On the meaning of low-dose ACTH(1–24) tests to assess functionality of the hypothalamic–pituitary–adrenal axis

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Abstract

To analyse further the ACTH(1–24) low-dose test, which is of clinical interest, we have examined the dose–response relationship between plasma ACTH(1–24) and cortisol concentrations after i.v. administration of increasing doses (1, 5 or 250 µg) of ACTH(1–24) as a bolus. In addition, we have measured plasma ACTH(1–39) and cortisol levels after an insulin tolerance test (ITT). Although there was a dose–response relationship between plasma ACTH(1–24) immunoreactivity and the dose injected, cortisol peaks were comparable, but lower than those reached after an ITT. Under these experimental conditions, an increase in plasma ACTH as low as 13 pmol/l (i.e. the increase obtained with the 1 µg dose) induced a near maximal cortisol response. Following injection of 1 µg ACTH(1–24), peak ACTH values were short lasting, similar to physiological daily bursts. After injection of 5 µg ACTH(1–24), plasma ACTH concentrations were higher than those reached during an ITT, but clearly shorter lasting. Injection of 250 µg ACTH(1–24) induced strikingly supraphysiological levels of plasma ACTH. We conclude that neither regular nor low-dose ACTH tests can fully reproduce the ITT. Our observations strongly suggest that the low-dose ACTH(1–24) test (1 µg) can be useful to estimate the adrenal sensitivity under basal, physiological conditions.

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Introduction

Assessment of the hypothalamo–pituitary–adrenal (HPA) axis status classically relies upon the gold standard insulin tolerance test (ITT), which is clearly unpleasant and could also be hazardous. It has been widely accepted, leaving out the case of recent hypothalamic and/or pituitary dysfunction, that a normal cortisol response to the harmless standard adrenocorticotrophin (ACTH(1–24)) (tetracosactrin, 250 µg) test faithfully predicts the integrity of the HPA axis (1, 2). However, many discrepancies between results obtained with the ITT and the standard ACTH(1–24) test have already been reported, some of them leading to misdiagnosis of central hypoadrenalism (3–6). A possible explanation for these discordant results could be that the administration of 250 µg synthetic ACTH provides extremely high plasma ACTH concentrations (7). This excessive stimulus may lead to false-positive cortisol responses and cause underdiagnosis of partial or subtle secondary hypoadrenalism.

Several investigators have recently confirmed the early work of Landon *et al.* (8), and reported that much lower doses of ACTH(1–24) – 0.5–5 µg – induce maximal cortisol release *in vivo* (9–11). These low doses of ACTH may improve the sensitivity of the test and be especially indicated in the screening of latent

hypocorticism (12–14). The clinical interest in the low-dose ACTH test is based on the hypothesis that ACTH(1–24) plasma levels during this test are comparable to those reached during an ITT. However, to the best of our knowledge, there has been no satisfying study dealing with this issue. In this study we have measured, using a specific and highly sensitive RIA, plasma ACTH(1–24) concentrations after i.v. injection of various doses of synthetic ACTH(1–24). Circulating cortisol levels have also been determined.

Subjects and methods

Subjects

A total of 18 healthy volunteers were included in our study after giving their informed consent. Eight volunteers (five male, three female, aged 26–54 years) were subjected to ACTH(1–24) injection. Ten volunteers (two male, eight female, aged 27–58 years) were subjected to an ITT. None of them was taking any medication known to affect the HPA axis.

Testing protocol

ACTH(1–24) tests All subjects underwent testing with three different doses of ACTH(1–24) (1, 5 or 250 µg) or

saline at 1000 h at 1 week intervals. An i.v. cannula was inserted in an antecubital vein, and after 30 min rest the substance was administered directly as a 1 ml bolus in a contralateral forearm vein using a plastic syringe attached to a 24 gauge needle. Blood samples were collected 15 min and immediately before, and 2, 5, 10, 20 and 30 min after the injection.

ITT An i.v. cannula was inserted in an antecubital vein, and after 30 min rest 0.1 IU/kg rapid insulin (Umuline, Eli Lilly, Saint-Cloud, France) was injected as a bolus. Blood samples were collected 15 min and immediately before, and 15, 30, 45, 60, 75 and 90 min after the injection.

For both tests blood samples were immediately centrifuged at 2000 *g* at 4°C for 15 min and the resulting plasma was stored at -20°C until cortisol and ACTH(1-24) or ACTH(1-39) measurements.

ACTH(1-24) preparation

One vial of 250 µg ACTH(1-24) (Ciba-Geigy, Basel, Switzerland) was diluted in sterile saline solution to concentrations of 1 or 5 µg/ml. The resulting solutions were used immediately after preparation.

Hormone assays

Plasma concentrations of ACTH(1-24) were measured in 100 µl of unextracted samples using an RIA developed in our laboratory. The antiserum was raised in a rabbit using ACTH(1-24) (Ciba-Geigy) coupled to BSA with carbodiimide. The cross-reactivity of the antiserum with ACTH(1-39) was 3-fold less (calculated on a molar basis) than with ACTH(1-24) (Fig. 1). Plasma ACTH(1-24) levels were calculated using a standard curve obtained with ACTH(1-24). The limit of detection of the assay was 0.34 pmol/l. The intra- and interassay coefficients of variation (CV) were 4.5 and 7.0% respectively. Plasma ACTH(1-39) levels were

Figure 1 Displacement curves obtained in ACTH RIA with ACTH(1-24) and ACTH(1-39).

measured in 200 µl of unextracted samples using an IRMA kit (Allegro, Nichols Institute Diagnostics, distributed by Mallinckrodt Médical, Bondoufle, France). The limit of detection of the assay was 1.1 pmol/l. The intra- and interassay CVs were 3 and 7.8% respectively. Plasma cortisol was measured using a solid phase chemiluminescent immunoassay (Immulite Cortisol, Behring, Reuil-Malmaison, France). The intra- and interassay CVs were 8 and 10% respectively.

Statistical analysis

All data are presented as the mean $\pm$ S.E. Statistical analysis was performed by ANOVA for repeated measures followed by Fisher's test for the kinetic data or by the Kruskal-Wallis followed by the Mann-Whitney test for the comparison of cortisol increments or peaks and ACTH peaks. Areas under the curves (AUCs) were calculated by the trapezoidal method and compared either by linear regression or by the Kruskal-Wallis followed by the Mann-Whitney test. Regression equations were fitted to the data (x =time; y =log [ACTH(1-24)]) by linear regression, and half-times for disappearance of immunoreactive ACTH(1-24) were calculated from the slopes.

Results

Cortisol response

Saline injection had no effect on cortisol levels (data not shown). Figure 2 depicts the variations of plasma cortisol levels after injection of increasing doses of ACTH(1-24) or during the ITT. Basal cortisol concentrations were not statistically different during the various tests. All the doses of ACTH(1-24) stimulated ($P < 0.0001$) plasma cortisol secretion that peaked 20 min after injection. However, there was no significant difference between mean incremental cortisol values (251 ± 26 , 296 ± 25 and 310 ± 34 nmol/l for the 1, 5 and 250 µg test respectively, $P = 0.5$) or peak cortisol levels (466 ± 16 , 524 ± 38 and 555 ± 36 nmol/l for the 1, 5 and 250 µg test respectively, $P = 0.17$) after injection of the different doses of ACTH(1-24). After insulin injection, plasma glucose fell below 2 mmol/l within 30 min. Cortisol secretion was significantly ($P < 0.0001$) stimulated and peaked at 45 min. Mean incremental cortisol value (472 ± 57 nmol/l) and peak cortisol levels (720 ± 71 nmol/l) were significantly ($P = 0.0358$ and $P = 0.0131$ respectively) different from those obtained after injection of the various doses of ACTH(1-24) (Table 1).

Kinetics of plasma ACTH(1-24) or ACTH(1-39) immunoreactivity

Plasma concentrations following ACTH(1-24) administration are shown in Fig. 2. Injection of various

Figure 2 Immunoreactive cortisol and ACTH following acute i.v. administration of increasing doses (1, 5 or 250 µg) of ACTH(1–24) or during an ITT in eight and ten normal subjects respectively. Results are given as means $\pm$ s.e.

amounts of ACTH(1–24) induced a linear ($r^2 = 0.951$, $P = 0.0001$) dose-related increase in plasma immunoreactive ACTH(1–24) (AUC: 7.3 ± 0.7 , 38.2 ± 6.6 and 1778.0 ± 126.0 pmol/l per min for the 1, 5 or 250 µg dose respectively). Peak values (12.9 ± 3.2 , 87.0 ± 19.0 and 5799.0 ± 705.0 pmol/l, for the 1, 5 or 250 µg dose respectively) were obtained at 2 min. Thereafter, there was a rapid decrease in plasma immunoreactive ACTH(1–24). When plotted semilogarithmically, the plasma ACTH(1–24) disappearance regression line appeared monophasic ($r^2 = 0.956$, $P = 0.0001$, $t_{1/2} = 4.91 \pm 0.23$ min). During the ITT, plasma ACTH(1–39)

increased 30 min after insulin injection and peaked (38.4 ± 5.1 pmol/l) at 45 min. This value was significantly higher ($P = 0.0009$) than the ACTH peak obtained after injection of 1 µg ACTH(1–24) and lower than the ACTH peaks obtained after injection of 5 or 250 µg ACTH(1–24) ($P = 0.0059$ and $P = 0.0006$ respectively). The AUC (calculated using the measurements obtained between 15 and 90 min after insulin injection) was 33.6 ± 5.9 pmol/l per min. This value was significantly different from those obtained with the 1 or 250 µg ACTH(1–24) test ($P = 0.0001$ and $P = 0.0004$ respectively) but comparable ($P = 0.477$)

Table 1 Effect of injection of increasing doses of ACTH(1–24) or of ITT on plasma cortisol increment or peak. Values are the mean $\pm$ s.e. obtained in eight and ten normal subjects respectively.

	ACTH(1–24) (µg)			ITT
	1	5	250	
Increment (nmol/l)	$251 \pm 26^*$	$296 \pm 25^*$	$310 \pm 34^*$	472 ± 57
Peak (nmol/l)	$466 \pm 16^*$	$524 \pm 38^*$	$555 \pm 36^*$	720 ± 71

* $P < 0.05$ vs ITT.

Table 2 Effect of injection of increasing doses of ACTH(1–24) or of ITT on plasma immunoreactive ACTH AUC or peak. Values are the mean $\pm$ s.e. obtained in eight and ten normal subjects respectively.

	ACTH(1–24) (μ g)			ITT
	1	5	250	
AUC (pmol/l per min)	7.3 $\pm$ 0.7*	38.2 $\pm$ 6.6	1778.0 $\pm$ 126.0*	33.6 $\pm$ 5.9
Peak (pmol/l)	12.9 $\pm$ 3.2*	87.0 $\pm$ 19.0*	5799.0 $\pm$ 705.0*	38.4 $\pm$ 5.1

* $P < 0.05$ vs ITT.

with the value obtained with the 5 μ g ACTH(1–24) test (Table 2).

Discussion

In our study, peak plasma ACTH(1–24) levels were very high after the 250 μ g dose and significant levels of this peptide were still detectable in plasma 30 min after injection. Such values may induce transient upregulation of adrenal ACTH receptors (15, 16) and as a consequence may lead to an overestimation of the adrenal sensitivity to ACTH. By comparison, the 1 μ g dose gave peak ACTH values within the physiological range. Indeed, plasma ACTH(1–24) levels reached after injection of 1 μ g of this substance were comparable to plasma ACTH diurnal changes (17) and may be used to investigate the physiological adrenal sensitivity. Peak plasma ACTH(1–24) levels following injection of 5 μ g of this substance were higher than circulating ACTH values observed during the ITT and therefore may not be used to test the adrenal response to a short-lasting stressful situation.

The kinetic properties of ACTH(1–24) and ACTH(1–39) in blood have been previously studied. Indeed, Graybeal & Fang (7), using an RIA that recognizes both ACTH(1–39) and (1–24), have measured plasma ACTH levels after a 10 min infusion of various doses of ACTH(1–24). The results obtained in this study are quite surprising since infusion of 0.05 μ g/kg ACTH(1–24) led to peak plasma ACTH levels as high as 1 ng/ml. In addition, plasma ACTH concentrations were similar after infusion of 0.05 or 0.2 μ g/kg ACTH(1–24), suggesting that the RIA used in this study was not suitable for measuring ACTH(1–24). Oelkers *et al.* (18) have described the dose–response relationship between plasma ACTH and delta cortisol after s.c. injection of different doses of human ACTH(1–39) or i.v. injection of 30 or 100 μ g human corticotrophin-releasing hormone. A near maximal cortisol response was obtained after s.c. injection of 10 μ g ACTH(1–39), which gave plasma ACTH levels as low as 60–70 pg/ml. These values are comparable with those reported in our study. In another study, Krishnan *et al.* (19) have measured plasma ACTH(1–24) levels after the infusion of 1 or 250 μ g of this peptide over a 30 min period. The maximum ACTH concentrations during this period

were 69 and 12 000 pmol/l, i.e. much higher than in our study. We have no explanation for this discrepancy. More recently, Mayenknecht *et al.* (20) have studied the kinetics of ACTH(1–24) in plasma after injection of 1 or 250 μ g ACTH(1–24) as a bolus. They found that plasma immunoreactive ACTH(1–24) rose to more than 60 000 pg/ml (i.e. 20 000 pmol/l) after injection of the 250 μ g dose and to 1900 pg/ml (i.e. 650 pmol/l) after injection of the 1 μ g dose. The discrepancy between these results and our observation is most probably related to the methodology used by Mayenknecht *et al.* to measure plasma ACTH(1–24). Those authors have used an RIA kit designed to measure ACTH(1–39). Although the antiserum used in the assay recognizes the N-terminal sequence of ACTH, its cross-reactivity with ACTH(1–24) could be higher than with ACTH(1–39). As a consequence, this may lead to an overestimation of immunoreactive ACTH(1–24). Our findings indicate that the mean half-life of ACTH(1–24) in human blood is about 5 min. This value is lower than the half-life of ACTH(1–39) (which ranges between 11 and 24 min (21)). As a consequence, injection of 5 μ g ACTH(1–24) leads to a short-lasting peak, which does not mimic the profile of endogenous ACTH(1–39) secretion obtained after an ITT. However, we demonstrated that although the ACTH peak was higher with the 5 μ g ACTH(1–24) test, the AUCs for ACTH were comparable after injection of 5 μ g ACTH(1–24) or during an ITT. This indicates that the design of a new test with i.v. administration of ACTH(1–24) as a bolus followed by a constant infusion may be necessary to reproduce the pattern of ACTH release during an ITT.

The validity of the low-dose ACTH(1–24) test has been studied and discussed by several authors (12–14) and was not addressed in our study. Like other investigators, we found that injection of 1 μ g ACTH(1–24) could induce maximal cortisol responses in normal volunteers.

In conclusion, our data indicate that low-dose ACTH(1–24) tests do not reproduce the amplitude and duration of plasma ACTH levels reached during an ITT. Since plasma ACTH(1–24) levels reached after injection of 1 μ g of this substance are comparable to plasma ACTH diurnal changes associated with increases in cortisol secretion, we propose that this low-dose ACTH(1–24) test may be used to investigate

the physiological adrenal sensitivity. The 5 µg ACTH(1–24) test, when injected as a bolus, does not reproduce a short-lasting stressful situation, such as an ITT. However, there is a need to standardize the injection procedure and, as recently published by Murphy *et al.* (22), the direct i.v. injection used in our study is a valid approach, since significant loss of ACTH(1–24) occurs when injections are given via long plastic devices (cannula or infusion set).

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