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Salivary Cortisol in Low Dose (1 μg) ACTH Test in Healthy Women: Comparison with Serum Cortisol

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Summary

To date, a single report has appeared on the use of salivary cortisol for adrenal function testing with a low dose ACTH, although 1 μg has become preferred as a more physiological stimulus than the commonly used 250 μg ACTH test. Our present study was aimed to obtain physiological data on changes of free salivary cortisol after 1 μg ACTH stimulation. This approach was compared with the common method based on the changes of total serum cortisol. Intravenous, low-dose ACTH test was performed in 15 healthy women (aged 22-40 years) with normal body weight, not using hormonal contraceptives, in the follicular phase of the menstrual cycle. Blood and saliva for determination of cortisol were collected before ACTH administration and 30 and 60 min after ACTH administration. Basal concentration of salivary cortisol (mean ± S.E.M., 15.9±1.96 nmol/l) increased after 1 μg ACTH to 29.1±2.01 nmol/l after 30 min, and to 27.4±2.15 nmol/l after 60 min. The differences between basal and stimulated values were highly significant ($p<0.0001$). The values of salivary cortisol displayed very little interindividual variability ($p<0.04$) in contrast to total serum cortisol values ($p<0.0001$) A comparison of areas under the curve (AUC) related to initial values indicated significantly higher AUC values for salivary cortisol than for total serum cortisol (1.89±0.88 vs. 1.22±0.19, $p<0.01$). Correlation analysis of serum and salivary cortisol levels showed a borderline relationship between basal levels ($r=0.5183$, $p=0.0525$); correlations after stimulation were not significant. Low-dose ACTH administration appeared as a sufficient stimulus for increasing salivary cortisol to a range considered as a normal adrenal functional reserve.

Key words
Low dose ACTH test • Salivary cortisol • Total serum cortisol • Free cortisol • Transcortin

Introduction

Determination of free cortisol in the serum is not part of routine tests due to its methodological and economical expensiveness (Gozansky *et al.* 2005). Therefore, for a routine evaluation of adrenal function, total serum cortisol is currently being used. The main disadvantage of this method is that the cortisol values are significantly influenced by the binding proteins (Le Roux *et al.* 2003, Hamrahiam *et al.* 2004, Gozansky *et al.* 2005, Ho *et al.* 2006). Their levels may be altered considerably under various clinical states. For instance, they are increased when using estrogen preparations or in pregnancy, whereas liver and kidney disorders or

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catabolic post-operation states generally have decreased cortisol binding globulin (CBG) concentrations (Landon *et al.* 1984, Meulenberg *et al.* 1987, Ho *et al.* 2006). Attempts were therefore made to obtain information about free cortisol indirectly, by using indexes calculated from total cortisol and CBG, or directly by analyzing urine or saliva (Le Roux *et al.* 2003).

The salivary cortisol, which reflects very well the free hormone fraction in the serum, appeared to be advantageous (Contreras *et al.* 2004, Gozansky *et al.* 2005). The follow up of free salivary cortisol has been successfully applied in diagnostics of disorders of the hypothalamo-pituitary-adrenal (HPA) axis under various situations, e.g. in the metabolic syndrome (Aardal and Holm 1995, Bjorntorp and Rosmond 1999, Vicennati and Pasquali 2000, Reynolds *et al.* 2001). The authors of these studies emphasize further advantages of salivary cortisol in evaluation of HPA function: namely the non-invasiveness of sample collection and its applicability in non-standard conditions outside an outpatient clinic (Aardal and Holm 1995, Contreras *et al.* 2004).

Free cortisol represents a hormone immediately available for use in tissues. Its response to a secretory stimulus is, in contrast to basal conditions, considerably influenced by sex. Furthermore, it depends on the phase of the menstrual cycle (Kirschbaum *et al.* 1999). In the case of total cortisol, these situations do not appreciably influence the magnitude of the response. Therefore, our study was focused on a well-defined group of healthy young women, all of them in a follicular phase of the cycle. Stimulation with 250 μg ACTH is now considered pharmacological in character and therefore a low dose (1 μg) variant has begun to be used, especially for the detection of subclinical forms of disorders of adrenal function or HPA axis (Dickstein *et al.* 1991, Dickstein 2003, Laureti *et al.* 2002). To the best of our knowledge, the values of salivary cortisol after a 1 μg (ACTH) stimulus have only been published by Marcus-Perlman *et al.* (2006).

The aim of this study was to obtain normal physiological data about salivary cortisol after stimulation of adrenal gland with a low dose of ACTH in population of young women in the follicular phase of menstrual cycle and to compare the data on salivary cortisol after the stimulation with the data on serum cortisol. The comparison with stimulated serum cortisol should provide further information about salivary cortisol.

Subjects and Methods

Subjects

Fifteen healthy, 22-40 years-old healthy women with normal body weight (BMI 20-25 kg/m^2), not using hormonal contraceptives, in a follicular phase of the menstrual cycle were investigated. Hormonal contraceptives or other medication were not used for at least three months. The study was approved by the Ethical Committee of the Institute of Endocrinology.

Low-dose (1 μg) ACTH test

The test was carried out in the Institute of Endocrinology, Prague, in a specialized Laboratory for Functional Tests, always in the morning at 9:00 h, after overnight fasting. Sixty min before the test the subjects were not allowed to smoke, drink liquids and brush their teeth. After a 30-min rest in bed with a cannula introduced into the cubital vein, blood and saliva were collected, and then 1 μg ACTH was intravenously administered (Time 0). The next sampling of blood and saliva was performed 30 and 60 min after ACTH administration in the supine position. Thirty min after withdrawal, blood was centrifuged at 3000 rpm for 15 min, and the serum was left frozen in plastic tubes and stored at –20 ˚C until analyzed. Saliva was collected by spitting into plastic tubes, the material was frozen at -20 ˚C and stored at this temperature. No saliva sample was contaminated with blood.

Solution preparation

Contents of the ampoule, 250 μg/ml ACTH (Synacthen, Novartis Pharma GmbH, Nurnberg, Germany) were added to 249 ml of sterile physiological solution. Each subject received 1 ml of the solution i.v., corresponding to 1 μg ACTH. The dose was prepared 10 min before injection.

Cortisol determination

Total serum cortisol was measured according to Bičíková *et al.* (1988). It was determined by non-extraction solid phase radioimmunoassay using coated tubes with rabbit polyclonal antiserum to cortisol-3-O(carboxymethyloxime) bovine serum albumin conjugate, homologous [^{125}I] tyrosine methylester derivative as a tracer. Frozen saliva samples were thawed and centrifuged at 3000 rpm for 10 min to clear off the debris and mucosa residues. The material was then pipetted by an automatic analyzer (Stratec, Immunotech,

Marseille, France). Salivary cortisol was determined by the same method as serum cortisol, but using 30 µl of saliva instead of 1.5 µl of the serum. Intra-assay coefficients of variation (c.v.) were 5.2 % and 7.4, inter-assay c.v. 9.8 % and 10.2 % for serum and salivary cortisol, respectively.

Statistical data treatment

Two linear models were used to compare the methods. The first, ANOVA model, was set up to evaluate the dissimilarity of inter-individual differences by the method based on salivary cortisol and the current approach based on cortisol levels in the serum. This model considered the time and subject as the variable factors. The F- and p-value of the latter factor express its contribution to the explanation of the total variability in cortisol levels. The second, general linear model (GLM), was aimed to compare the dissimilarity between time profiles of the salivary and blood cortisol levels after ACTH-stimulation. The Bonferroni multiple comparisons following the testing by GLM were used to compare the distinctiveness of the responses to ACTH stimulation in the methods based on the salivary and blood cortisol. To approximate Gaussian distribution and constant variance, the original data were transformed by power transformation. Wilcoxon test was used for comparison of areas under the curve (AUC) between the methods and Spearman correlations were employed to evaluate the relationships between the basal and stimulated levels. Statistical software Statgraphics Plus version 5.1 was used for the analysis.

Results

The basal levels of total cortisol 486±122.7 nmol/l (mean ± S.D.) increased after administration of 1 µg ACTH to 607.2±92.4 nmol/l in the 30th min and to 610.9±131.27 nmol/l in the 60th min. The difference between basal value and values in 30th and 60th min were significant (Fig. 1).

Basal concentration of salivary cortisol (15.9 ± 7.6 nmol/l) increased after 1 µg ACTH to 29.1±7.8 nmol/l in the 30th and to 27.4 ± 8.3 nmol/l in the 60th min. The differences between basal and stimulated values were highly significant (Fig.1).

A comparison of AUC related to initial values showed significantly higher AUC for salivary cortisol than AUC for total serum cortisol: 1.89±0.88 vs. 1.22± 0.19, p<0.01 (Wilcoxon´s test).

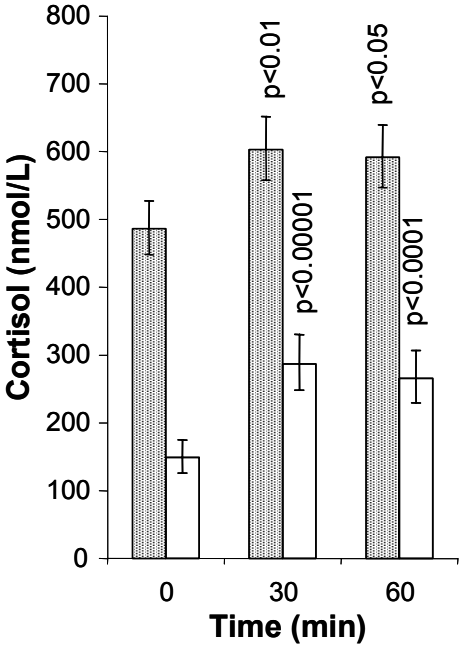

Fig. 1. Evaluation of ACTH tests for serum (shaded bars) and salivary cortisol (open bars). These bars with respective error bars represent retransformed mean values and their 95 % confidence intervals in individual stages of the ACTH test for serum and salivary cortisol, respectively. The salivary cortisol is displayed as ten times the amount. The F-values and significances in the GLM (general linear model) consisting of the factors Matrix, Subject, and Time were as follows: Matrix: F=6208, p<0.0001; Subject: F=4.19, p<0.0001; Time: F=29.7, p<0.0001; Matrix×Time interaction: F=1.22, NS. The model was highly significant (F=300, p<0.0001) explaining 98.8 % of the variability in cortisol levels. The p-values above the error bars denote the significance of difference between basal and stimulated values as found using Bonferroni multiple comparisons following the GLM. When using the ANOVA model consisting of Subject and Time as factors separately for serum and saliva, the F-values and significances were as follows: Serum.-Subject: F=4.19, p<0.0003; Time: F=12.3, p<0.0001; Saliva.-Subject: F=2.06, p<0.03, Time: F=22.1, p<0.0001.

The individual results showed high inter-individual variability in the case of serum cortisol (p<0.0001), while only borderline inter-individual differences were found when analyzing the salivary cortisol (p<0.04).

A correlation analysis of serum and salivary cortisol levels showed only a marginal relationship between the basal levels (r=0.5183, p=0.0525), while correlations at all times after stimulation were not significant.

Although the Matrix × Time interaction indicating a different response to ACTH stimulation in the serum and saliva was not significant, Bonferroni

multiple comparisons found more significant differences between basal and stimulated cortisol levels in saliva than in the serum (Fig. 1).

Discussion

A comparison of changes in total serum cortisol with results obtained by measuring salivary cortisol response to 1 μg ACTH stimulus pointed to an important difference. There was a high interindividual variability in the case of serum cortisol ($p<0.0001$), while only borderline interindividual differences were found when analyzing the salivary cortisol ($p<0.04$). This means that the between-subject variance is substantially lower in salivary cortisol compared to serum cortisol as documented by the different F-and p-values in the ACTH test in serum and saliva (Fig. 1).

On the other hand, as expected, the differences between basal and stimulated values of both parameters were highly significant ($p<0.0001$). The post-stimulatory response of salivary cortisol was considerably higher in comparison with total serum cortisol, as demonstrated by the significant difference between AUC for salivary and serum cortisol (Fig. 1). This finding is in agreement with some recent studies, describing more pronounced increase of free cortisol in comparison to the total values, as a consequence of CBG changes. The assumed changes of CBG concentrations and its saturation could explain not only the high variability of post-stimulatory values of total serum cortisol, but also the fact that stimulated values of serum cortisol did not correlate with salivary concentrations at individual times during the test (Contreras *et al.* 2004, Wong *et al.* 2004, Gozansky *et al.* 2005). However, we did not follow CBG levels in our study.

It may be of interest that a maximum response of cortisol in saliva as well as in serum after l μg stimulus occurred in all the subjects at the same time, i.e. in the 30th or 60th min. This confirms earlier findings that actual levels of salivary cortisol react almost immediately to changes in the free fraction of circulating hormone (Landon *et al.* 1984).

According to Kirschbaum *et al.* (1999), the poststimulatory values of free salivary cortisol are markedly influenced by sex, and in women also by a phase of the menstrual cycle, probably as a result of different adrenal sensitivity. Therefore, it is difficult to compare our results from healthy women with normal body weight with recent data of Marcus-Perlman *et al.* (2006) who studied the effect of 1 μg ACTH stimulus on salivary cortisol in a mixed control group (males and females). Furthermore, the body weight and the phase of menstrual cycle in women were not specified in their study.

From our results and the data in the literature it is obvious that the measurement of salivary cortisol is less dependent on other factors than the total serum cortisol and therefore reflects the adrenal functional reserve more closely (Contreras *et al.* 2004). In its diagnostic use, the salivary cortisol appears to be a better marker of adrenal function because the individual response to a stimulus does not vary significantly in healthy subjects in contrast to the wide range of responses seen in serum total hormone levels. The results about salivary cortisol in low-dose ACTH test in young women shown in this study can be used as physiological reference in the diagnostic decision of adrenal insufficiency exclusion.

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