

EXPERIMENTAL STUDY

Genetic influence of an ACTH receptor promoter polymorphism on adrenal androgen secretion

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Abstract

Objective: Adrenocorticotrophic hormone (ACTH) is the primary secretagogue stimulating secretion of adrenal androgens (AA). Yet, genetic and environmental factors are assumed to play a determining role in the regulation of their biosynthesis and thus might explain the high variability of AA levels. Here we investigate the influence of an ACTH receptor promoter polymorphism affecting ACTH receptor gene transcription on ACTH-dependent dehydroepiandrosterone (DHEA) secretion.

Design: We recently reported a polymorphism within the transcription initiation site of the ACTH receptor gene promoter that alters the consensus sequence from CTC to CCC at –2 bp. This results in lower promoter activity *in vitro* and is associated with impaired cortisol response to ACTH stimulation *in vivo*. We now studied 14 normal, lean volunteers aged 20–35 years (eight CTC/CTC and six CCC/CCC carriers) in a 6-h ACTH stimulation test.

Methods: After overnight dexamethasone suppression, ACTH1-24 was administered continuously in each subject with hourly increasing doses (120–3840 ng/m² body surface area/h) within a 6-h period. On a separate day, baseline DHEA samples were collected.

Results: In the 6-h ACTH stimulation test, CTC/CTC carriers showed a significantly higher DHEA response than CCC/CCC carriers (area under the curve: 19 367 ± 2919 vs 11 098 ± 1241 nmol/l per min; $P < 0.04$, Mann–Whitney U-test). In contrast, baseline DHEA concentrations did not differ between groups.

Conclusion: These data demonstrate that genetic variations within the ACTH receptor promoter result in decreased DHEA secretion. Thus, we might have identified one of the genetic factors responsible for variation in ACTH-dependent DHEA secretion.

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Introduction

The adrenal androgens (AA) are mainly synthesized in the inner zona reticularis of the adrenal gland, and are secreted in higher amounts than both glucocorticoids and mineralocorticoids (1). Only lipophilic dehydroepiandrosterone (DHEA) can be converted to estrogens and androgens intracellularly, while dehydroepiandrosterone sulfate (DHEAS) is its hydrophilic storage form in the blood (2). In contrast to the other major adrenal steroids, cortisol and aldosterone, DHEA secretion follows a characteristic, age-related pattern throughout life. Gradually rising in prepuberty (the adrenarche), it reaches peak concentrations in the third decade of life, before declining again in old age. The concentrations at 70–80 years of age are only 20–30% of those in young adults (the adrenopause) (1–3). In addition to these age-related DHEA variations, a broad intersubject

variability has been reported (4, 5). While the regulation of cortisol and aldosterone biosynthesis is well-defined, the mechanisms of AA production remain elusive. As indicated by *in vitro* and *in vivo* results, androgen synthesis in the zona reticularis is mainly regulated by adrenocorticotrophic hormone (ACTH) (6, 7). ACTH, after binding to its G-protein-coupled receptor, activates the adenylate cyclase (cAMP) pathway with subsequent activation of protein kinase A. DHEA synthesis requires only two steroidogenic enzymes, cholesterol-side-chain cleavage enzyme (CYP11A) and 17 α -hydroxylase (CYP17), both of which are also utilized for cortisol synthesis in the zona fasciculata (8–10).

However, differential synthesis of cortisol and AA, as well as the high degree of interindividual variation of DHEA secretion, indicates that, besides ACTH, other, genetic or environmental, factors modulate AA biosynthesis (1, 8, 9, 11–16).

We hypothesize that observed differences in adrenal responsiveness to fixed doses of ACTH during a stimulation test (5) depend upon molecular differences within the ACTH-dependent pathway of steroid synthesis.

Our study shows that the recently described polymorphism within the ACTH receptor promoter transcription initiation site located at -2 bp (17) is associated with a reduced DHEA response to ACTH in healthy subjects. This adds new evidence of the control of adrenal androgen synthesis, and may indicate an important mechanism underlying the ACTH-dependent variation of AA concentrations.

Subjects and methods

Subjects

Genotyping, recruitment of the study population, and the test characteristics have been described previously (17). For genotyping, DNA from blood samples (DNA isolation by the QIAamp DNA Blood Mini Kit; Qiagen) from male donors received from the blood bank of the University Hospital Freiburg was amplified by PCR and restriction enzyme fragment polymorphism identified by *SacI* digestion. The upstream and the downstream primers were 5'-GCG CGC GCA GAT CTA AGC AGG AAC TTT CTG GG-3' and 5'-CGG GGT ACC GGG ATG ACA TTT ATT CAA GG-3', binding at positions -89 and $+23$ (with regard to the transcription initiation site) respectively. PCR was performed with 5 U Ampli Taq (PE Applied Biosystems, Darmstadt, Germany). Cycle conditions were 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 56°C) and extension (1 min at 72°C). As the CCC polymorphism disrupts an existing *SacI* restriction site, PCR products were digested for 3 h with *SacI* (20 U, New England Bio-Labs, Frankfurt, Germany). Fragments were separated on a 2% agarose gel, stained with ethidium bromide, and photographed.

Participants in the ACTH stimulation test were aged 20–35 years, with a normal BMI of 20–25 and a body surface area of roughly 2 m^2 without significant difference between the genotypes. Exclusion criteria were bronchial asthma; pituitary, renal or liver disease; acute or chronic stress; alcohol abuse; depressive disorders; current or former treatment with glucocorticoids; and known allergies to ACTH 1–24. The ethics committee of the University of Freiburg approved the study protocol, and all subjects gave written, informed consent.

Baseline DHEA and DHEAS concentrations

The baseline DHEA and DHEAS serum samples were collected at 1600 h. For this purpose, we used pooled baseline samples from a corticotropin-releasing hormone (CRH) stimulation test (-15 and 0 min) (17).

ACTH stimulation test

In this study, for DHEA measurements after ACTH stimulation, serum samples from eight homozygous CTC and six CCC carriers were used. The ACTH test was performed on a separate occasion at least 3 days after collection of the baseline samples. The applied ACTH infusion test was modified from a test described by Komindr *et al.* (18) and Slayden *et al.* (19).

All participants were pretreated with dexamethasone orally to suppress endogenous ACTH secretion: 2 mg at 2300 h the day before, and 1.5 mg at 0800 h, 1200 h and 1600 h respectively on the day of the ACTH stimulation test. Doses and timing of dexamethasone administration were determined empirically in preliminary tests. Low plasma ACTH levels (≤ 1.1 pmol/l) in all subjects indicated adequate suppression.

Participants arrived at 1545 h in the outpatient clinic after fasting since lunch (1300 h). At 1600 h, two i.v. lines were inserted, one for continuous ACTH infusion, doubled hourly ($120\text{--}3840\text{ ng/m}^2$ body surface area/h), and the other for collection of blood samples. As the cortisol response between CTC/CTC and CCC/CCC individuals was not different during hour 1 of the test (17), we selected the time period of 60–340 min for DHEA determination.

Hormone assay

All samples were centrifuged at 4000 r.p.m. for 10 min and stored at -20°C until assayed. All serum samples for DHEA were measured at the end of the study in one run. Serum DHEA was determined by a commercial radioimmunoassay (Diagnostic Systems Laboratories, Sinsheim, Germany). The cross-reactivity of the antibody was as follows: DHEAS, 0.02%; 4-androstene-3,17-dione, 0.46%; and testosterone, 0.03%. Intra-assay variabilities were 2.7–3.8%, and interassay variabilities 3.8–8.6%.

DHEAS samples were also measured at the end of the study in one run. Serum DHEAS was determined by a commercial chemiluminescence immunoassay (Nichols Advantage dehydroepiandrosteronsulfat test of Nichols Institute Diagnostics, Bad Vilbel, Germany). The intra-assay variabilities were 6.7–7.3% depending on different mean concentrations of DHEAS. The interassay variabilities were 6.0–10.0%.

Cross-reactivity of the antibody to DHEA, androstenedione, testosterone, estradiol, progesterone, hydrocortisone, estrone sulfate and aldosterone sulfate is not measurable; to estronsulfate, it was 0.3%, and to androsterone sulfate 0.4%.

Statistical analyses

All data are reported as mean \pm S.E.M. Statistical analysis was performed with StatView 5 (SAS Institute, Cary, NC, USA). The area under the concentration-time

curve (AUC) was calculated by means of trapezoidal integration at 60–340 min and expressed as nmol/l per min. Statistical comparisons were analyzed by the Mann–Whitney U-test for unpaired samples. Significance was defined as $P < 0.05$.

Results

Baseline DHEA and DHEAS concentrations

Baseline serum DHEA and DHEAS values were not affected by the polymorphism (DHEA: CTC/CTC 43.3 ± 5.2 nmol/l; CCC/CCC 46.1 ± 14.2 nmol/l (Fig. 1); DHEAS: CTC/CTC 11.1 ± 1.3 μ mol/l; CCC/CCC 10.7 ± 0.8 μ mol/l (data not shown)).

ACTH-stimulated DHEA and DHEAS concentrations

To investigate differences of DHEA levels after ACTH stimulation, a prolonged ACTH stimulation test was performed. DHEA responses in CTC/CTC subjects were higher than those in CCC/CCC subjects (Fig. 2A). At 60 min, DHEA concentrations were 25.0 ± 3.1 nmol/l in CTC/CTC and 14.9 ± 1.4 nmol/l in CCC/CCC subjects, and after 340 min 119.3 ± 14.2 nmol/l and 76.3 ± 6.2 nmol/l respectively. The integrated serum DHEA concentration was 43% higher in CTC/CTC subjects than in CCC/CCC subjects during the 60–340-min interval ($P < 0.04$, Mann–Whitney U-test) (Fig. 2B).

There was no difference of DHEAS levels in the ACTH stimulation test, probably because of the much longer half-life of DHEAS (data not shown).

Discussion

While the mechanisms of cortisol and aldosterone regulation have been characterized in some detail, the molecular basis of the synthesis of adrenal androgens is not well understood. In many aspects, AA synthesis is unique. For example, in interspecies comparison, significant secretion of AA is a characteristic feature

only of nonhuman primates and man. Thus, rodent studies are of limited value concerning AA regulation (9). Furthermore, while glucocorticoid and mineralocorticoid levels remain more or less stable throughout human life and are not sex-specific, AA levels change drastically during development and aging and show clear gender differences, with higher circulating levels in men than in women. Besides, in both genders, investigators have noted a broad, probably genetically determined variation in DHEA secretion. It has also been hypothesized that this heterogeneity in AA levels is due to a variable response of AA to ACTH stimulation (1, 5, 9).

In our study, subjects homozygous for the CCC polymorphism showed lower DHEA levels after ACTH stimulation than homozygous CTC subjects. Although a variety of factors may modulate AA levels, our data clearly support the hypothesis that – probably among other factors – there is a genetically determined ACTH-dependent variability of adrenal DHEA secretion. As we have demonstrated previously, the described polymorphism within the transcription initiation site of the ACTH receptor promoter results in lower promoter activity *in vitro* (17). In addition, the impaired cortisol response to ACTH as reported earlier is in line with a lower expression of the ACTH receptor in the adrenal zona fasciculata *in vivo* (17). The present set of data suggests that the observed ACTH receptor polymorphism also leads to a reduced number of binding sites within the zona reticularis, resulting in a blunted activation of the ACTH-dependent pathway and thus lower DHEA secretion after ACTH stimulation.

Discordances of AA and cortisol secretion by gender, aging and stress response indicate non-ACTH-dependent components of AA regulation. It has been demonstrated that insulin, gonadal androgens, growth hormone, prolactin, CRH and peptides derived from pro-opiomelanocortins other than ACTH are linked to AA secretion. In addition, cytokines and a variety of growth factors also have been shown to play a role in AA regulation, and recent research has focused on intra-adrenal regulatory mechanisms of DHEA secretion (1, 8, 9, 11–15).

Despite the broad spectrum of various AA regulation factors, a single base exchange in the promoter of the ACTH receptor gene results in significantly different DHEA serum levels during ACTH infusion, thus emphasizing the determining role of ACTH in controlling AA production. The differences in DHEA levels in response to ACTH stimulation are already obvious at low stimulating ACTH1–24 doses, indicating that this polymorphism is functionally relevant within normal circadian ACTH concentrations.

Azziz *et al.* noted that poststimulatory intersubject variance of cortisol is significantly lower than that of DHEA (5). Interestingly, and consistent with the findings of Azziz *et al.*, in our studies, we also observed a greater difference between the different genotypes in DHEA

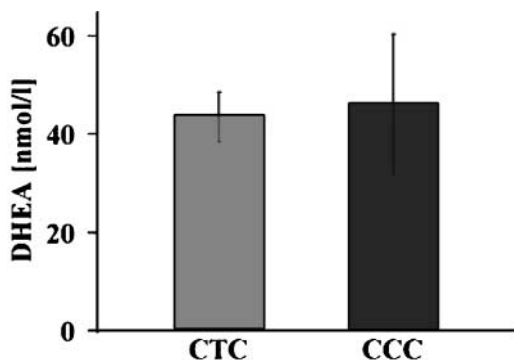


Figure 1 Mean baseline DHEA levels in homozygous CTC and CCC individuals.

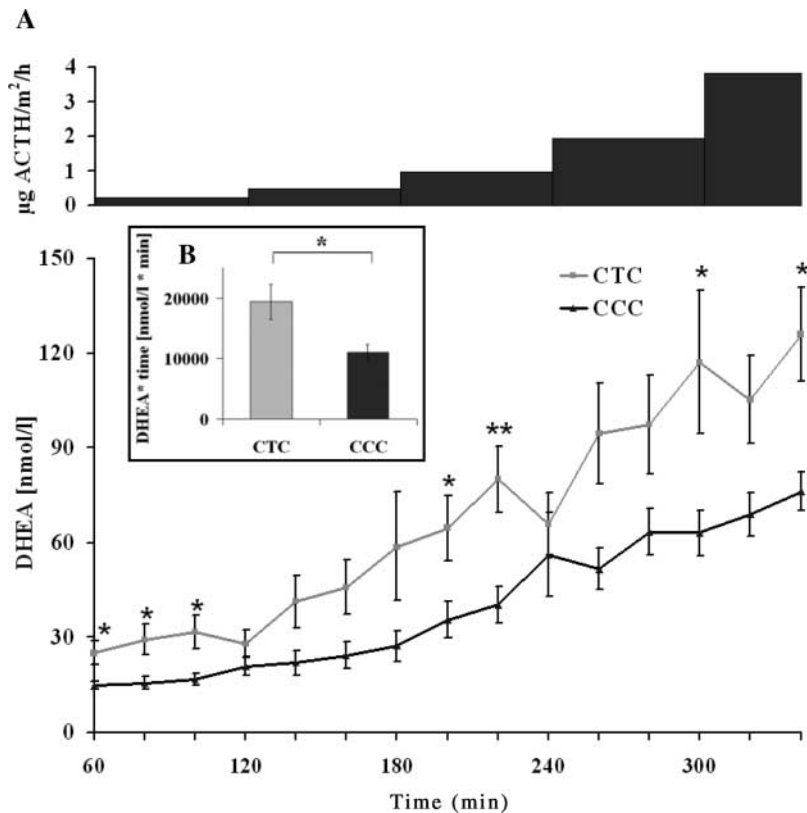


Figure 2 (A) Mean stimulated serum DHEA concentrations of homozygous CTC and homozygous CCC subjects during hourly doubling ACTH administration (starting dose: 120 ng/m² body surface area/h) after overnight dexamethasone suppression. This demonstrates lower adrenal responsiveness in individuals with the CCC/CCC genotype. * $P < 0.05$, ** $P < 0.01$. (B) Area under the concentration-time curve of DHEA concentration during the ACTH stimulation test for homozygous CTC and homozygous CCC subjects. * $P < 0.04$.

than in cortisol concentrations in response to ACTH. The difference of the integrated serum DHEA concentrations was 43% for the two genotypes investigated in this study (60–340-min interval after initiation of ACTH infusion), whereas the difference in time-integrated cortisol secretion was 26% for the same period of time (17). The extremely sensitive response of DHEA to ACTH stimulation, being more sensitive than either cortisol or aldosterone, has also been noted by Arvat *et al.* (20). This might amplify the effects of the polymorphism on DHEA secretion and thus be one explanation for greater intersubject variability of DHEA than of cortisol to ACTH (20). The high responsiveness of the reticular adrenal zone to ACTH coincides with clinical experience suggesting that DHEA is a very sensitive marker of ACTH secretion (10).

As expected, we did not find a difference of DHEAS levels in the ACTH stimulation test, probably due to the much longer half-life of DHEAS than DHEA (10, 21). Another reason for the lack of increase of DHEAS in our study may be the initially suppressed levels of DHEA by dexamethasone. If the equilibrium concentration ratio of DHEA to DHEAS is roughly 1:100, any increase of DHEAS has to be preceded by a significant increase of DHEA beyond basal physiologic serum concentrations. These were reached only at the end of our study. Under basal conditions, differences in DHEA values were also not discernible. This is not

in contrast to the results during ACTH stimulation, as many factors, including ACTH concentrations, may compensate for differences in ACTH sensitivity under baseline conditions caused by the endogenous ACTH receptor promoter polymorphism. In line with this hypothesis are our data of the CRH stimulation test (17), which showed a compensating higher ACTH response to CRH in CCC/CCC subjects.

In our study, we investigated healthy men in their twenties to early thirties who have DHEA peak values in this period of life. The observed difference in DHEA concentrations between the two genotypes could be less obvious in other age groups. However, as ACTH secretion does not show any significant changes throughout a lifetime, a lowered response to ACTH by the described polymorphism should affect DHEA secretion throughout life. The mechanism responsible for age-related differences in DHEA levels is still unknown, but is per se independent of the described polymorphism.

In conclusion, we describe an ACTH receptor promoter polymorphism (CCC) that results in lower responsiveness of the zona reticularis and, thus, leads to impaired DHEA concentrations to ACTH in young, healthy men. Although a variety of factors contribute to AA regulation, the effects of the ACTH receptor polymorphism might play a key role in the variable response of DHEA to ACTH.

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