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Glucocorticoid receptor gene polymorphisms and glucocorticoid sensitivity of subdermal blood vessels and leukocytes

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ABSTRACT

A considerable variability in the sensitivity to glucocorticoids (GCs) exists between individuals and these differences have been implicated in the etiology of psychiatric diseases such as depression. Glucocorticoid receptor (GR) gene polymorphisms might account in part for variability in GC responsiveness. We assessed the association between four common GR gene (*NR3C1*) polymorphisms (ER22/23EK, N363S, *Bcl*I, 9beta) and markers of glucocorticoid sensitivity in two target tissues (subdermal blood vessels, peripheral leukocytes) in 206 healthy individuals. The *Bcl*I GG genotype group showed the least degree of skin blanching, reflecting a lower GC sensitivity of subdermal blood vessels ($p = .01$). No association between GR genotype and GC sensitivity of peripheral leukocytes was observed. In the same subjects we previously observed an association between GR genotype and GC sensitivity of the pituitary. Polymorphism of the GR gene might constitute a vulnerability or protection factor for stress related disorders and altered GC sensitivity.

1. Introduction

The hypothalamus–pituitary–adrenal (HPA) axis, one of the major stress response systems of the organism, critically depends on adequate regulation in order to prevent overactivity or insufficient activation. Cortisol, the major glucocorticoid (GC) in humans, is secreted upon activation of the HPA axis and has important negative feedback effects following HPA axis activation. Binding to the glucocorticoid receptor (GR) terminates the stress response via inhibition of CRH and ACTH secretion (de Kloet et al., 2005). Both hypo- and hyperactivity of the HPA axis and concurrent alterations in GC signaling have been associated with the etiology of psychosomatic and psychiatric disorders. Hypocortisolemic states have been reported in fibromyalgia, chronic fatigue syndrome (Heim et al., 2000), and PTSD (Yehuda et al., 2004) while in depressed patients HPA axis hyperactivity and relative GC resistance have been observed (Heuser et al., 2000; Pariante, 2003). In depression, both *in vivo* tests such as the dexamethasone suppression test (DST) and *in vitro* studies evaluating the effect of glucocorticoids on cellular function have shown reduced responses to GCs (reviewed in Pariante, 2004).

GCs have vital functions in development, energy metabolism and behavior and they modulate a number of physiological systems, including the immune system and the vasculature (Munck et al., 1984). Alterations in GC sensitivity in peripheral target tissues have been related to symptoms in some stress related disorders. In patients with PTSD, chronic fatigue syndrome or fibromyalgia, a relative hypocortisolemic state can be associated with a disinhibition of immune functions and may result in increased inflammatory responses due to impaired suppressive effects of low cortisol levels (Fries et al., 2005; Heim et al., 2000).

Within the normal population, a considerable variability in the sensitivity to glucocorticoids across individuals has been observed (Baxter and Rousseau, 1979; Huizenga et al., 1998a,b). Furthermore, it has been shown that GC sensitivity of one target tissue does not reflect GC sensitivity of other organs in patients receiving GC treatment (Corrigan et al., 1991, 1996; Sher et al., 1994) and in healthy individuals (Ebrecht et al., 2000; Vasiliadi et al., 2002).

GCs exert their effects mainly via the glucocorticoid receptor (GR), a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors. The GR mediates transactivation or repression of GC responsive genes by direct DNA binding or by protein–protein interactions with other transcription factors. The magnitude and efficacy of GC action depends, besides other factors, on characteristics of the GR. It has been hypothesized that genetic variations of the GR are associated with the observed variability in GC responsiveness. Rare clinical abnormalities in GC

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sensitivity, such as the generalized inherited GC resistance syndrome, have been linked to mutations of the GR (review see Charmandari et al., 2004). More common polymorphisms of the GR gene, namely the ER22/23EK (rs6189&6190), N363S (rs6195), the intronic *BclI* (rs41423247), and the A3669G variation in exon 9beta (rs6198) have been associated with variability in sensitivity to exogenous GCs in the general population (DeRijk et al., 2001; Huizenga et al., 1998a,b; Koper et al., 1997; Panarelli et al., 1998; Stevens et al., 2004; van Rossum et al., 2002, 2003; Wüst et al., 2004).

The aim of the present study was to determine the association between these four common GR gene polymorphisms and sensitivity to GCs of relevant target tissues. We recently observed a sex-specific and significant association between the 9beta A3669G variant and GC sensitivity of the pituitary. Male 9beta AG carriers displayed relative non-suppression of ACTH and salivary cortisol levels after administration of a low dose of dexamethasone (Kumsta et al., 2007). Here, we report on associations between GR gene variants and two further important targets of GCs, namely blood vessels and peripheral leukocytes. On blood vessels, GCs and mineralocorticoids interact with vascular receptors and indirectly influence vascular tone by increasing vascular sensitivity to noradrenalin. In the immune system GCs have complex effects including an inhibition of the release of the proinflammatory cytokine interleukin-6 (IL-6) from monocytes and macrophages. In our study we assessed (a) the intensity of skin blanching in response to topically applied GCs as a marker of GC sensitivity of subdermal blood vessels and (b) the inhibition of LPS-stimulated proinflammatory cytokine production by leukocytes after coincubation with increasing levels of dexamethasone, providing a marker of GC sensitivity of circulating leukocytes.

Given the pronounced target tissue specificity of GC effects along with the lack of intraindividual consistency of GC sensitivity across tissues, effects of GR SNPs might be differentially pronounced in different tissues. The simultaneous examination of four common SNPs of the GR gene – combined with our previous findings on dexamethasone suppression in the same population – can add important insights into the relevance of genetic variability of the GR for GC sensitivity.

2. Methods

2.1. Subjects and study design

An initial sample of 601 healthy subjects from a Trier-based community sample and from students of the University of Trier was genotyped for the GR variants under investigation. Subsequently, 206 subjects were selected according to their GR genotype, which resulted in a stratified sample of about equally sized comparison groups (see Section 3). The actually phenotyped study sample consisted of 118 females and 88 males (mean age 25.1 with standard error of mean (S.E.M.) of ±.26 years, BMI 24.2 ± .28). Participants were non-smokers and of central European descent. Only females using ethinyl-estradiol containing oral contraceptives (OC) were included in order to avoid a potential menstrual cycle phase dependent modulation of HPA axis regulation. Except for the use of OC, all subjects reported to be medication free. Before the first experimental session, the absence of acute or chronic diseases was confirmed in a medical exam. The protocol was approved by the ethics committee of the German Psychological Association, and written informed consent was obtained from all participants. The study presented here was part of a larger research project on the association between GR gene polymorphism and HPA axis regulation (Kumsta et al., 2007). For experiments on GC sensitivity, subjects reported to the laboratory two times. On the first day, a blood sample was drawn between 1400 and 1500 h to assess sensitivity in peripheral leukocytes. Thereafter, skin blanching was induced. Subjects reported the next day and the degree of skin blanching was rated.

2.2. Skin vasoconstriction assay

Solutions of beclomethasone dipropionate (Sigma) were prepared in ethanol/water (95:5, vol/vol) at concentrations of 0, 0.2, 1, 5, 10, and 20 μg/ml. Six circles with a 25-mm diameter were outlined on the volar aspect of the subject's forearm. Fifty microliters of each solution was applied to a corresponding circle between 1600 and 1700 h in randomized order. After evaporation of the ethanol, the forearm was covered with polyethylene vacuum foil. The occlusive dressing was removed the following day between 1400 and 1500 h. Thirty minutes later the intensity of the skin blanching was rated for each circle. The test areas were examined by two trained, blinded raters under standardized light conditions. Scores on a standardized rating scale ranged from 0 (no blanching), 1 (faint blanching), 2 (obvious blanching not extending the circle), to 3 (intense blanching extending over the margin of the circle). Interobserver agreement showed a reliability of $r = .81$. This method was previously used in different studies (Walker et al., 1997, 1998) and blanching score ratings have been validated against objective recordings with reflectance spectrophotometry (Noon et al., 1996). A sum score was computed for each subject's response to beclomethasone as the total of the six blanching scores.

2.3. Dexamethasone suppression of IL-6 production in peripheral leukocytes

Venous blood was collected in heparinized sterile tubes (Braun, Melsungen, Germany) and diluted 10:1 with saline. The blood was then coincubated with lipopolysaccharide (LPS, E. Coli, Difco, Augsburg, Germany) and six different concentrations of dexamethasone (Sigma, Deisenhofen, Germany) on a 24-well plate (Greiner, Nuertingen, Germany). Diluted whole blood (400 μl) was added to 50 μl of LPS and 50 μl of dexamethasone. The final concentrations were 30 ng/ml LPS and 0, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-5} M dexamethasone, respectively. After 6 h of incubation at 37 °C and 5% CO_2, the plates were centrifuged for 10 min at 2000 × *g* at 4 °C. The plasma supernatant was collected and stored at −80 °C until analysis.

2.4. Biochemical analyses

IL-6 was measured using ELISA employing the multiple antibody sandwich principle (BD Pharmingen, San Diego, CA, USA). Interassay and intraassay coefficients of variance were below 10 and 12%, respectively.

2.5. DNA extraction and genotyping

DNA was extracted from 10 ml peripheral venous blood following a standard NaCl salting out method according to the protocol of Miller et al. (1988). Genotyping was performed using the allelic discrimination technique, with custom designed primers and probes (Assay by Design service, Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands, primer and probe sequences available on request), using TaqMan Universal PCR master mix (Applied Biosystems). Reaction components and amplification parameters were based on the manufacturer's instructions.

2.6. Statistical analyses

IL-6 levels were log transformed to yield unskewed outcome variables. For analysis of cytokine production, General Linear Models (GLMs) were computed with the repeated measure *concentration of dexamethasone*. In order to reveal possible *sex* by *genotype* interactions, *sex* was also included as a predictor in the GLMs. Greenhouse–Geisser corrections were applied where appropriate, and only adjusted results are reported. Skin blanching scores with rising beclomethasone concentration and the sum score were compared using the Kruskal–Wallis. Post hoc analyses for comparisons of the sum scores between the genotype groups were performed with individual Mann–Whitney *U*-tests. All results shown are the mean ± S.E.M. Individual haplotype assignments for the four polymorphisms were determined using SNPHAP. Linkage disequilibrium among the four markers was estimated with D' and r^2 using Haploview.

3. Results

3.1. Genotyping

Genotyping of 601 subjects for the 4 SNPs revealed 5 haplotypes (see Fig. 1). Observed haplotype frequencies corresponded to those previously reported and all SNPs were in Hardy Weinberg Equilibrium. The haplotype with the highest frequency (45%) will be referred to as the 'Most Common Haplotype' (MCH, Haplotype 1). The GAGAGG to GAAAAG transition at position 22/23 (2.5%) always occurred together with the G allele in exon 9beta (Haplotype 2). In 13.5% of the subjects, A/G variation in exon 9beta (Haplotype 5) was also observed independently from ER22/23EK and the other SNPs. The base changes from A to G at position 363 (4%, Haplotype 3) and the intronic change from C to G (*BclI*, 35%, Haplotype 4) also occurred independently of the other