Establishing Normal Ranges of Basal and ACTH-Stimulated Serum Free Cortisol in Children

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Key Words

Free cortisol · Total cortisol · ACTH test · Normal values

Abstract

Background: Normative data have been established for stimulated serum total cortisol in children but not for serum free cortisol. **Methods:** Children who were referred for ACTH testing to rule out adrenal insufficiency were enrolled. Only children with normal response and normal androgen levels were included. Total cortisol was determined by a chemiluminescence method, and free cortisol was measured by the same method following equilibrium dialysis. **Results:** The study group consisted of 85 subjects (28 male; 57 female) with a median age of 8.5 years (range 0.6–17.7). The mean basal and peak total cortisol levels were 11.5 ± 5.7 and 32.9 ± 6.2 $\mu\text{g/dl}$, respectively. The mean basal and peak free cortisol levels were 0.4 ± 0.3 and 1.8 ± 0.6 $\mu\text{g/dl}$, respectively. There was a negative correlation between peak total cortisol and age but not between peak free cortisol and age. The 3rd and 97th percentile values for peak free cortisol were 0.94 $\mu\text{g/dl}$ (26 nmol/l) and 2.97 $\mu\text{g/dl}$ (82 nmol/l), respectively. **Conclusions:** Measurement of free cortisol has the advantage of being independent of cortisol-binding globulin lev-

els. This study provides reference ranges for stimulated free cortisol in children, with a cutoff value of 0.9 $\mu\text{g/dl}$ (25 nmol/l) as a normal response to a standard ACTH test.

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Introduction

More than 90% of the serum cortisol is protein bound. Between 80 and 90% of the circulating cortisol is bound to cortisol-binding globulin (CBG), 10–15% is bound to albumin and 5–10% circulates as free and active hormone. Changes in binding proteins can alter measured serum total cortisol without influencing serum free cortisol concentrations [1–3]. Estrogen, oral contraception, pregnancy, and liver disease increase CBG, while malnutrition, nephrotic syndrome, and dilutional states, such as heart failure, decrease both albumin and CBG and may cause subnormal ACTH-stimulated total cortisol levels, leading to the erroneous diagnosis of adrenal insufficiency and unnecessary glucocorticoid therapy [4]. Although total cortisol generally correlates well with the free fraction, there are clinical conditions, such as major surgery, severe illness, or acute phase of septic shock and stress, in

which large changes in CBG and albumin concentration take place, thus raising a real need for the measurement of free cortisol concentration [4–8]. Hypoalbuminemia and alterations in the binding of cortisol may lead to sub-normal total cortisol responses to ACTH, while the free cortisol response can remain entirely normal in subjects with severe acute illness [9]. Other examples in which free cortisol measurement becomes particularly important are the rare cases of inactive corticosteroid-binding globulin, such as CBG-Lyon [10] or congenital CBG deficiency [11, 12]. The current consensus is that the free rather than the protein-bound fraction of cortisol is responsible for its physiological function [4].

It is therefore desirable to establish reference ranges for free cortisol in order to prevent misdiagnosis and unnecessary treatment with corticosteroids. While normal free cortisol levels have been established for adults [13], there are no comparable data for children. In this study, we aimed to assess normal values for free cortisol levels in relation to total cortisol, age, and the pubertal stage in children who had been referred for evaluation of their hypothalamus-pituitary-adrenal (HPA) axis in order to rule out adrenal insufficiency/enzymatic defect. The study group consisted of children in whom altered adrenal function was suspected but cortisol deficiency was not likely and ultimately excluded.

Materials and Methods

Subjects

Children (age 0.5–18 years) who were referred for ACTH testing at our unit in order to rule out adrenal insufficiency or adrenal enzymatic defect were invited to participate in the study. All tests were performed between 8 and 9 a.m. in order to measure morning basal cortisol. The reasons for referral were: precocious pubarche ($n = 42$), advanced puberty ($n = 20$), adrenarche before gonadarche ($n = 9$), hirsutism ($n = 7$), acne ($n = 3$), advanced bone age ($n = 2$), and parental nonclassical 21 hydroxylase deficiency ($n = 2$). Puberty was assessed clinically according to the criteria of Marshall and Tanner [14, 15] by an experienced pediatric endocrinologist. In this study, we defined the Tanner stage by breast stage in girls and testicular volume in boys [14, 15]. Only children with normal ACTH-stimulated total cortisol and 17OHP findings and normal testosterone and androstenedione levels were included. The cutoff value was set at $>20 \mu\text{g/dl}$ ($>550 \text{ nmol/l}$) for total cortisol [16, 17] and at $<330 \mu\text{g/dl}$ ($<10 \text{ nmol/l}$) for 17OHP [18]. Testosterone and androstenedione levels were measured and matched for age, sex, and pubertal stage [19].

Methods

Synacthen (Sigma-Tau, Rome, Italy) was injected intravenously at a dose of $250 \mu\text{g/m}^2$ (up to a maximal dose of $250 \mu\text{g}$). Blood was drawn for the evaluation of cortisol and free cortisol at baseline and at 30 and 60 min after the injection. Total cortisol was measured by

Table 1. Basal and ACTH-stimulated serum total and free cortisol levels in 85 subjects aged between 0.6 and 17.7 years, showing a higher fraction of peak serum free cortisol compared with basal serum free cortisol

	Total cortisol, $\mu\text{g/dl}$	Free cortisol, $\mu\text{g/dl}$	Fraction of free cortisol, %
Baseline			
Mean $\pm$ SD	11.53 ± 5.74	0.42 ± 0.30	3.95 ± 1.73
95% CI	10.29–12.77	0.36–0.49	3.57–4.33
Peak			
Mean $\pm$ SD	32.95 ± 6.22	1.82 ± 0.63	6.69 ± 2.06
95% CI	31.61–34.29	1.68–1.96	6.23–7.13
% Increase			
Mean $\pm$ SD	258 ± 180	504 ± 426	87 ± 70
95% CI	218–297	410–597	72–102

$p < 0.001$ for % increase between free cortisol and total cortisol.
To convert total and free cortisol $\mu\text{g/dl}$ to nmol/l , multiply by 27.6.

electrochemiluminescence (Cobas A 411; Roche, Indianapolis, Ind., USA). The intra- and interassay coefficients of variation were 1.4 and 1.6%, respectively. Free cortisol was determined by equilibrium dialysis. The equilibrium dialysis cells Slide-A-Lyzer mini dialysis units 7,000 molecular weight cutoff used (Pierce, Rockford, Ill., USA) were disposable dialysis cups made of polypropylene and low-binding regenerated cellulose membranes. After 15 min of soaking in 1 liter of water for glycerol removal, the dialysis unit was placed in a microtube filled with 1 ml dialysate buffer. The human serum for dialysis was placed in the unit cell ($500 \mu\text{l}$). Each liter of dialysate buffer contained sodium chloride (5.265 g), 60% syrup of DL-lactic acid as the sodium salt (1 ml), L-glutamic acid as the monosodium salt (561 mg), potassium chloride (224 mg), monopotassium phosphate (180 mg), calcium chloride dihydrate (275 mg), magnesium sulphate heptahydrate (246 mg), urea (300 mg), HEPES sodium salt (5.891 g), HEPES acid (7.19 g), penicillin (100,000 USP units), streptomycin (100 mg), amphotericin B (0.25 mg), gentamicin (100 mg), sodium azide (520 mg), gelatin (500 mg), and rabbit IgG (200 mg). The chemicals were obtained from Sigma Chemical Co., Saint Louis, Mo., USA. The equilibrium dialysis was performed in a water bath at 37°C for 24 h with continuous agitation. The cortisol was then measured in the dialyzed serum by electrochemiluminescence. The intra- and interassay coefficients of variation of the assay were 8 and 12%, respectively [13].

Statistical analysis was done using the BMDP program [20]. Descriptive statistics are given as mean (SD) for continuous variables and as frequency for categorical variables. Discrete variables were compared using Pearson's χ^2 test or Fisher's exact test as appropriate. Comparisons between continuous variables were performed using one-way ANOVA. We tested for equal variances using Levene's test. If Levene's test was significant, we used the equality of means test Welch or Brown-Forsythe. Pearson's correlation was applied to detect correlations between different parameters. A p value of ≤ 0.05 was considered significant.

Table 2. Basal and stimulated serum free cortisol by gender and Tanner breast stage in girls and genital stage in boys

	Girls			Boys		
	B1	B2–3	B4–5	G1	G2–3	G4–5
Number of children	30	10	12	16	6	3
Basal free cortisol ^a	0.40±0.26	0.47±0.20	0.52±0.43	0.27±0.22	0.52±0.32	0.64±0.66
% free cortisol at baseline ^b	3.4±1.32	4.4±1.74	4.6±1.97	3.2±1.51	5.2±2.18	4.7±0.93
Peak free cortisol ^c	1.88±0.66	1.79±0.56	1.87±0.58	1.65±0.45	1.87±1.17	2.08±0.74
% free cortisol at peak ^d	6.3±1.98	6.5±1.35	7.6±2.39	6.5±1.95	7.1±3.20	8.4±1.31

Values are expressed as mean ± SD, unless otherwise indicated. There was a significant difference in basal serum free cortisol levels and the fraction of serum free cortisol at baseline between the three Tanner stage groups in both sexes, but this difference was not found for peak serum free cortisol and the fraction of serum free cortisol at peak. No difference was found between the sexes. Free cortisol is given in µg/dl. To convert to nmol/l, multiply by 27.6. B1–5, G1–5 = Tanner stages. ^a p = 0.86 for gender, p = 0.04 for Tanner stage, p = 0.4 for interaction between gender and Tanner stage. ^b p = 0.5 for gender, p = 0.003 for Tanner stage, p = 0.6 for interaction between gender and Tanner stage. ^c p = 0.94 for gender, p = 0.66 for Tanner stage, p = 0.55 for interaction between gender and Tanner stage. ^d p = 0.39 for gender, p = 0.09 for Tanner stage, p = 0.9 for interaction between gender and Tanner stage.

Statement of Ethics

Written informed consent was obtained from the parents or guardians on behalf of their child. The study was approved by the medical Ethics Committee of Tel Aviv Sourasky Medical Center.

Results

The study group consisted of 85 subjects (57 girls and 28 boys) whose median age was 8.5 years (range 0.6–17.7). Forty-six of them were prepubertal, 16 were Tanner breast/genital stages 2 or 3, and 15 were stages 4 or 5. Pubertal stage data were missing for 8 patients. Basal and peak total and free cortisol levels and free cortisol fractions are given in table 1.

Basal Total and Free Cortisol Levels

The 3rd percentile for basal total cortisol was 3.5 µg/dl (96 nmol/l), and the 97th percentile was 25.6 µg/dl (706 nmol/l). The 3rd percentile for basal free cortisol was 0.07 µg/dl (2 nmol/l), and the 97th percentile was 1.2 µg/dl (33 nmol/l).

Baseline and peak total cortisol and free cortisol were positively correlated ($r = 0.8$, $p < 0.001$ and $r = 0.5$, $p < 0.001$, respectively). There was a negative correlation between the peak total cortisol level and age ($r = -0.3$, $p = 0.001$) but not between peak free cortisol and age.

Total and Free Cortisol Levels in Relation to Age Groups

The subjects were divided into three age groups (<5 years, 5–10 years, and >10 years). There was no difference

in the mean basal total cortisol levels among the three age groups, but there was a significant difference in the mean peak total cortisol levels [36.2 ± 5.63 µg/dl (999 ± 155 nmol/l), 33.25 ± 6.67 µg/dl (917 ± 184 nmol/l), and 30.21 ± 4.5 µg/dl (833 ± 124 nmol/l), ANOVA $p < 0.01$]. The mean free cortisol baseline level was similar for the three age groups [0.41 ± 0.28 µg/dl (11 ± 8 nmol/l), 0.41 ± 0.27 µg/dl (11 ± 7 nmol/l), and 0.45 ± 0.37 µg/dl (12 ± 10 nmol/l)]. However, there was a trend for higher peak free cortisol values in children <5 years of age [2.21 ± 0.62 µg/dl (61 ± 17 nmol/l), 1.70 ± 0.52 µg/dl (47 ± 14 nmol/l), and 1.85 ± 0.78 µg/dl (51 ± 22 nmol/l), ANOVA $p = 0.06$].

Stimulated Total and Free Cortisol Level

The 3rd percentile for peak total cortisol was 24.3 µg/dl (670 nmol/l), and the 97th percentile was 44.4 µg/dl (1225 nmol/l). The 3rd percentile value for the mean peak free cortisol level was 0.94 µg/dl (26 nmol/l), and the 97th percentile was 2.97 µg/dl (82 nmol/l). Based on these values, we propose a cutoff value of 0.9 µg/dl (25 nmol/l) as the normal response to a standard ACTH test.

Total and Free Cortisol Levels in Relation to Gender and Pubertal Stage

There were no significant gender differences in total cortisol and free cortisol levels even when the pubertal stage was taken into account. There was a significant difference in basal free cortisol levels between the three pubertal stage groups in both sexes (table 2). This difference was not found for the basal and peak total cortisol levels and the peak free cortisol levels (table 2). There was a pos-

itive correlation between basal free cortisol values and the pubertal stage ($r = 0.25$, $p = 0.03$) but none was found between basal total cortisol and the pubertal stage. There was a negative correlation between peak total cortisol and age ($r = -0.345$, $p = 0.001$), as well as a positive correlation between the fraction of free cortisol at baseline and at peak and the pubertal stage ($r = 0.312$, $p = 0.006$ and $r = 0.247$, $p = 0.03$, respectively). No correlation was found between basal and stimulated total and free cortisol levels and body mass index standard deviation score.

Discussion

Alterations in CBG and albumin concentrations might cause misinterpretation of dynamic tests of the HPA axis due to their influence on total cortisol levels. This, in turn, can lead to an erroneous diagnosis of adrenal insufficiency and unnecessary glucocorticoid therapy. Therefore, assessment of the free fraction of circulating cortisol has been attempted, using indirectly calculated free cortisol or directly measured free cortisol. The free cortisol index, defined as serum total cortisol divided by the CBG level is a reliable measure of serum free cortisol [21]. However, it ignores both potential alternations in CBG-binding properties and the influence of albumin on the actual free cortisol fraction. The latter difficulty has been addressed by Dorin et al. [22] who showed that the measurement of serum albumin as well as that of CBG followed by a modified equation better approximates free cortisol as determined directly through ultrafiltration. Based on the results of this study, we propose normal values for basal and post-ACTH stimulation levels of free cortisol in children and adolescents. We demonstrated that the relative increase in free cortisol as a result of ACTH administration was significantly higher than the relative increase in total cortisol. This finding had not been demonstrated after a low-dose ACTH stimulation test in adults [13]. This difference might be explained by CBG saturation having been reached after a high-dose ACTH stimulation test. Specifically, CBG can bind up to 25 µg/dl of the circulating cortisol. As CBG becomes saturated, a larger proportion of circulating cortisol will be albumin bound and unbound [4]. The 6-fold increase in the free cortisol level during a standard ACTH test that was documented for our pediatric group is similar to the 7- to 10-fold increase in free cortisol concentration previously reported in a random sample taken from critical ill adult patients [9].

As expected in healthy subjects, there was a highly significant correlation between the total cortisol and free

cortisol levels. Interestingly, there was a negative correlation between peak total cortisol and age but not for peak free cortisol. This finding for total cortisol was reported earlier by Lashansky et al. [19]. The reason for this is probably that CBG levels decrease with age [23]. Of note, the basal free cortisol and the basal and peak fraction of free cortisol increased with pubertal stage in both sexes, but no comparable increase was observed for total cortisol levels. These findings are compatible with the results on salivary cortisol reported by Kiess et al. [24]. Those authors found that salivary cortisol concentrations correlate significantly with pubertal stage. The increase in free cortisol levels with pubertal stages might reflect a higher overall activity of the adrenal gland during puberty [24]. Unlike Kiess et al. [24], however, we did not find any positive correlations between free cortisol levels with BMI SDS and age.

Eighty-one (95%) of our study patients had baseline free cortisol levels of 0.07–1.2 µg/dl (0.2–3.3 nmol/l) and peak levels of 0.94–2.97 µg/dl (2.6–8.3 nmol/l). Based on these values, we propose a cutoff value of 0.9 µg/dl (2.5 nmol/l) as normal response to a standard ACTH test. This cutoff point is the same as that found for adult subjects following low-dose ACTH testing [13] standard dose ACTH testing [25].

We used the standard ACTH test to assess the free cortisol response in children and adolescents. We have previously shown that the standard ACTH test is equivalent to a low-dose ACTH test and that it is highly correlated to insulin-induced hypoglycemia (the gold standard test) in detecting HPA sufficiency and insufficiency in children with either multiple pituitary hormone deficiency or isolated growth hormone deficiency [26]. In addition, a low-dose (1 µg) ACTH test might show a high rate of abnormal responses due to technical problems [27]. There is no consensus regarding the cutoff value for total cortisol response to ACTH. Lindholm [17] proposed a cutoff of 18 or 20 µg/dl (500 or 550 nmol/l, respectively). We had previously shown [26] that the cortisol response to standard ACTH stimulation test for children was >22 µg/dl (>597 nmol/l). Since all of our subjects had peak levels >23 µg/dl (>636 nmol/l), selecting a lower value as the threshold used would not have changed the results.

Our study has several limitations: first, the carrier rate of mild *CYP21A2* mutation is relatively high among Ashkenazi Jews (1:10 in Israel) [28]. We did not carry out a molecular analysis of the gene in our study group. However, by including only those participants with a peak 17OHP response to ACTH below 10 nmol/l, we ensured that the vast majority of the patients were noncarriers [29,

30]. In addition, since our study population was of a mixed origin, the rate of carriers was even lower than 1:10 [28]. Secondly, current assays for determining free cortisol concentrations are difficult to perform, time-consuming, and labor-intensive. It is likely that rapid assays for measurements of free cortisol will become available in the near future, just as serum-free T4 assays became widely available for clinical use more than a decade ago [4]. Thirdly, since all tests were done in an outpatient setting, the baseline levels of cortisol and of free cortisol were obtained between 8 and 9 a.m., which could be after the circadian peak of cortisol. However, the time of the day does not have any impact on the ACTH-stimulated total and free cortisol levels [31], and they are the main outcome of this study. Lastly, although the study group was selected from all children who were referred for evaluation of adrenal function due to suspected adrenal dysfunction, only those with normal test results were included in the study.

In summary, we present normal reference values for free cortisol levels at baseline and after ACTH administration in healthy children and adolescents. These values will be helpful in evaluating adrenal reserve in pediatric subjects with conditions that affect total cortisol levels, such as in CBG deficiency, hypoproteinemia, dilutional states, or increased estrogen levels.

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Disclosure Statement

The authors declare that there are no conflicts of interest associated with this paper.

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