	F	23	1	NI	NI	**LOH**	**LOH**	retained	cyt C

Grade: 1. Micro adenoma
2. Macroadenoma, no invasion
3. Macroadenoma with invasion
4. Macroadenoma with intra/extracranial spread

LOH : loss of heterozygosity
NI : not informative
ND : not determined
retained : informative, no LOH

P53: nuc: nuclear staining
cyt : cytoplasmic staining
A:75-100% B:50-75%
C: 25-50 % D: <25 %

Taking patient 6 as an example, a difference in the SSCP pattern in leucocyte and tumor PCR products from this patient was observed. Leucocyte DNA shows pattern AB (heterozygous), while tumor DNA reveals pattern AA, suggesting that the variant allele was not present in the tumor. A total of 16 patients showed SSCP pattern AB in leucocyte DNA, six of whom had an AA pattern in their tumor DNA, indicating that in 6 out of the 16 patients who were heterozygous for the polymorphism in exon V, LOH occurred at this specific part of the GR. Six other patients were homozygous in leucocyte DNA (5 of them showed pattern AA, one pattern BB), so the polymorphism was not informative in these patients with respect to LOH (Table 1).

With respect to PCR fragment exon IX, ten patients were heterozygous for the polymorphism (AB) in leucocyte DNA, two of whom showed LOH in the tumor (SSCP pattern AA, Table 1). The 12 other patients showed the homozygously normal pattern (AA) in leucocyte DNA not allowing a conclusion about a possible LOH in this part of the GR gene could be reached in these patients.

It can be concluded that six patients show LOH at the GR locus. Three of them in PCR fragment of exon V (patients 6, 10 and 18) and two of them in both exons V and IX (patients 9 and 22). Patient 12 shows LOH at the N-terminus of exon II and at exon V. Overall LOH was identified in 6 patients, heterozygosity was maintained in 12 patients in sofar as the polymorphisms were informative in these patients, and 4 patients were completely uninformative.

Tumor grading in patients in whose tumors LOH for the GR gene was found, showed that none of these tumors were invasive (Table 1). Only one patient (number 5) showed LOH on chromosome 11 at the PYGM locus. This subject had a malignant corticotroph carcinoma and has been reported in detail previously (14). All the other Cushing's adenomas examined (n=21) retained the PYGM allele at 11q13. In sofar as the data on LOH were informative, tumors showing LOH for the GR gene locus do not show LOH on chromosome 11. Similarly, for chromosome 11 LOH, heterozygosity in the informative parts of the GR gene was retained.

Of 11 clinically non-functioning pituitary adenomas, 6 were heterozygous for the polymorphism in exon V (showing the AB phenotype), none of these tumors showed LOH. Five were heterozygous for the polymorphism in exon IX, 4 of which also were heterozygous

for the exon V polymorphism. The tumors exhibited no LOH for exon IX. The polymorphisms II/I and II/5 were not present in the DNA from leucocytes and the adenomas.

Of 8 prolactinomas, 3 were heterozygous for the polymorphism in exon V; one of these tumors showed LOH (the AB pattern was changed into a BB pattern). Two of these tumors were also heterozygous for the exon IX polymorphism without showing LOH. The II/I and II/5 polymorphisms were not present in the DNA from leucocytes and the adenomas. Of 9 GH-producing adenomas, 2 were heterozygous for the exon V and IX polymorphisms but showed no LOH in the tumor, and 1 was heterozygous for the polymorphism in exon IX without showing LOH. No II/1 polymorphisms were present in the DNA from leucocytes and the adenomas, in one case the II/5 polymorphism was present in the leucocyte DNA and remained present in the adenoma DNA.

In conclusion in 23 of these 28 pituitary tumor types were heterozygous for at least one of the polymorphisms investigated, while LOH was detected only in one of them. Statistical analysis using the Fisher exact test on LOH in the GR-gene in corticotrophinomas versus pituitary adenomas of other origin showed a P-value of <0.02.

DISCUSSION

Hypercortisolism in Cushing's disease is caused by semiautonomous ACTH secretion by a pituitary corticotrophinoma, in most cases a microadenoma (17). Corticotrophinomas are characterized by a decreased sensitivity of the tumor cells to the negative feedback action of cortisol on ACTH secretion (1,2), which is mediated by the GR. Hormone-bound GRs regulate the expression of glucocorticoid responsive genes, by activating or repressing the transcription of glucocorticoid regulated genes by binding to "positive" and "negative" glucocorticoid response elements (GRE and nGRE) (18). The pro-opio-melanocortin (POMC) gene has been shown to contain an nGRE.

With respect to the diminished sensitivity to the inhibitory feedback action of cortisol, corticotrophinomas show similarity with the syndrome of cortisol resistance. This syndrome is characterized by increased serum concentrations of cortisol due to a decreased inhibitory feedback by cortisol on pituitary ACTH production, without clinical stigmata of Cushing's

syndrome. The increased ACTH production results in secondary overproduction of adrenal androgens and mineralocorticoids (19). It has been demonstrated that mutations in the hormone binding domain of the GR gene result in the syndrome of cortisol resistance in man (5-8). In the present study we investigated by PCR/SSCP analysis whether somatic mutations in the GR gene in ACTH-secreting pituitary adenoma cells might be responsible for the relative cortisol resistance observed in these tumors. In only one patient (number 18), a silent point mutation in exon VI was identified (both in leucocyte and tumor DNA), indicating that small de novo mutations in the GR gene in corticotrophic cells are not frequently involved in the relative cortisol resistance of ACTH secreting tumor cells in the pituitary. In a recent paper also no point mutations in the GR gene were found in 17 corticotrophinomas, while additional studies did not point at a differential relative expression of the two GR isoforms (20).
We have recently described five polymorphisms in the GR-gene (10). These polymorphisms were used as markers for the detection of LOH at the GR locus in these 22 corticotrophinomas. We found LOH in 6 out of the 18 tumors where at least one of the polymorphisms was informative. In four cases no informative polymorphisms were present. Our data indicate that the deletions that have occured may be relatively small. Thus, in tumors 6 and 10, LOH was found with respect to exon V, while for exon IX heterozygosity was retained. Moreover, in patient 18, LOH in the tumor was found in exon V, while the silent point-mutation in exon VI was present in both leucocyte and tumor DNA.For an adequate GC response, all parts of the GR are essential. Deletions such as those observed here will undoubtedly inactivate the gene product, if, indeed any is made. Moreover, GR-sensitivity has been shown to be gene-dosage dependent. Thus, Karl et al (7) described a family in which inactivation of one copy of the GR-gene, through a splice-site mutation, resulted in a reduced GR-number and in glucocorticoid resistance. Since the present polymorphisms are located within the gene itself and observations such as in tumors 6, 10 and 18 suggest that deletions might be relatively small it is unlikely that these results could have been reached using extragenic micro-satellite markers. LOH of the GR gene was found in 6 of the 18 informative corticotrophinomas studied, and was found in only one of the 23 informative non-functioning adenomas, prolactinomas and somatotrophinomas. This does imply some specificity of this LOH to corticotrophinomas making it more likely that it contributes to the glucocorticoid

resistance of this state.

From the data we obtained so far, it seems that adenomas showing LOH at the GR gene locus are non-invasive tumors which show no LOH on chromosome 11q13. Although this was the only allele examined, the finding that the most frequent allelic deletion in pituitary adenomas (12,13) was not detected here, further supports the tumor subtype specificity of GR LOH in corticotrophinomas.

It would have been interesting to see if the observed LOH at the GR gene locus is parallelled by reductions in the levels of GR-mRNA and GR protein. Unfortunately, the nature (paraffin embedded tissues) and quantity of the material makes extensive quantitative studies at the protein and mRNA level impossible. Other studies have demonstrated that homozygous point-mutations in the hormone binding domain of the GR gene cause the clinical syndrome of generalized cortisol resistance in man (5,6). The present data as well as the results from these previous studies suggest that there might be a decreased GR function in the pituitary tumors from the 6 patients in whom LOH was demonstrated. Consequently, these cells are relatively resistant to the inhibitory feedback action of cortisol on ACTH secretion, which might explain the increased ACTH production by these tumors.

Hypothesizing on the basis of the present data, the following model can be put forward: in a differentiated corticotrophic pituitary cell, a spontaneous genetic alteration, LOH at the GR locus, occurs. Consequently, this cell is relatively insensitive to cortisol. As the mutated corticotrophic cell is less sensitive to cortisol, this could be seen as a relative shortage of inhibitory hormones, and it provides an advantage for clonal selection, as previously proposed by Karl et al (8). A striking example of the enormous clonal expansion due to discontinuation of the physiological inhibitory feedback by cortisol is the Nelson tumor which is an aggressive, fast growing ACTH-secreting pituitary adenoma. Nelson tumors appear in 10-30% of patients who have previously undergone bilateral adrenalectomy for Cushing's disease. In a recent study by Karl et al (9), a somatic frame shift mutation in the GR gene was found in one out of 4 Nelson tumors investigated. It was concluded that the GR defect might have played a pathophysiological role in the tumorigenesis of this corticotrophinoma. Another example in favour of the theory that clonal expansion of a single genetically altered corticotrophic cell occurs as a result of diminished negative feedback by cortisol is given by a patient described by

Karl et al (8). This patient had cortisol resistance due to a dominant negative GR gene mutation. During the treatment for cortisol resistance with low doses of DEX, the patient developed signs and symptoms of Cushing's disease. Despite discontinuation of the DEX treatment, the symptoms of Cushing's disease progressed. Eventually, the patient underwent bilateral adrenalectomy, and subsequently, when a pituitary magnetic resonance scan became positive for a tumor, a corticotrophinoma was removed. It was suggested that in this patient the exposure of the corticotroph to enhance stimulation by increased production of hypothalamic corticotropin releasing hormone and arginine vasopressine or a decreased sensitivity of the corticotroph to the negative feedback by cortisol may have led to adenoma formation.

From the data presented here it can be concluded that simple somatic point mutations in the GR gene in corticotrophinomas do not seem to be the cause of the relative cortisol resistance in these tumor cells. However, we demonstrate for the first time that there is a frequent loss of heterozygosity at the GR gene locus in ACTH secreting pituitary adenomas. As deletions of (part of) the GR-gene leads to glucocorticoid resistance, this is a possible explanation for their relative resistance to the inhibitory feedback of cortisol on the ACTH secretion. Furthermore LOH at the GR gene locus may play a pathophysiological role in the initiation of corticotrophinoma formation, although our data are insufficient to relate this to the size of the tumor.

List of references

1. Liddle GW. 1960 Tests of pituitary-adrenal suppressibility in the diagnosis of Cushing's syndrome. J Clin Enocrinol and Metab. 20:1539-1560.
2. James VHT, Landon J , Wynn V. 1965 Oral and intravenous suppression tests in the diagnosis of Cushing's syndrome. J Clin Endocrinol and Metab. 33:515-524.
3. Giguere V, Hollenberg SM, Rosenfeld MG et al. 1986 Functional domains of the human glucocorticoid receptor. Cell. 46:645-652.
4. Wright AP, Zilliacus J, McEwan IJ et al. 1993 Structure and function of the glucocorticoid receptor. J Steroid Biochem Mol Biol. 47:11-19.
5. Hurley DM, Accili D, Stratakis CA et al. 1991 Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucocorticoid resistance. J Clin Invest. 87:680-686.
6. Malchoff DM, Brufsky A, Reardon G et al. 1993 A mutation of the glucocorticoid receptor in primary cortisol resistance. J Clin Invest. 91:1918-1925.
7. Karl M, Lamberts SW, Detera-Wadleigh SD et al. 1993 Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. J Clin Endocrinol Metab. 76:683-689.
8. Karl M, Lamberts SWJ, Koper JW et al. 1996 Cushing's disease preceded by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proc Ass Am Phys. 108:296-307.
9. Karl M, Von Wichert G, Kempter E et al. 1996 Nelson's syndrome associated with a somatic frame shift mutation in the glucocorticoid receptor gene. J Clin Endocrinol Metab. 81:124-129
10. Koper JW, Stolk RP, de Lange P et al. 1977 Lack of association between five polymrphisms in the glucocorticoid receptor gene and glucocorticoid resistance. Human Genet. 99:663-668.
11. Buckley N, Bates AS, Broome JC et al. 1994 p53 Protein accumulates in Cushings adenomas and invasive non-functional adenomas. J Clin Endocrinol Metab. 79:1513-1516.
12. Boggild MD, Jenkinson S, Pistorello M et al. 1994 Molecular genetic studies of sporadic pituitary tumors. J Clin Endocrinol Metab. 78:387-392.
13. Bates AS, Farrell WE, Bicknell EJ et al. 1997 Allelic deletion in pituitary adenomas reflects aggressive biological activity and has potential value as a prognostic marker. J Clin Endocrinol and Metab. 82: 818-824.
14. Burke CW, Adams CBT, Esiri MM et al. 1990 Transsphenoidal surgery for Cushing's disease: does what is removed determine the endocrine outcome? Clin Endocrinol 33: 525-537.
15. Pearce SHS, Trump D, Wooding C et al. 1996. Loss of heterozygosity studies at the retinoblastoma and breast cancer susceptibility (BRCA2) loci in pituitary, parathyroid, pancreatic and carcinoid tumours. Clin Endocrinol 45: 195-200.

16. Orita M, Iwahara H, Kanazawa H et al. 1989 Detection of polymofphisms of human DNA by gel electroforesis as single strand conformation. Nath Acad Sci USA. 86:2766-2770.
17. Krieger DT. 1983 Physiopathology of Cushing's disease.Endocr Rev. 4:22-43.
18. Drouin J, Trifiro MA, Plante RK et al. 1989 Glucocorticoid receptor binding to a specific DNA sequence is required for hormone-dependent repression of pro-opiomelanocortin gene transcription. Mol Cell Biol 9:5305-5314. .
19. Lamberts SW, Koper JW, Biemond P et al. 1992 Cortisol receptor resistance: the variability of its clinical presentation and response to treatment. J Clin Endocrinol Metab. 74:313-32 1
20. Dahia PLM, Honneger J, Reincke M et al. 1997 Expression of glucocorticoid receptor gene isoforms in corticotropin secreting tumors. J Clin Endocrinol Metab. 82:1088-1093

CHAPTER 8

DECREASED LIGAND AFFINITY RATHER THAN GLUCOCORTICOID RECEPTOR DOWN-REGULATION IN PATIENTS WITH ENDOGENOUS CUSHING'S SYNDROME.

Nannette A.T.M. Huizenga, Wouter W. De Herder, Jan W. Koper, Pieter de Lange,
Aart Jan v.d. Lely, Albert O. Brinkmann, Frank H. de Jong and Steven W.J. Lamberts.

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ABSTRACT

Glucocorticoids (GCs) serve a variety of important functions throughout the body. The synthesis and secretion of GCs are under the strict influence of the hypothalamo-pituitary-adrenal axis (HPA-axis). The mechanisms of action of GCs are mediated by the intracellular glucocorticoid receptor (GR). Over the years, many studies have been performed concerning the regulation of GR expression by GC concentrations.

In the present study, we determined the characteristics of the GR in peripheral mononuclear blood leukocytes (PBML) from thirteen patients with endogenous Cushing's syndrome and fifteen controls, using a whole cell dexamethasone binding assay. Furthermore, cortisol concentrations were determined in order to investigate a possible relationship between serum cortisol levels and receptor characteristics.

There were no differences in mean receptor number between patients and controls. On the other hand, a significantly lower ligand affinity was identified in cells from patients with Cushing's syndrome compared to controls. A complete normalisation of the ligand affinity was observed after treatment, in the only patient tested in this respect, whereas the receptor number was not affected. In patients, there was a statistically significant negative correlation between cortisol concentrations and ligand affinity, which was not found in controls.

Receptor down-regulation does not occur in PBML from patients with endogenous Cushing's syndrome. On the other hand, there seems to be a diminished ligand affinity which possibly reflects receptor modification in response to exposure to the continuously high cortisol levels in patients with Cushing's syndrome. This assumption is substantiated by the fact that in one patient a normalisation of the ligand affinity after complete remission of the disease was seen.

INTRODUCTION

Glucocorticoids (GCs) serve a variety of important functions throughout the body. GCs affect metabolism by maintaining plasma glucose levels. They are important in the regulation of fat metabolism, mediate stress response, influence the immune and central nervous system and have numerous effects on development and differentiation (1). The regulation of serum GC concentrations is under the influence of the hypothalamo-pituitary-adrenal axis (HPA-axis) (2). Hypothalamic corticotropin releasing hormone (CRH) is transported to the pituitary, which, in response, secretes corticotrophin (ACTH) into the hypophysial portal system. The adrenal gland is stimulated by ACTH to synthesize and secrete cortisol. Cortisol in its turn exerts a negative feedback on both the hypothalamic and the pituitary level in order to complete a negative feedback loop. In this way, a perfect balance between cortisol requirement and cortisol secretion can be achieved. The HPA-axis is under the influence of many other systems. In cases of stress for example, the HPA-axis is activated, resulting in higher concentrations of GC (2).

GCs exert their effects via the cytoplasmic glucocorticoid receptor (GR), which is a member of the family of intracellular steroid hormone receptors to which receptors for vitamin D, retinoic acid and thyroid hormone belong as well (3, 4). The structural organisation of the GR is characterized by a short and highly conserved cysteine rich central region constituting the DNA binding domain, a relatively well conserved carboxy terminal domain which is important for both hormone binding and translocation, and a poorly conserved amino terminal region containing the transactivation domains responsible for gene activation (5). It is now well established that the ability of GCs to exert their biological effects requires the presence of a sufficient amount of intact receptor molecules (4, 6). There is evidence that GR undergo down-regulation after the exposure to ligand *in vitro*, in animals and men (7,8). This receptor down-regulation is supposed to be an additional form of negative feedback regulation of GC action, apart from the regulation of GC serum levels by the HPA-axis (6). Nevertheless, the mechanisms of possible receptor down-regulation are poorly understood, and many discrepancies in different studies were reported. Moreover, most studies investigating receptor down-regulation were performed *in vitro* or, when performed *in vivo*, used pharmacological

amounts of GCs. In the present study, we investigated GR characteristics in patients with endogenous Cushing's syndrome. The aim was to identify whether GR down-regulation in peripheral blood mononuclear leukocytes (PBML) from these patients with longterm hypercortisolism who lack a diurnal rhythm of serum cortisol concentrations does occur. We found no receptor down-regulation, but a statistically significant decrease in ligand affinity for the receptor, which appeared to be closely related to the serum cortisol concentrations. Furthermore, the ligand affinity returned to normal in a patient after successful treatment for Cushing's disease.

PATIENTS AND METHODS

Patients and control subjects.

Thirteen patients, seven females and six males, with clinical and biochemical Cushing's syndrome were included. In all patients 24 hours urinary cortisol excretion was above the upper limits of normal. Furthermore, they showed insufficient adrenal cortisol suppression in the overnight 1 mg dexamethasone suppression test, and diurnal rhythms of serum cortisol concentrations were absent in all patients. Nine of the patients had Cushing's disease, two had an adrenal cortisol producing carcinoma, one had ectopic ACTH secretion and one had an adrenal cortisol producing adenoma.

Control subjects were fourteen healty volunteers, eight females and six males, without Cushing's syndrome or any other endocrine disorder. None of the female volunteers was using oral anticonceptive drugs at the time of investigation.

Peripheral blood mononuclear cells.

40 ml of blood was drawn into heparinized tubes between 8.00h and 9.00h by vena puncture. PBMLs were isolated as described previously (9). The blood was diluted twofold with saline and layered over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). The PBML enriched interphase was isolated and washed twice with saline. The final cell pellet was resuspended in 15 ml RPMI-1640 medium (Gibco Europe, Breda, the Netherlands), containing 15 mM Hepes, 10% charcoal adsorbed fetal calf serum (Amstelstad/Flow, Zwanenburg, the Netherlands), 2

mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 1.5 mg/ml fungizone. The cells were incubated for 30 minutes at 37°C in a shaking waterbath in order to remove endogenous cortisol. The cell suspension was centrifuged and resuspended in 15 ml medium. This procedure was repeated twice more. Finally, the cells were resuspended at a density of 2.5-10 x 10^6 cells per ml in the medium.

Whole cell dexamethasone binding assay.

The whole cell dexamethasone binding assay was performed as described previously by Molijn et al (9). Briefly: incubation was started in a volume of 240 μl (0.5-2 x10^6 cells) containing ^{3}H-dexamethasone at concentrations of 1.3 to 40 nM without (total binding) and with (specific binding) a 400-fold excess of unlabelled dexamethasone. Two tubes whitout labeled dexamethasone were incubated under the same conditions for determination of cell number and viability at the end of the procedure. The tubes were incubated during 1 hour at 30 °C in a shaking water bath. The incubation was stopped by the addition of 2 ml cold saline, followed by centrifugation and two washing steps. Finally, the cells were resuspended in 250 μl medium. Radioactivity in 200 μl of this suspension was counted in a liquid scintillation counter. Specific binding was calculated by subtracting non-specific binding from total binding. Receptor number and ligand affinity ($1/K_d$) were calculated from the data using the method of Scatchard (10).

Cortisol determinations.

At the same time blood was drawn for the whole cell dexamethasone binding assay, extra blood was drawn for cortisol determinations. In the patients, two more blood samples were taken at 17.00h and 22.00h in order to investigate the circadian rhythm of cortisol concentrations. Patients were at basal rest during the day the samples were taken. Serum cortisol concentrations were determined using RIA-kits obtained from DPC (Los Angeles, CA). Intra- and interassay variations were below 8.0% and 9.5%.

Statistical analysis

The results for serum cortisol concentrations, number of receptors and K_d are reported as mean

± SEM. To asses the relationships between cortisol concentrations and number of receptors or K_d, linear regression analysis was used.

RESULTS

There was a statistically significant higher early morning serum cortisol concentration in patients with Cushing's syndrome compared to controls (Table 1). Although not all individual patients had early morning cortisol concentrations above the upper normal level (800 nmol/l), none of the patients with Cushing's syndrome had a diurnal rhythm of serum cortisol concentrations (data not shown).

Table 1. Differences in serum cortisol concentrations and cortisol receptor characteristics between Cushing patients (n=13) and controls (n=14).

	Cushing		Controls		
	Mean	**SEM**	**Mean**	**SEM**	**P-value**
serum cortisol (nmol/l)	822	64.5	382	37.6	<0.001*
number of receptors per cell	6339	417	6184	211	0.72
Kd (nmol/l)	17.4	1.9	9.3	0.5	<0.001*

Table 1 also shows that there were no differences in the number of receptors per cell between the two groups. On the other hand, there was a statistically significantly higher K_d in the patient group compared to controls, indicating a lower affinity of the receptor for its ligand

As shown in table 2 neither in the patients group nor in the control group, there was a statistically significant correlation between number of receptors per cell and serum cortisol concentrations. On the other hand, as shown in Figure 1, there was a significant positive correlation between K_d and serum cortisol concentrations (so a negative correlation between ligand affinity and serum cortisol concentrations) in patients with Cushing's disease, which was not present in the control group.

Table 2. Correlations between serum cortisol concentrations and cortisol receptor characteristics in controls (n=14) and patients with Cushing's syndrome (n=13)

	Cushing		**Controls**	
	r	**P-value**	*r*	**P-value**
number of receptors per cell	0.12	0.68	0.50	0.07
Kd (nmol/l)	0.59	0.03*	-0.02	0.95

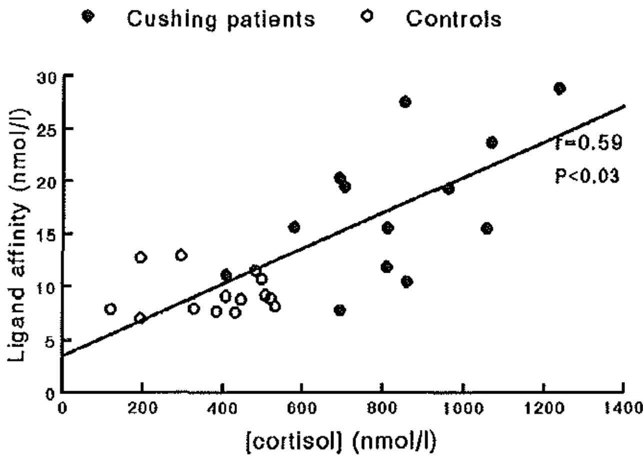

Figure 1. Relationship between serum cortisol concentrations and GR ligand affinity in PBMLs in 13 patients with Cushing's syndrome (black dots) and 14 healthy controls (open dots).

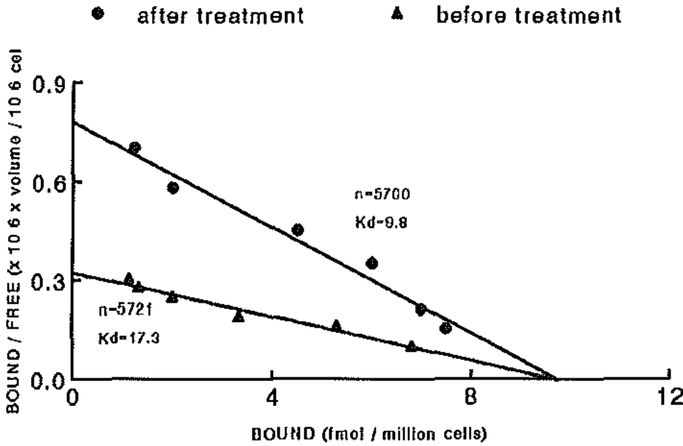

Figure 2. Whole cell dexamethasone binding assays of PBML in a patient with Cushing's disease before (triangles) and after (dots) succesful treatment.

Figure 2 shows the results of Scatchard analyses of the GR in one of the patients with pituitary dependent Cushing's disease before and after succesfull transsphenoidal adenomectomy of the ACTH-secreting microadenoma. Although the basal morning serum cortisol concentration after remission was not much lower than during disease (704 nmol/l versus 510 nmol/l, respectively), the serum cortisol concentrations after treatment showed a diurnal rhythm in contrast to those before treatment. The data in figure 2 indicate that the treatment did not influence the number of receptors per cell measured in PBML from this patient, but that the ligand affinity normalized after treatment.

DISCUSSION

To our knowledge, GR down-regulation in patients with endogenous Cushing's syndrome has never been investigated. Therefore, we investigated 13 patients with endogenous Cushing's syndrome with respect to GR characteristics. We found no receptor down-regulation, but a

significantly lower ligand affinity in patients compared to controls. A possible explanation could be that high concentrations of GC influence the outcome of the whole cell dexamethasone binding assay. However, a previous study by our group (9) especially investigated the effect of exposure to high cortisol concentrations on the number of receptors and the ligand affinity in this assay. It was shown that only 3.3% of endogenous cortisol remained specifically bound to the receptor. Moreover, incubation in the presence of high doses of cortisol affected both receptor number ("down-regulation") and ligand affinity ("decreased affinity"). In contrast, the results from this study show an isolated lowering in ligand affinity, whithout effects on receptor number. In addition, the absolute cortisol concentrations in the patients with Cushing's syndrome were much lower than the concentrations administered in the *in vitro* experiments performed by Molijn et al. Since the lowered ligand affinity does not seem to be caused by the presence of cortisol in the whole cell dexamethasone binding assay, these results possibly reflect receptor modification in response to the exposure to continuously high cortisol levels, as present in patients with Cushing's syndrome who lack a normal diurnal rhythm of serum cortisol concentrations. This assumption is substantiated by the fact that in one patient a normalisation of the ligand affinity was observed after complete remission of the disease.

The ability of GCs to act on target tissues requires the presence of intact and sufficient numbers of GRs (3). Many studies investigating GR numbers have been performed, on basis of the hypothesis that receptor down-regulation might be an additional form of negative feedback, protecting against the continued signal elicited by ligand in cases of hypercorticolism or other forms of GC excess (6). In several studies a direct correlation between GR number and the cell's sensitivity to GCs was found (11, 12). Furthermore, a receptor down-regulation in reaction to GC therapy was demonstrated in cell cultures and animals including humans (7,8). The possible mechanisms of this receptor down-regulation is poorly understood. There is evidence for an enhanced receptor degradation (13,14) *in vitro* but nothing is known about accelerated GR turnover *in vivo*. Furthermore, many investigations were performed on GR mRNA expression levels. There is evidence that GC treatment modulates GR expression in a number of tissues and cell types, and that down-regulation occurs at both transcriptional, post-transcriptional and/or post-translational levels (6, 13, 15). Moreover, most of the data available

at present concern *in vitro* studies or results obtained after administration of pharmacological amounts of exogenously GC.

Little is known about the physiological actions of GC on receptor number or affinity. An elegant example in this respect would be the syndrome of generalized GC resistance. GC resistance is a rare disease, in which an extreme insensitivity of the target tissues to GC action leads to a clinical syndrome characterized by signs and symptoms of secondary overproduction of adrenal androgens and mineralocorticoids. Up till now, in only four kindreds the molecular basis of the clinical syndrome has been elucidated. In three of these four (16-18), different mutations in the hormone binding domain of the GR gene were found, while in the fourth kindred (19) a heterozygous splice site deletion at the 3' boundary of exon 6 of the GR gene appeared to be the cause of the syndrome. In the latter kindred, the splice site deletion resulted in an instable mRNA with only half of the number of receptors on PBMLs as a final result. In all of these patients, the HPA-axis was set at a higher level, resulting in higher ACTH and cortisol concentrations. None of these patients showed any signs or symptoms mimiking an Addisonian clinical picture, meaning that there was a sufficient compensation of cortisol concentrations as a result of the increased HPA-activity. In none of the patients receptor up-regulation was demonstrated, especially not in the patient with only half of the number of receptors as a result of the splice site deletion in the GR gene. In these cases, it can be concluded that receptor up-regulation is not an additional feedback system in cases of relative cortisol shortage.

On the other hand, one might ask why people treated with GCs develop Cushing's syndrome; sufficient receptor down-regulation should protect a patient from developing side-effects of GC treatment. Nevertheless, many patients treated with GCs have serious adverse effects.

It can be concluded that there is no GR down-regulation in patients with endogenous Cushing' syndrome, but that a diminished ligand affinity of yet unknown cause might partially protect the cells from the high cortisol levels. Nevertheless, this protecting mechanism seems to be insufficient, because all patients showed clinical signs and sympoms of GC excess.

List of references

1. Munck A, Guyre PM. 1986 Glucocorticoid physiology, pharmacology and stress. In: Steroid Hormone Resistance: Mechanism and Clinical Aspects. Advances in Experimental Medicine and Biology. Vol.196. 81-96 Eds Chrousos GP, Loriaux DL, & Lipsett MB. New York: Plenum Press.
2. Chrousos GP. 1995 The Hypothalamic-pituitary-adrenal-axis and immune-mediated inflammation. New England J Med 322:1351-1362.
3. Bamberger CM, Schulte HM, Chrousos GP. 1995 Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocrine Rev 17:245-261.
4. Evans RM. 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889-892.
5. Giguere V, Hollenberg SM, Rosenfeld MG et al. 1985 Functional domains of the human glucocorticoid receptor. Cell 46:645-652.
6. Brönnegård M. 1996 Steroid receptor number, individual variation and downregulation by treatment. Am J Resp Crit Care Med 154:528-533.
7. Silvia CM, Powell-Oliver FE, Jewell CM et al. 1994 Regulation of the human glucocorticoid receptor by long-term and chronic treatment with glucocorticoids. Steroids 59:436-442.
8. Knutsson U, Stierna P, Carlstedt-Duke J et al. 1995 Effects of intranasal glucocorticoids on endogenous glucocorticoid peripheral and central function. J Endocrinol 144:301-310.
9. Molijn GJ, Koper JW, van Uffelen CJC et al. 1995 Temperature induced down-regulation of the glucocorticoid receptor in peripheral blood mononuclear leukocyte in patients with sepsis or septic shock. Clin Endocrinol 43:197-203.
10. Scatchard G. 1949. The attractions of proteins for small molecules and ions. Ann NY ac Science 51:660-672.
11. Gehring U, Mugele K, Ulrich J. 1984 Cellular receptor levels and glucocorticoid responsiveness of lymphoma cells. Mol Cell Endocrinol 36:107-112.
12. Vanderblid JN, Miesfeld R, Yamamoto KR. 1987 Intracellular receptor concentration limits glucocorticoid-dependent enhancer activity. Mol Endocrinol 1:68-76.
13. Dong Y, Poelinger L, Gustafsson JA et al. 1988 Regulation of glucocorticoid receptor expression: evidence for transcriptional and posttranslational mechanisms. Mol Endocrinol 2:1256-1264.
14. Okret S, Dong Y, Brönnegård M et al. 1991 Regulation of glucocorticoid receptor expression. Biochimie 73: 51-59
15. Burnstein KL, Bellingham DL, Jewell CM et al. 1991 Autoregulation of the glucocorticoid receptor gene expression. Steroids 56: 52-58
16. Hurley DM, Accili D, Stratakis CA et al. 1991 Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucocorticoid resistance. J Clin Invest 87:680-686.

17. Malchoff DM, Brufsky A, Reardon G et al. 1993 A mutation of the glucocorticoid receptor in primary cortisol resistance. J Clin Invest 91:918-1925.
18. Karl M, Lamberts SWJ, Koper JW et al. 1996 Cushing's disease preceded by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proc Ass Am Phys 108:296-307.
19. Karl M, Lamberts SWJ, Detera-Wadleigh SD Enico IJ et al. 1993 Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. J Clin Endocrinol Metab 76: 683-689.

CHAPTER 9

GENERAL DISCUSSION

In this thesis, studies on several aspects of the variability of the sensitivity to GCs in the normal population as well as in disease are described.GCs serve a variety of important functions in eukaryotic cells and tissues including mediation of the stress response, induction of gluconeogenesis in the liver, regulation of the fat metabolism, influences on the immune and central nervous systems as well as the development and function of numerous other organs. In man, the major circulating GC is cortisol. Under basal conditions, serum cortisol concentrations are under the influence of the HPA-axis. Hypothalamic CRH and AVP are secreted into the hypophysial portal system to stimulate the secretion of ACTH by the pituitary. ACTH stimulates the adrenal glands to synthesize and secrete cortisol. Cortisol in its turn exerts a negative feedback action on the secretion of both hypothalamic CRH and pituitary ACTH in order to complete a negative feedback loop. In this way, a perfect balance between cortisol requirement and cortisol secretion can be ascertained. The HPA-axis is under the influence of a circadian rhythm: because of the diurnal rhythm in the rate of ACTH secretion, plasma cortisol levels are higher in the morning than at night. Both negative feedback and diurnal rhythm may be overcome by stress which causes increases in CRH, AVP and ACTH output resulting in plasma cortisol levels considerably above those found under basal conditions.

Interperson variability versus personal stability of cortisol concentrations.

Large individual variations in both baseline and stimulated cortisol concentrations have been reported in the normal population (1). Little is known concerning the question whether cortisol levels are stable within normal subjects. The only studies available with this respect are twin studies, which indicate that genetic factors may be of importance (2, 3). The variation in baseline serum cortisol concentrations between persons is thought to be a function of many influences including the individual feedback sensitivity of the HPA-axis, circadian rhythm and episodic secretion, whereas cortisol binding globulin (CBG) levels also affect cortisol concentrations (3). In chapter 2 of this thesis, a study is described in which early morning cortisol concentrations were determined twice in a large group of healthy elderly individuals, at an interval of two and a half years. Serum cortisol concentrations showed a wide range between subjects but a strong individual stability, indicating that the HPA-axis is set at a stable

and reproducible set point for a given individual.

The role for the DST in the determination of differences in GC sensitivity

Another aspect of the study described in chapter 2 was to get an insight in the possible role of the 1 mg overnight DST in the determination of differences in sensitivity to GC in the normal population. It was shown that 96% of 216 healthy elderly individuals showed a post DEX cortisol concentration below the level of 50 nmol/l. However, 20 out of these 216 volunteers showed diminished adrenal suppression in reaction to 1 mg of DEX compared to the other individuals in the group (post DEX cortisol concentrations above 50 nmol/l). Nevertheless, only 5 subjects showed a post DEX cortisol concentration higher than 140 nmol/l, which is the normal cut-off point in our clinic when the test is used as a screening for endogenous Cushing's syndrome.

As mentioned, the DST is normally used in the screening procedure for patients suspected of endogenous Cushing's syndrome (4). It is known that in depressed patients an insufficient adrenal suppression in reaction to 1 mg DEX can occur as well (5). Also, many drugs may affect the outcome of the 1 mg DST (6) by altering DEX metabolism by the liver. For example anti-epileptic drugs induce hepatic enzyme activity, thereby increasing the metabolic clearance of GCs including DEX (7). In a study in psychiatric patients, abnormal results of the DST were closely associated with low plasma levels of DEX (8). In a study comparing patients with endogenous depression and normal controls, it was shown that serum DEX levels were significantly lower in non-suppressors than in suppressors (9). Therefore, in the study described in Chapter 2, DEX concentrations were determined in order to get an insight in the role of the DEX metabolism as a confounder in the interpretation of the results of the DST if used as an indicator for GC sensitivity. Only two out of the five subjects showing a lowered adrenal suppression in the 1 mg DST, showed a serum DEX concentration above a level of 2 nmol/l. The other three individuals showed serum DEX levels varying from 0.8 to 1.6 nmol/l, which were among the lowest levels measured in the whole group. One of them was using anti-epileptic drugs. Unfortunately, these three persons did not participate in the 0.25 mg DST (see below). The capacity of the liver to metabolize DEX appeared to be related to liver function parameters in males and to BMI in females. This influence of liver function disturbances, BMI

and several drugs on the metabolism of DEX might therefore contribute to the occurrence of false positive or false negative outcomes of the 1 mg DST in clinical endocrinology.
However, it is clear that the 1 mg DST is an accurate test to detect individuals with a lowered sensitivity to GCs. On the other hand, it became clear from these results that a dose of 1 mg of DEX is far too high to detect individuals with an increased sensitivity to GCs, or individuals with subtly diminished sensitivity.
Therefore, a 0.25 mg DST was performed two and a half years later in the same group of healthy volunteers. Post DEX cortisol concentrations showed a much broader range compared to those in the 1 mg DST. Furthermore, a group of individuals with an increased sensitivity to GCs could be identified using the results of the 0.25 mg DST (see below). Interestingly, it became clear that subjects with the highest baseline cortisol concentrations also showed the highest post 0.25 mg DEX cortisol concentrations. This indicates that in a given individual, there is a set-point of HPA-activity which is defined before as well as after a low dose of DEX. It can be concluded that the 0.25 mg DST seems to be a more sensitive indicator for subtle GC sensitivity differences, whereas the 1 mg DST still remains the golden standard for excessive sensitivity changes as in Cushing's disease or CR.

Effect of age on the HPA-axis activity

Over the years the effects of age and gender on the function of the HPA-axis axis have been extensively investigated. These studies, however, have resulted in contradictory results (10). Both circadian patterns of cortisol and ACTH secretion and random morning plasma cortisol concentrations were reported to be unaltered in the elderly (11-17). On the other hand, an age-related trend towards increased levels of evening plasma cortisol was reported (18), and in elderly males increased mean cortisol levels derived from 24-h frequent sampling were found, which were not present in elderly females (19). Furthermore, it was reported that aging in humans is not accompanied by a significant increase in spontaneous ACTH or cortisol secretion or by a decrease in HPA sensitivity to GC feedback suppression (15). In Chapter 2 of this thesis, we found no relation between age or gender with baseline cortisol concentrations or with post 1.00- or 0.25 mg DEX cortisol concentrations, indicating that neither age nor gender has any influence on the activity of the HPA-axis.

(Lack of) Association between GR gene polymorphisms and glucocorticoid sensitivity.

The mechanisms of action of steroid hormones, thyroid hormone, vitamin D and retinoic acid are mediated specific intracellular receptor proteins (20). Within this superfamily, the GC, mineralocorticoid, progesteron and androgen receptors constitute a subfamily (21). These receptor proteins regulate expression of specific target genes by ligand-dependent transcriptional activation, i.e. ligand-dependent activation of the receptor with subsequent dimer formation and DNA-binding. The structure of the GR consists of a poorly conserved amino-terminal region containing the transactivation domains responsible for gene activation, a highly conserved cysteine-rich central DNA-binding domain, and a relatively well conserved carboxy-terminal domain important for hormone binding. It is well established that the ability of GC to exert their biological effects *in vivo* require the presence of intact receptor molecules (21). CR in the cases that have been described previously (22-24), was found to be caused by mutations in the hormone binding domain of the GR gene. Thus in "classical CR" the GR seems to be the key modulator of GC sensitivity. The results from the studies described in Chapter 2 and 3 indicate that biochemical cortisol resistance is probably more common than was expected up till now: As mentioned before, 20 subjects out of the 216 individuals that participated in the 1 mg DST showed post DEX cortisol concentrations above the level of 50 nmol/l. Five of them showed post DEX cortisol concentrations above 140 nmol/l. However, none of these subjects showed signs or symptoms of clinical CR. Using PCR-SSCP analysis, five polymorphisms in the GR-gene were identified in this group of healthy elderly individuals. None of these polymorphisms was associated with clinical CR in this group of volunteers, nor was there an overall association with diminished adrenal suppression. However, one of the polymorphisms found in this screening procedure, the double point mutation in codons 22 and 23 (Figure 1), was found several times in patients with obvious clinical and biochemical CR as well. In two of the patients described in Chapter 5 of this thesis who showed clear clinical and biochemical pictures of CR, the double poin-mutation in codons 22 and 23 was found. From one of the patients, a young girl with severe steroid resistant asthma, we screened the family with respect to this polymorphism. It appeared that her healthy, unaffected grandmother, mother and brother were positive for the mutation as well. In the study described in Chapter 3, the double point-mutation was found in the individual with the highest post DEX cortisol

concentration (256 nmol/l). Moreover, two other "non-suppressors" (post DEX cortisol concentration were 110 and 241 nmol/l, respectively) were found to carry the mutation. However, eight other subjects out of the whole group of volunteers we tested, also showed this polymorphism, in combination with a normal cortisol suppression in reaction to 1 mg of DEX. In *in vitro* expression experiments, this polymorphism showed no alterations in the efficacy of the GR to activate transcription compared to the wild-type receptor (25). Possibly, in the non-suppressors we found in this study and in the patients with CR carrying this polymorphism, the mutation is linked to another mutation / polymorphism in or near the GR gene that was not detected using the PCR/SSCP approach in this study.
No study mentioned in this thesis so far has distinguished between the two different GR isoforms termed GRα and GRβ, that are formed by alternative splicing of the human GR pre-mRNA (26, 27). Recent studies by Bamberger et al (28) however, indicate that the distinction between the GRα and GRβ form might be more important than previously thought. These two protein isoforms have the first 727 amino acids in common, and, thus, both contain the transactivation and the DNA-binding domains. GRβ differs from GRα only in its C-terminus with replacement of the last 50 amino acids of the latter with a unique 15-amino acid sequence (26, 27). Because of this difference in the hormone binding domain, GRβ was thought to be unable to bind GCs and thus to be transcriptionally inactive (29). In a recent study by Bamberger et al (28), it was demonstrated that overexpression of GRβ could antagonize the effect of hormone activated GRα on a GRE gene *in vitro*. This dominant negative effect of GRβ on GRα mediated gene transcription was dose-dependent with overexpression of GRβ leading up to 90 % reduction of reporter gene activity. GRβ mRNA (28) and protein (30) were shown to be expressed in many tissues suggesting that this isoform might be physiological relavant (20). The existance of at least two GR isoforms that apparantly exert opposite effects makes the picture of GR mediated transcription more complex. It demands careful reevaluation of previous studies that did not distinguish between the two isoforms. The polymorphisms described in Chapter 3 in combination with the observed differences in GC sensitivity will be interesting to investigate with respect to these different GR isoforms.

Another polymorphism described in Chapter 3, an asparagine to serine change in codon 363 as

a result of an A to G point mutation in the N-terminal part of the GR gene (Figure 1), was found in subjects with a normal adrenal suppression in reaction to DEX , rather than in subjects with a diminished cortisol suppression. This prompted us to reevaluate the whole study population with respect to this polymorphism. We found that in 6% of the individuals tested, the polymorphism was present. This mutation has been described before in a family with CR (in combination with a splice-site deletion) and in SCLC cell lines DMS097 (31) and COR L24 (32). In *in vitro* experiments, no significant difference was observed in the capacity of this variant GR relative to the wild type to activate transcription of the CAT gene in an MMTV driven reporter construct, after transfection into COS-1 cells. Our own results using the more sensitive MMTV-LUC system also do not indicate a difference between N363S and wild type GR in this respect. Apart from transcriptional activation, GR is also involved in transcriptional repression of target genes. It does so either by binding directly to so-called negative glucocorticoid responsive elements (nGREs) (33, 34), or via interactions with factors such as activating protein-1 (AP-1) (35, 36) or nuclear factor (NF)-κB (37-39), thereby preventing these factors from activating their target genes. Co-transfection experiments have shown that the codon 363 variant has an unaltered capacity to repress target genes via nGREs or via the above mentioned transcription factors (40). Nevertheless, by comparing *in vivo* indicators for an increased sensitivity to GCs between subjects carrying this mutation and controls, it appeared that the individuals carrying the mutation showed a statistically significant increased adrenal suppression and insulin response in reaction to a low (0.25 mg) dose of DEX as indicators of short term CG effects. A dose of 1 mg of DEX seemed to be too high to detect the differences in cortisol suppression. The increased insulin response, however, became apparant after both DSTs. Furthermore, an increased BMI and a decreased BMD compared to age- and sex-related controls as indicators of long term GC effects were found in this group. The apparent discrepancy between these *in vivo* effects and our inability to confirm this increased sensitivity to GCs *in vitro* is likely to be due to the broad spectrum of regulatory mechanisms in which GCs and their receptors are involved. We showed earlier that GR mutations can effect transcriptional activation and -repression differently (25). It could well be that COS-1 cells lack the transcription factors determining the effect of the N363S mutation *in vivo* and that the presence of endogenous GR in these cells is an impediment for the study of

subtle effects of this mutation *in vitro*. Finally, our results may indicate that this polymorphism is linked to an additional genetic alteration, as was proposed for the double point mutation in codons 22 and 23. This yet unknown factor might interact with the GR protein to exert its effect. However, for diagnostic purposes, the recognition of a point mutation in the GR gene linked to a phenotypic difference may prove very useful, irrespective of the fact whether or not this may solely, in part or not at all be a consequence of altered GR function. A next step will be to investigate whether the codon 363 polymorphism might predict an increased sensitivity to the development of early and/or serious side effects during therapy with GCs. If this would be true, we might have found an elegant way to predict the appearance of side effects during GC therapy.

(Lack of) Association between clinical CR and GR gene polymorphisms/point mutations.
In cases of the extremes of the spectrum of GC sensitivity, these differences in GC sensitivity may lead to clinical syndromes like cortisol resistance or the cortisol hyperreactivity syndrome. Ever since the first description 1976, only about 30 patients and (asymptomatic) familie members with CR have been described. Up till now, the molecular basis for CR has been described in three patients and affected family members (22-24). In all three cases, mutations in the hormone binding domain of the GR gene were responsible for the clinical manifestation of CR. Recent reports of a significant prevalence of possible abnormalities in the GR in patients attending the endocrine clinic for hypokalemia, hypertension, acne, hirsutism and menstrual disorders (40, 41), prompted us to carry out a thourough investigation with respect to GR protein and GR gene in many patients attending the endocrine clinic for a broad spectrum of complaints suggesting a possible CR. As described in Chapter 5, four of these patients showed, characteristics in the GR protein in PBML in concordance with biochemical CR, in combination with the suspicious clinical picture. Two of them showed a diminished ligand affinity, in one patient only half of the number of receptors in PBML became apparent and in two of the patients, an inability to inhibit mitogen induced cell proliferation by DEX in the *in vitro* bioassay was shown. Moreover, all patients showed an insufficient cortisol suppression in the 1 mg DST. Screening of the GR gene of these patients using PCR/SSCP analysis, however, revealed no new point mutations. As mentioned before, two of the patients described carried

the double point mutation at codon 22 and 23 that was also found in the general population screening described in Chapter 3. Afetr PCR/SSCP analysis, the whole ligand binding domain was reevaluated using sequence analysis, but again no alterations in the GR gene were found. It is clear that there is a discrepancy between the clinical and biochemical picture in these patients on one side, and the molecular characterization on the other. In order to investigate the GR gene more extensively, a next step will be to look at the transcriptional level by studying mRNA expression. These investigations are currently under study.

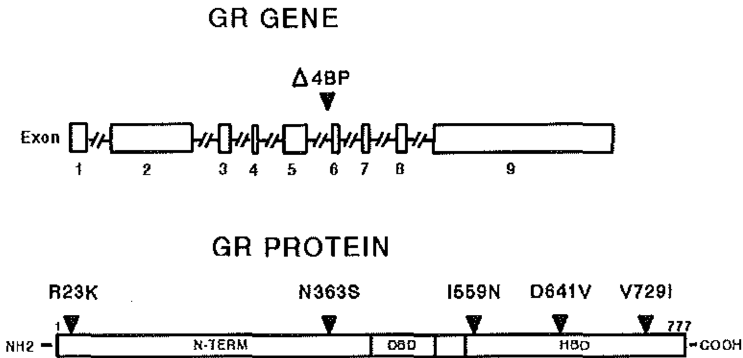

Figure 1. The GR gene with indicated all known mutations / delations in humans

Analysis of the GR gene of the patient described in Chapter 6 disclosed a novel point mutation (T to A) in exon 5 at cDNA position 1808 (26, 27). This base change predicted the substitution of the neutral and hydrophobic amino acid isoleucine by the neutral and polar asparagine at codon 559 in the proximal region of the hormone binding domain (Figure 1). By sequencing the cDNA transcripts of the patient's lymphoblasts and fibroblasts, the expression of both wild type and mutant recptor in the patients's tissues was confirmed. It became apparent that this mutant receptor had a dominant negative effect on the wild type receptor.

Apart from extreme cases of differences in GC sensitivity which give rise to real clinical syndromes, clinical observations show that there is a considerable variability in sensitivity to GC therapy as well. Many patients recieve GC therapy for a variety of non-endocrine diseases. Some patients show a quick and good response to GCs, while others need higher dosages causing only minor responses. One of the biggest disadvantages of GC therapy is the unpredictable occurence of side effects. As described above, many studies have been performed searching for alterations in the GR gene to explain these differences. Nevertheless, up till now, only four point mutations in the hormone binding domain of the GR gene have been described, all of which were accompanied with the syndrome of severe CR. Little is known about the role of GC metabolism. As described in Chapter 2, there was a very wide range between individuals in serum DEX concentrations following the administration of 1.00 or 0.25 mg of DEX. Moreover, there was a strong intra-individual correlation between the serum DEX concentration after 1.00 and 0.25 mg of DEX. As mentioned before, it became clear that there is a relationship between liver function parameters, BMI, and administration of several drugs on the one hand, and the eventual DEX concentration after oral administration of DEX on the other. Also from the literature it is known that a number of factors may alter the hepatic metabolism of GCs. The metabolism includes reduction-, oxidation-, hydroxylation- and conjugation-steps, all of which are catalyzed by different enzymes. It is known that hormonal factors (e.g. thyroid hormone affects 5α- and 5β reductase activity) (42), obesity (accelerared cortisol turnover) (43), several diseases (e.g. selctive loss of 5α- and 5β reductase activity in liver cirrhosis) (44) and many drugs (e.g. phenytoin and rifampicin accelerate GC turnover) (45, 46) influence GC metabolism. Therefore it is possible that endogenous factors like for example enzyme mutations could influence GC metabolism. It might well be that the observed differences in sensitivity to GC therapy are a reflection of differences in GC catabolism rather than a reflection of target tissue sensitivity to GCs. In order to investigate this assumption, we will have to study a group of healthy volunteers with respect to DEX catabolism e.g. after 1 mg of DEX, and compare DEX concentrations with the corresponding cortisol concentrations. In this way, one might get an insight in the role GC metabolism plays in the occurence of side effects.

GR, CR and Cushing's disease

In Chapter 6 the development of a corticotroph adenoma in a patient with CR was reported. During treatment for CR with high doses of DEX, but many years after the onset of the therapy, the patient developed signs and symptoms of Cushing's syndrome. Despite discontinuation of the GC therapy, the symptoms of Cushing's syndrome worsened. Eventually, the patient underwent bilateral adrenalectomy and subsequently, when a pituitary magnetic resonance imaging scan became positive for a tumor, he had transsphenoidal surgery in order to remove a histologically verified corticotropinoma.

Corticotropinomas are characterized by a decreased sensitivity of the tumor cells to the negative feedback action of cortisol on ACTH secretion (47, 48), which is mediated by the GR. Hormone-bound GRs regulate the expression of glucocorticoid responsive genes, by activating or repressing the transcription of glucocorticoid regulated genes after binding to "positive" and "negative" glucocorticoid response elements (GRE and nGRE) (34). The pro-opio-melanocortin (POMC) gene, of which the gene product, the POMC-molecule, is the precursor molecule of ACTH, contains an nGRE.

With respect to the diminished sensitivity to the inhibitory feedback action of cortisol, corticotropinomas show similarity with the syndrome of CR. This observation together with the development of Cushing's disease in our patient with CR suggested the hypothesis that mutations in the GR gene in corticotropinomas might be the cause of the diminished GC sensitivity in these tumors. From the study described in Chapter 7, it became apparent that somatic mutations do not seem to be the cause of the relative CR in corticotropinomas. On the other hand, the polymorphisms in the GR gene described in Chapter 3 could be used as markers for the detection of LOH at the GR gene locus. It was found that LOH at the GR gene locus was a common phenomenon in corticotropinomas, in contrast to other pituitary adenomas that were investigated as controls in this study. From the data presented here it can be concluded that simple somatic point mutations in the GR gene in corticotrophinomas do not seem to be the cause of the relative cortisol resistance in these tumor cells. However, we demonstrated for the first time that there is a frequent loss of heterozygosity at the GR gene locus in ACTH secreting pituitary adenomas. As deletions of (part of) the GR-gene leads to glucocorticoid resistance, this is a possible explanation for their relative resistance to the

inhibitory feedback of cortisol on the ACTH secretion. Consequently, these cells are relatively resistant to the inhibitory feedback action of cortisol on ACTH secretion, which might explain the increased ACTH production by these tumours.
Studies using X-chromosome inactivation analysis (49) have shown that the majority of pituitary adenomas are monoclonal, suggesting that genetic alteration in one single cell is the initiating step in the development of these tumours. Faglia (50) proposed a multistage model of tumorigenesis of pituitary adenomas involving initiation and promotion. Initiation implies pituitary cells undergoing spontaneous or acquired mutations, while promotion assumes the expansion of the mutated cell sustained by intrinsic or extrinsic promoting factors. If we apply this model to the data obtained from our study, the following hypothesis can be put forward: in a differentiated corticotrophic pituitary cell, a spontaneous genetic alteration, LOH at the GR locus, occurs. Consequently, this cell is relatively insensitive to cortisol. One of the possible promoting influences proposed by Faglia, is a defect in inhibitory hormones. As the mutated corticotrophic cell is less sensitive to cortisol, this could be seen as a relative shortage of inhibitory hormones, and it provides and advantage for clonal selection. A striking example of this is the report by Karl et al on a somatic frame shift mutation in the GR gene in one of four patients with a Nelson tumor (51). The mutation was restricted to the pituitary tumor tissue, presumably leading to a decrease to half the number of receptors in the aggressively growing adenoma. These data, together with the data from our patient described in Chapter 6 and the LOH studies from Chapter 7 suggest that decreased sensitivity of the corticotroph to the negative feedback of GCs may lead to adenoma formation. It can be concluded that GR defects might have played a pathogenetic role in the tumorigenesis of these corticotropinomas.

The last Chapter of this thesis describes the effect of hypercortisolism in endogenous Cushing's disease on GR number. Many studies investigating GR number have been performed, on basis of the hypothesis that receptor down-regulation might be an additional form of negative feedback, protecting against the continued signal elicited by the ligand in cases of hypercortisolism (52-58). In several studies a direct correlation between GR number and the cell's sensitivity to GCs was found (54, 55). Furthermore, a receptor down-regulation in reaction to GC therapy was demonstrated in cell cultures and animals including humans (52,

53). The possible mechanisms of this receptor down-regulation is poorly understood. There is evidence for an enhancement of receptor degradation (56, 57) *in vitro* but nothing is known about accelerated GR turnover *in vivo*. Furthermore, many investigations were performed on GR mRNA expression levels. There is evidence that GC treatment modulates GR expression in a number of tissues and cell types, and that down-regulation occurs at both transcriptional, post-transcriptional and/or post-translational levels (58). Nevertheless, we found no evidence for a receptor down-regulation on PBML from patients with endogenous Cushing's syndrome compared to controls. We did, however, find a significantly diminished ligand affinity of yet unknown cause that might partially protect the cells from the high cortisol levels. Nevertheless, this protecting mechanism seems to be inefficient, because all patients showed clinical signs and sympoms of GC excess.

The studies described in this thesis tried to clarify the differences in GC sensitivity in both disease and in the normal population, with a central role for the GR. Although several questions were answered, also many new questions came up. There are many discrepancies between GR alterations and clinical or biochemical CR. Moreover, in only a few cases of clinical CR, a GR defect proved to be the direct cause of the clinical syndrome. Further studies investigating the GR more extensively will have to clarify whether the GR indeed plays such an important role or, if other ways should be chosen in order to elucidate the remaining questions about differences in GC sensitivity.

List of references

1. Krieger DT, Allen W, Rizzo F et al.1971 Characterization of the normal temporal pattern of plasma corticosteroid levels. J Clin Endocrinol Metab. 32:266-284.
2. Maxwell JD, Boyle JA, Greig WR et al. 1969 Plasma corticosteroids in healthy twin pairs. J Med Genet. 6:294-297.
3. Meikle AW, Stringham JD, Woodward MG et al. 1988 Heritability of variation of plasma cortisol levels. Metabolism. 37:514-517.
4. Nugent CA, Nichols T, Tyler FH. 1965 Diagnosis of Cushing's syndrome. Single dose dexamethasone suppression test. Archives of Internal Medcine. 116:172-76.
5. Carroll BJ. 1984 Dexamethasone suppression test for depression. Adv Biochem Psychopharmacol. 39:179-188.
6. Guthrie S. 1991 The impact of dexamethasone pharmacokinetics on the DST: a review. Psychopharmacol Bull. 27:565-576.
7. Jubiz W, Meikle AW, Levinson RA et al. 1970 Effect of diphenylhydantoin on the metabolism of dexamethasone. N Engl J Med. 283:11-14.
8. Arana GW, Workman RJ , Baldessarini RJ. 1984 Association between low plasma levels of dexamethasone and elevated levels of cortisol in psychiatric patients given dexamethasone. Am J Psychiatry. 141:1619-1620.
9. Poland RE, Rubin RT, Lesser IM et al. 1987 Neuroendocrine aspects of primary endogenous depression. II. Serum dexamethasone concentrations and hypothalamic-pituitary-adrenal cortical activity as determinants of the dexamethasone suppression test response. Arch Gen Psychiatry. 44:790-795.
10. Seeman TE, Robbins RJ. 1994 Aging and hypothalamic-pituitary-adrenal response to challenge in humans. Endocr Rev. 15:233-260.
11. Colucci CF, D'Alessandro B, Bellastella A et al. 1975 Circadian rhythm of plasma cortisol in the aged (Cosinor method). Gerontol Clin. 17:89-95.
12. Touitou Y, Sulon J, Bogdan A et al. 1982 Adrenal circadian system in young and elderly human subjects: a comparative study. J Endocrinol. 93:201-210.
13. Blichert-Toft M. 1971 Assessment of serum corticotrophin concentration and its nyctohemeral rhythm in the aging. Gerontol Clin. 13:215-220.
14. Friedman M, Green MF, Sharland DE. 1969 Assessment of hypothalamic-pituitary-adrenal function in the geriatric age group. J Gerontol. 24:292-297.
15. Waltman C, Blackman MR, Chrousos GP et al 1991 Spontaneous and glucocorticoid-inhibited adrenocorticotropic hormone and cortisol secretion are similar in healthy young and old men. J Clin Endocrinol Metab. 73:495-502.

16. Vermeulen A, Deslypere JP, Schelfhout W et al. 1982 Adrenocortical function in old age: response to acute adrenocorticotropin stimulation. J Clin Endocrinol Metab. 54:187-191.

17. West CD, Brown H, Simons EL et al. 1961 Adrenocortical function and cortisol metabolism in old age. J Clin Endocrinol Metab. 21:1197-1207.

18. Pavlov EP, Harman SM, Chrousos GP et al. 1986 Responses of plasma adrenocorticotropin, cortisol, and dehydroepiandrosterone to ovine corticotropin-releasing hormone in healthy aging men. J Clin Endocrinol Metab. 62:767-772.

19. Zumoff B, Fukushima DK, Weitzman ED et al. 1974 The sex difference in plasma cortisol concentration in man. J Clin Endocrinol Metab. 39:805-808.

20. Bamberger MB, Schulte HM, Chrousos GP. 1996 Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocrine Rev 17:245-261.

21. Brönngård M, Stierna P, Marcus C. 1996 Glucocorticoid resistant syndromes- Molecular basis and clinical presentation. J. Neuroendocrinol 8:405-415.

22. Malchoff DM, Brufsky A, Reardon G et al. 1993 A mutation of the glucocorticoid receptor in primary cortisol resistance. J Clin Invest 91:1918-1925

23. Karl M, Lamberts SW, Detera-Wadleigh SD et al. 1993 Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. J Clin Endocrinol Metab 76:683-689

24. Hurley DM, Accili D, Stratakis CA et al. 1991 Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucocorticoid resistance. J Clin Invest 87:680-686

25. De Lange L, Koper JW, Huizenga NATM et al. 1997 Differential hormone-dependent transcriptional activation and -repression by naturally occurring human glucocorticoid receptor variants. Mol Endocrinol 11:1156-1164

26. Enico LJ. Detera-Wadleigh SD. 1991 The genomic structure of the human glucocorticoid receptor. J. Biol Chem. 266:7182-7188.

27. Hollenberg SM, Weinberger C, Ong ES et al. 1985 Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 318:635-641

28. Bamberger CM, Bamberger AM, De Castro M et al.1995 Glucocorticoid receptor ß, a potential endogenous inhibitor of glucocorticoid action in humans. J Clin Invest 95:2435-2441

29. Giguere V, Hollenberg SM, Rosenfeld MG et al. 1986 Functional domains of the human glucocorticoid receptor. Cell 46:645-652.

30 De Castro M, Elliot S, Kino T et al. 1996 The non-ligand binding ß-isoform of the human glucocorticoid receptor (hGRß): tissue levels, mechanism of action, and potential physiologic role. Mol Med 2:597-607

31. Gaitan D, DeBold CR, Turney MK et al. 1995 Glucocorticoid receptor structure and function in an adrenocorticotropin-secreting small cell lung cancer. Mol Endocrinol. 9:1193-1201.

32. Ray DW, Littlewood AC, Clark AJ et al. 1994 Human small cell lung cancer cell lines expressing the proopiomelanocortin gene have aberrant glucocorticoid receptor function. J Clin Invest. 93:1625-1630.
33. Sakai DD, Helms S, Carlstedt-Duke J et al. 1988 Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Genes Dev. 2:1144-1154.
34. Drouin J, Sun YL, Nemer M. 1989 Glucocorticoid repression of pro-opiomelanocortin gene transcription. J Steroid Biochem. 34:63-69.
35. Heck S, Kullmann M, Gast A et al. 1994 A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. Embo J. 13:4087-4095.
36. Liu W, Hillmann AG, Harmon JM. 1995 Hormone-independent repression of AP-1-inducible collagenase promoter activity by glucocorticoid receptors. Mol Cell Biol. 15:1005-1013.
37. Rebuffe-Scrive M, Krotkiewski M, Elfverson J et al. 1988 Muscle and adipose tissue morphology and metabolism in Cushing's syndrome. J Clin Endocrinol Metab. 67:1122-1128.
38. Rebuffe-Scrive M, Bronnegard M, Nilsson A, Eldh J et al. 1990 Steroid hormone receptors in human adipose tissues. J Clin Endocrinol Metab. 71:1215-1219.
39. Reid IR, Gluckman PD, Ibbertson HK. 1989 Insulin-like growth factor 1 and bone turnover in glucocorticoid-treated and control subjects. Clin Endocrinol. 30:347-353.
40. Lamberts SWJ, Koper JW, Biemond P et al. 1992 Cortisol receptor resistance: the variability of its clinical presentation and response to treatment. J Clin Endocrinol Metab. 74:313-321.
41. Werner S, Thoren M, Gustafsson JA et al. 1992 Glucocorticoid receptor abnormalities in fibroblasts from patients with idiopathic resistance to dexamethasone diagnosed when evaluated for adrenocortical disorders. J Clin Endocrinol Metab 75:1005-1009
42. McGuire JS, Tomkins GM. 1959 The effsts of thyroxine administration on the enzymatic reduction of delta-3-ketosteroids. J. Biol. Chem. 234:791-794.
43. Migeon CJ, Green OC, Eckert JP. 1963 Study of the adrenocortisac function in obesity. J Clin Endocrinol Metab 12:718-739.
44. Peterson RE. 1972 Metabolism of adrenocortical steroids. In: Christy NP Ed. The human adrenal cortex. New York: Harper & Row, 87-189.
45. Werk EE, McGee J, Sholiton LJ. 1964 Effect of diphenylhydantoin on cortisol metabolism in men. J. Clin. Invest. 43:1824-1853.
46. Burnsein S, Klaiber EL. 1965 Phenobarbital induced increase in 6ß-hydroxycortisol excretion: clue to its significance in human urine. J Clin Endocrinol Metab 25:293-296.
47. Liddle GW. 1960 Tests of pituitary-adrenal suppressibility in the diagnosis of Cushing's syndrome. J Clin Enocrinol and Metab. 20:1539-1560.
48. James VHT, Landon J , Wynn V. 1965 Oral and intravenous suppression tests in the diagnosis of Cushing's syndrome. J Clin Endocrinol and Metab. 33:515-524.

49. Herman V, Fagin J, Gonsky R et al. 1990 Clonal origin of pituitary adenomas J. Clin Endocrinol Metab 71:1427-1433.
50. Faglia G. 1993 Epidemiology and pathogenesis of pituitary adenomas. Acta Endocrinol 129 Suppl 1: 1-5.
51. Karl M, Von Wichert G, Kempter E et al. 1996 Nelson's syndrome associated with a somatic frame shift mutation in the glucocorticoid receptor gene. J Clin Endocrinol Metab. 81:124-129.
52. SilviaCM, Powell-Oliver FE, Jewell CM et al. 1994 Regulation of the human glucocorticoid receptor by long-term and chronic treatment with glucocorticoids. Steroids 59:436-442.
53. Knutsson U, Stierna P, Carlstedt-Duke J et al. 1995 Effects of intranasal glucocorticoids on endogenous glucocorticoid peripheral and central function. J Endocrinol 144:301-310.
54. Gehring U, Mugele K, Ulrich J. 1984 Cellular receptor levels and glucocorticoid responsiveness of lymphoma cells. Mol Cell Endocrinol 36:107-112.
55. Vanderblid JN, Miesfeld R, Yamamoto KR. 1987 Intracellular receptor concentration limits glucocorticoid-dependent enhancer activity. Mol Endocrinol 1:68-76.
56. Dong Y, Poelinger L, Gustafsson JA et al. 1988 Regulation of glucocorticoid receptor expression: evidence for transcriptional and posttranslational mechanisms. Mol Endocrinol 2:1256-1264.
57. Okret S, Dong Y, Brönnegård M, Gustafsson JA. 1991 Regulation of glucocorticoid receptor expression. Biochimie 73: 51-59
58. Burnstein KL, Bellingham DL, Jewell CM et al. 1991 Autoregulation of the glucocorticoid receptor gene expression. Steroids 56: 52-58

SUMMARY

GC hormones serve a variety of important functions throughout the body. In man, the major circulating GC is cortisol. Under basal conditions, serum cortisol concentrations are under the influence of the HPA-axis to keep a perfect balance between cortisol requirement and cortisol secretion. The mechanisms of action of steroid hormones, thyroid hormone, vitamin D and retinoic acid are mediated by specific intracellular receptor proteins.These receptor proteins regulate expression of specific target genes by ligand-dependent transcriptional activation, i.e. ligand-dependent activation of the receptor with subsequent dimer formation and DNA-binding. The structure of the GR consists of a poorly conserved amino-terminal region containing the transactivation domains responsible for gene activation, a highly conserved cysteine-rich central DNA-binding domain, and a relatively well conserved carboxy-terminal domain important for hormone binding. From clinical observations in patients who are treated with GC therapy for non-endocrine diseases, it is known that there is a considerable variation in the sensitivity to GC in the normal population. In extreme cases, this variation may lead to clinical syndromes like CR or cortisol hypersensitivity. In all cases of CR described so far, mutations in the hormone binding domain of the GR gene appeared to be the cause of the disease.

In this thesis, studies on several aspects of variability in GC sensitivity in the normal population as well as in disease are described.

Chapter 1 provides a general introduction. It opens with a short description of GC synthesis, secretion and effects. As the GR plays a central role in this thesis, an extensive description of the structure, function and mechanism of action of the GR is given. Finally, several aspects of differences in GC sensitivity in disease and in the normal population are discussed.

Chapter 2 describes a cohort study in 216 healthy elderly individuals. In these subjects, two DSTs with 1.00 and 0.25 mg of DEX, respectively, were performed at an interval of two and a half years. It appeared that serum cortisol concentrations showed a wide range between subjects, but a strong individual stability. Moreover, it became clear that subjects with the highest baseline cortisol concentrations also showed the highest post 0.25 mg DEX cortisol concentrations. This indicates that the HPA-axis is set at a stable and reproducible set point for a given individual. Furthermore, it was concluded that a dose of 1.00 mg of DEX is too high to

detect small differences in GC sensitivity.The 0.25 mg DST gives a much broader range in post DEX cortisol concentrations and should be used to detect small differences in GC sensitivity, especially when increased sensitivity is suspected.

Chapter 3 describes the general screening of the GR gene in a subgroup of the study-population described in Chapter 2. This screening revealed the existence of five polymorphisms in the GR gene in the normal population. None of these polymorphisms, however, was associated with clinical or biochemical CR. Nevertheless, it became clear that a point mutation at cDNA position 1220 resulting in an A to G change in codon 363 might be associated with increased sensitivity to GC. This prompted us to re-evaluate the whole study population of 216 healthy elderly individuals with respect to this polymorphism. The mutation was found in 6% of the normal population.

Chapter 4 describes that subjects carrying this polymorphism seem to be more sensitive to GC, both with respect to short-term effects of exogenously administered GCs (increased cortisol suppression and increased insulin response), and with respect to long-term effects of endogenous GCs (higher BMI, lower BMD). However, in *in vitro* assays, this increased sensitivity could not be proven. It could well be that COS-1 cells, which were used in these experiments lack the transcription factors determining the effect of the N363S mutation *in vivo* or that the presence of endogenous GR in these cells is an impediment for the study of subtle effects of this mutation *in vitro*. Another explanation might be that this polymorphism is linked to an additional genetic alteration. This yet unknown factor might interact with the GR protein to exert its effect. A next step will be to investigate whether the codon 363 polymorphism might predict an increased sensitivity to the development of early and/or serious side effects during therapy with GCs.

Recent reports of a significant prevalence of possible abnormalities in the GR in patients attending the endocrine clinic for hypokalemia, hypertension, acne, hirsutism and menstrual disorders, prompted us to carry out a thourough investigation with respect to the GR protein and the GR gene in many patients attending the endocrine clinic for a broad spectrum of complaints suggesting a possible CR.

In **Chapter 5** we describe 5 patients who showed a combination of a suspicious clinical

picture, together with functional characteristics of the GR protein in PBML which were in concordance with biochemical CR. Two of these patients showed a diminished ligand affinity, in one patient the number of receptors in PBML was reduced by 50%, and two of the patients showed an inability to inhibit mitogen induced cell proliferation in an *in vitro* bioassay. Moreover, all patients showed a decreased cortisol suppression in reaction to 1 mg of DEX. Screening the GR gene of these patients using PCR/SSCP analysis, however, revealed no new point mutations or any other GR gene alterations. A next step will be to investigate the GR at the RNA level.

Chapter 6 contains the patient history of a man who presented initially with hypokalemia and hypertension, which was caused by severe CR. During treatment for CR with high doses of DEX, he developed Cushing's disease. Analysis of the GR gene of the patient disclosed a novel point mutation (T to A) in exon 5 at cDNA position 1808. This base change predicted the substitution of the neutral and hydrophobic amino acids isoleucine by the neutral and polar asparagine at codon 559 in the proximal region of the hormone binding domain. By sequencing the cDNA transcripts of the patient's lymphoblasts and fibroblasts, the expression of both wild type and mutant recptor in the patients's tissues was confirmed. It became apparant that this mutant receptor had a dominant negative effect on the wild type receptor.

Chapter 7 describes a study concerning the role of the GR in Cushing's disease. Continuing along the line provided by Chapter 6, in combination with the observation that corticotropinomas show a strong resemblance to CR with respect to the diminished sensitivity to GCs, it was hypothesized that mutations in the GR gene in corticotropinomas might be the cause of the decreased GC sensitivity in these tumors. However, it became apparent that somatic mutations did not cause the relative CR in corticotropinomas. On the other hand, the polymorphisms in the GR gene described in Chapter 3 could be used as markers for the detection of LOH at the GR gene locus. It was found that LOH at the GR gene locus is a common phenomenon in corticotropinomas, in contrast to other pituitary adenomas that were investigated as controls in this study. Furthermore, it was concluded that the GR defect might have played a pathogenetic role in the tumorigenesis of these corticotropinomas.

Chapter 8 describes the effect of hypercortisolism in endogenous Cushing's disease on GR

number. Many studies investigating GR number have been performed, on basis of the hypothesis that receptor down-regulation might be an additional form of negative feedback, protecting the cell against the continued signal elicited by ligand in cases of hypercorticolism or other forms of GC excess. We found, however, a diminished ligand affinity rather than a receptor down-regulation in PBMLs from patients with endogenous Cushing's syndrome. This diminished ligand affinity of yet unknown cause might partially protect the cells from the high cortisol levels. Nevertheless, this protecting mechanism seems to be insufficient, because all patients showed clinical signs and sympoms of GC excess.

Chapter 9 is a general discussion of the results described in Chapters 2-8.

It is concluded that the studies described in Chapter 2-8 further clarified the role of the GR in the complex problem of differences in GC sensitivity in both the normal population and disease.

SAMENVATTING

Glucocorticoiden (GCn) zijn steroid hormonen die naast androgenen en mineralocorticoiden geproduceerd worden door de bijnierschors. Cortisol, de belangrijkste glucocorticoid bij de mens, vervult vele belangrijke functies in het lichaam zoals de regulatie van de glucose huishouding, het behouden van de homeostase en in mindere mate de regulatie van de water en zout huishouding. Daarnaast is cortisol een krachtige regulator van de immuunrespons. Onder normale omstandigheden wordt de concentratie van het serum cortisol gereguleerd door de hypothalamus-hypofyse-bijnier-as (HPA-as), waardoor een perfecte balans bestaat tussen de cortisol behoefte van het lichaam en de cortisol secretie door de bijnieren.
Cortisol oefent zijn werking uit door zich specifiek te binden aan de glucocorticoid receptor (GR), een eiwit dat tot expressie komt in vrijwel alle cellen van het lichaam. De GR is, evenals de receptoren voor andere steroid hormonen, zoals onder andere de geslachtshormonen, lid van de familie van intracellulaire ligand afhankelijke transcriptie regulatoren. Deze eiwitten zijn zijn structureel verwant en bevatten een aantal functionele domeinen, waaronder een N-terminaal transactivatie domein, een centraal gelegen DNA-bindend domein, en een C-terminaal hormoon bindend domein. De GR is in afwezigheid van hormoon in het cytoplasma aanwezig als onderdeel van een complex, dat naast het receptor eiwit onder meer bestaat uit "heat-shock" eiwitten. Binding van cortisol aan de receptor resulteert onder andere in dissociatie van dit complex, dimerisatie van hormoon gebonden receptoren en verplaatsing van dit hormoon-receptor complex naar de celkern. Het cortisol-GR complex is daar in staat zich te binden aan specifieke DNA gedeelten, de zogenaamde glucocorticoid respons elementen (GREs), die gelokaliseerd zijn in promotor gebieden van doelwit genen. Via deze DNA binding en interactie met andere eiwitten in de celkern reguleert het cortisol-GRcomplex de modulatie van de expressie van deze genen.
Het is bekend uit klinische observaties dat er binnen patiënten groepen die worden behandeld met GCn in verband met zeer uiteenlopende aandoeningen, er een aanzienlijke variatie bestaat in de gevoeligheid voor deze middelen. In extreme gevallen kunnen deze verschillen in gevoeligheid zelfs leiden tot op zichzelf staande ziektebeelden, zoals het syndoom van de gegeneraliseerde cortisol resistentie (CR), of het cortisol hypersensitiviteit syndroom. CR is een zeldzaam ziektebeeld dat wordt gekenmerkt door een gegeneraliseerde verminderde gevoeligheid voor cortisol, hetgeen gecompenseerd wordt door een hogere cortisol produktie

door de bijnieren ten gevolge van een hogere activiteit van de HPA-as. In alle gevallen van CR die tot nu toe in de literatuur beschreven zijn, bleek de verminderde gevoeligheid voor cortisol te berusten op mutaties in het hormoon bindende domein van de GR.

Dit proefschrift beschrijft een aantal studies over de verschillen in gevoeligheid voor cortisol zoals die gezien worden binnen de normale populatie. Daarnaast wordt aandacht besteed aan sensitiviteits verschillen voor GCn in bepaalde ziektebeelden. Hierbij wordt met name onderzocht welke rol de GR speelt in het ontstaan van deze verschillen in gevoeligheid.

Hoofdstuk 1 is een algemene inleiding over de synthese, secretie en werking van GCn. Omdat de GR een centrale rol speelt in dit proefschrift, wordt een uitgebreide beschrijving gegeven van de structuur, de functie en het werkingsmechanisme van de GR. Tenslotte worden een aantal aspecten van de variabiliteit in GC gevoeligheid beschreven, zowel binnen de normale populatie als bij bepaalde ziektebeelden.

Hoofdstuk 2 van dit proefschrift beschrijft een studie naar de variatie in gevoeligheid voor GCn binnen de normale oudere bevolking. In een groep van 216 gezonde proefpersonen van 55 jaar en ouder werden, met een interval van 2.5 jaar twee dexamethason (DEX) suppressie testen (DST) verricht, met respectievelijk 1.00 en 0.25 mg DEX. Er bleek een aanzienlijke variabiliteit te bestaan in de basale serum cortisol concentraties van de verschillende proefpersonen. Er was echter een duidelijke stabiliteit te zien in serum cortisol concentraties binnen proefpersonen over het interval van 2.5 jaar. Daarnaast bleek dat proefpersonen met de hoogste serum cortisol concentraties voor DEX, ook de hoogste cortisol concentraties vertoonden na de toediening van 0.25 mg DEX. Dit duidt erop dat de HPA-as van een persoon is afgesteld op een individueel, stabiel "setpoint".

Een andere conclusie uit dit hoofdstuk is dat een DST waarbij gebruik gemaakt wordt van 1.00 mg DEX geen goede test is voor het aantonen van verschillen in gevoeligheid voor GCn binnen de normale populatie; 96% van alle proefpersonen vertoonde een bijna totale onderdrukking van de HPA-as. Een dosering van 0.25 mg DEX toonde een veel grotere spreiding in post-DEX cortisol concentraties, omdat bij deze dosering de HPA-as niet volledig onderdrukt blijkt te worden. Deze test is dan ook te prefereren voor het detecteren van verschillen in cortisol gevoeligheid binnen de normale populatie, met name indien een

verhoogde gevoeligheid verwacht wordt.

Hoofdstuk 3 beschrijft de screening op mutaties in het gen dat codeert voor de GR in een deel van de populatie zoals die werd beschreven in hoofdstuk 2. Bij deze screening, waarbij gebruik werd gemaakt van PCR/SSCP-analyse, werden binnen de normale populatie vijf verschillende polymorphismen aangetoond in het GR gen. Echter, geen van deze polymorphismen bleek te zijn geassocieerd met een klinische of biochemische CR. Wel kon worden aangetoond dat één van de polymorphismen, veroorzaakt door een puntmutatie op cDNA positie 1220, resulterend in een A naar G verandering in codon 363 (N363S), geassocieerd lijkt met een verhoogde gevoeligheid voor GCn. Derhalve werd de gehele groep van 216 proefpersonen gescreend op het bestaan van dit polymorphisme. Bij 13 mensen uit de groep (6%) werd de mutatie gevonden.

In **Hoofdstuk 4** wordt beschreven dat personen die drager zijn van deze mutatie een verhoogde gevoeligheid voor GCn lijken te bezitten. Dit geldt zowel voor de korte termijn effecten van exogeen toegediende GCn (waardoor een toegenomen cortisol suppressie en insuline respons), als voor de lange termijn effecten van endogeen cortisol (hogere body mass index, lagere bot mineraal dichtheid). Echter, deze verhoogde gevoeligheid kon *in vitro*, waarbij gebruik werd gemaakt van transfectie studies in COS-1 cellen, niet worden aangetoond. Een mogelijke verklaring hiervoor is dat in COS-1 cellen de transcriptie factoren ontbreken die voor het effect van de N363S mutatie *in vivo* verantwoordelijk zijn, of dat de aanwezigheid van endogeen GR in de cellen het onmogelijk maakt subtiele effecten van de mutatie aan te tonen. Een andere verklaring kan zijn dat de N363S mutatie geassocieerd is met een andere, nog niet gedetermineerde genetische verandering. Nader onderzoek zal in de toekomst moeten aantonen of de N363S mutatie in verband gebracht kan worden met het ontstaan van snel optredende en/of ernstige bijwerkingen van GC therapie.

De afgelopen jaren is een aantal publicaties verschenen over mogelijke afwijkingen in de GR bij patiënten die gezien worden op de polikliniek endocrinologie met een hypokaliemie, een hypertensie, klachten van acné, hirsutisme en menstruatie stoornissen. Daarom werd bij een aantal van deze patiënten met klachten die zouden kunnen duiden op het bestaan van een mogelijke CR onderzoek verricht met betrekking tot het GR eiwit en het GR gen.

In **Hoofdstuk 5** worden vijf patiënten beschreven bij wie een klinisch beeld verdacht voor CR werd gezien, in combinatie met afwijkingen in het GR eiwit zoals bepaald bij bindingsstudies in perifere mononucleaire leukocyten. Bij twee van de patiënten werd een verminderde ligand binding gevonden. Leukocyten van een andere patiënt toonden slechts de helft van het normale aantal GRn. De leukocyten van de twee laatste patiënten toonden een onvermogen tot inhibitie van mitogeen geïnduceerde cel proliferatie in een *in vitro* bioassay. Daarnaast vertoonden alle patiënten een verminderde suppressie van cortisol in een 1 mg DST. Echter, bij analyse van het GR gen met behulp van PCR/SSCP/sequentie analyse kon bij geen van deze patiënten een afwijking worden aangetoond. Daarom zal bij volgend onderzoek bekeken worden of er afwijkingen zijn op RNA niveau.

In **Hoofdstuk 6** wordt de casus beschreven van een patiënt die zich aanvankelijk presenteerde met een zeer ernstige hypokaliemie en hypertensie, hetgeen bleek te berusten op CR. Tijdens behandeling voor CR met hoge doseringen GCn ontwikkelde hij de ziekte van Cushing. Analyse van het GR gen van deze patiënt toonde een punt mutatie (T naar A) in exon 5 op cDNA positie 1808. Deze verandering is verantwoordelijk voor de vervanging van het neutrale en hydrofobe aminozuur isoleucine door het neutrale en polaire asparagine in codon 559, aan het begin van het hormoon bindende domein van de GR. Met behulp van sequentie analyse van het cDNA uit lymphoblasten en fibroblasten van de patiënt konden zowel het wild type als de gemuteerde receptor worden aangetoond. Nadere *in vitro* analyse met co-transfectie studies bracht een dominant negatief effect van de gemuteerde receptor op het wild type receptor aan het licht, hetgeen een goede verklaring kan zijn voor het zeer ernstige klinische beeld van de patiënt.

Hoofdstuk 7 beschrijft een studie naar de rol die de GR speelt in de ziekte van Cushing. De casus zoals beschreven in hoofdstuk 6 heeft voor de eerste maal heeft aangetoond dat patiënten met een gegeneraliseerde CR de ziekte van Cushing kunnen ontwikkelen. Daarnaast vertonen ACTH producerende hypofyse adenomen een sterke gelijkenis met CR voor wat betreft de verminderde gevoeligheid voor cortisol. De werkhypothese was derhalve dat mutaties in het GR gen in ACTH producerende hypofyse adenomen de oorzaak zouden kunnen zijn van de verminderde gevoeligheid voor de negatieve feed back van cortisol in deze

tumoren. Het bleek echter dat somatische mutaties niet de oorzaak zijn voor de CR van ACTH producerende hypofyse adenomen. In deze studie werden tevens de polymorphismen, zoals beschreven in hoofdstuk 3, gebruikt als markers voor het optreden van "loss of heterozygosity" (LOH) in deze tumoren. Het werd duidelijk dat LOH ter plaatse van het GR gen een frequent voorkomend fenomeen is in ACTH producerende hypofyse adenomen. Dit in tegenstelling tot andere hypofyse tumoren die als controle preparaten werden gebruikt in deze studie. Er kan worden geconcludeerd dat het defect in de GR een mogelijke pathogenetische rol kan spelen in het ontstaan van deze tumoren.

Hoofdstuk 8 beschrijft een studie naar het effect van hypercortisolisme op het GR aantal in perifere leukocyten bij patiënten met een endogeen syndroom van Cushing. Er zijn reeds vele studies verricht naar GR aantal in geval van hypercortisolisme op basis van de hypothese dat receptor down regulatie een extra vorm van negatieve feedback kan bieden ter bescherming van de cel tegen de continue cortisol druk. Echter, deze studie heeft aangetoond dat in geval van hypercortisolisme in het syndroom van Cushing geen receptor down regulatie ontstaat, maar dat de affiniteit van de receptor voor het hormoon aanzienlijk daalt. Het is mogelijk dat deze verminderde hormoon affiniteit, waarvan het mechanisme nog onduidelijk is, de cel een extra bescherming tegen cortisol biedt. Als dit het geval zou zijn, dan zou dat beschermings mechanisme echter onvoldoende zijn, aangezien alle onderzochte patiënten duidelijke klinische symptomen van hypercortisolisme vertoonden.

Hoofdstuk 9 tenslotte is de algemene discussie over wat in hoofdstukken 2-8 beschreven is.

Concluderend wordt in dit proefschrift beschreven welke rol de GR speelt in de verschillen in gevoeligheid voor GCn zoals die gezien worden in de normale bevolking, in geval van cortisol resistentie en in het syndroom van Cushing.

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CURRICULUM VITAE

16 november 1966	Geboren te Breda.
1979-1985	Voorbereidend Wetenschappelijk Onderwijs, Mencia de Mendoza Lyceum, Breda.
1985-1991	Doctoraal Geneeskunde, Erasmus Universiteit Rotterdam. Afstudeer onderzoek: Het voorkomen van hepatitis B op Curaçao, Lands-Laboratorium, Curaçao.
1993	Arts-examen, Erasmus Universiteit Rotterdam.
1993-1997	Onderzoeker in opleiding, Laboratorium Inwendige Geneeskunde III, Erasmus Universiteit Rotterdam.
1997-heden	Assistent geneeskundige in opleiding tot internist, afdeling inwendige geneeskunde III, Academisch Ziekenhuis Rotterdam-Dijkzigt.