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ACUTE ADMINISTRATION OF ALPRAZOLAM, A BENZODIAZEPINE ACTIVATING GABA RECEPTORS, INHIBITS CORTISOL SECRETION IN PATIENTS WITH SUBCLINICAL BUT NOT OVERT CUSHING'S SYNDROME

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ABSTRACT. The purpose of this study is to verify whether acute pre-treatment with alprazolam (ALP), a benzodiazepine that inhibits HPA secretion in normal subjects, could better characterize patients with subclinical Cushing's syndrome (SCS) than the 1-mg dexamethasone test (DST).

In 22 patients with SCS, 10 with overt Cushing's syndrome (CS), 11 with non-functioning adrenal incidentalomas (NF) and 14 normal subjects (NS) we studied the effect of ALP (1 mg, p.o. at 2300 hours) on cortisol levels after 1-mg DST. Cortisol levels (mean ± SEM) after DST were lower (P = 0.012) in SCS (3.9 ± 0.3 μg/dl) than in overt CS (10.4 ± 1.9 lg/dl), while they were higher (P = 0.0005) than in NF (1.1 ± 0.1 μg/dl) and NS (1.5 ± 0.1 μg/dl). After ALP pre-treatment, cortisol levels further decreased (P = 0.004) in SCS (3.0 ± 0.3 lg/dl), but neither in CS (9.3 ± 1.3 lg/dl) nor in NF (1.3 ± 0.1 μg/dl) and in NS (1.3 ± 0.1 μg/dl). In SCS, cortisol levels after ALP +1-mg DST persisted lower (P = 0.0005) than those in CS, but higher (P = 0.0005) than those in NF and NS. Considering individual cases, ALP pre-treatment reduced cortisol levels <3 and a1.8 lg/dl in 50 and 23 % of SCS patients, respectively. ALP amplifies the cortisol inhibition exerted by 1-mg DST in patients with SCS but not in those with CS. The clinical usefulness of ALP to increase the sensitivity of 1-mg DST to identify true autonomous cortisol release in patients with adrenal incidentalomas as well as to predict different clinical outcomes remains to be clarified.

Introduction

Subclinical Cushing's syndrome (SCS) is defined as a status of altered hypothalamic–pituitary–adrenal (HPA) axis secretion characterized by an autonomous and unreg- ulated cortisol secretion, not fully restrained by pituitary feedback, in the absence of the classical signs or symptoms of overt cortisol excess [1–5].

Subclinical Cushing's syndrome was initially reported around 30 years ago in patients studied for incidentally discovered adrenal masses [6, 7], the so called adrenal incidentalomas, that have become a common finding in clinical practice. In fact, the widespread use of abdominal ultrasonography, computed tomography (CT) and magnetic resonance imaging (MRI), have led to an increase in the detection of adrenal incidentalomas in recent years, rang- ing between 1 and 8.7 % [8]; the interest of SCS in this population is due to its high prevalence, ranging between 5 and 30 % of different series [4, 5].

Among the various tests used for the diagnosis of SCS, the 1-mg dexamethasone test (DST) is considered the most valuable test to screen for SCS, either alone or in combi- nation with other parameters [4, 5]. Dexamethasone is a synthetic glucocorticoid (GC) with selectivity for gluco- corticoid receptor (GR), that preferentially activates pitui- taric GRs and has been extensively employed to assess the integrity of the HPA-axis feed-back [9, 10]. It is used to evaluate the status of the HPA axis in adrenal inciden- talomas, applying concepts derived by screening of overt cortisol excess [11]. The 1-mg DST cortisol cut-off is still a matter of debate, being the cut-off of 1.8 lg/dl (50 nmol/l) considered as having the highest sensitivity [4, 5, 8, 12–14], while the cut-off of 3.0 lg/dl (83 nmol/l) has been demonstrated by some Authors as having the best com- promise between sensitivity and specificity [4]. On the other hand, the Endocrine Society Clinical Guidelines for the diagnosis of Cushing's syndrome recommended the cut-off for of 1.8 lg/dl for the initial hormonal evaluation of patients with suspected overt cortisol excess [11].

Alprazolam (ALP), a benzodiazepine (BDZ) activating c-amino butyric acid (GABA)-ergic receptors, exhibits a clear inhibitory effect on HPA axis secretion [15, 16] through an inhibitory influence on CRH- and/or AVP secreting neurons [15, 16]. In fact, in normal subjects ALP inhibits the ACTH and cortisol responses to several stimulations, such as vasopressin, metabolic and mental stress, naloxone and synthetic GH-secretagogues [17–22]. Moreover, although ALP failed to modify CRH-induced ACTH secretion from rat pituitary [15] and it does not inhibit the ACTH and cor- tisol responses to exogenous CRH in humans [19, 23], the existence of GABA/BDZ receptors in the pituitary gland suggested a direct modulatory role of

GABA and/or BDZ on corticotroph secretion induced by ACTH secretagogues other than CRH [15, 16]. Moreover, ALP did show a marked inhibitory effect even on ACTH rise induced by metyrapone or insulin-induced hypoglycemia, the most potent stimula- tions of corticotroph secretion [24, 25], as well as on HPA response to the blockade of mineralocorticoid receptors [26], clearly indicating a primary role of GABA-ergic pathways in the modulation of HPA activity in physiological conditions. There is evidence that ALP still inhibits, though with less potency, HPA response to different ACTH secreta- gogues in clinical conditions characterized by functional HPA hyperactivity, such as obesity [22], while it does not modify ACTH and cortisol responses in patients with overt Cushing's syndrome [22, 27], suggesting that the inhibitory influence of GABA/BDZ is lost in patients with organic hypercortisolism.

Interestingly, ALP has been demonstrated to also bind to the peripheral benzodiazepine receptors (PBR), structurally and functionally different to GABA receptors, that have been shown to play a critical role in steroidogenetic pro- cesses leading to the synthesis of both neurosteroids and steroid hormones in adrenal gland; moreover, they also exert a modulator influence on the activity of the anterior pituitary gland [28, 29].

Based on these premises, the aim of the present study was to verify the effect of acute ALP pre-treatment on cortisol response to 1-mg DST in patients with SCS, comparing them with patient with overt CS, non-functioning adrenal adenomas and normal subjects.

Subjects and methods

Thirty-three consecutive patients (21 F and 12 M; age 57.4 ± 1.2 years) with adrenal masses incidentally dis- covered by abdominal CT or MRI, 10 patients with overt Cushing's syndrome (CS, 9 F and 1 M; age 53.0 ± 3.2 years) due to either cortisol-secreting adenomas (n = 5) or ACTH-secreting pituitary adenomas (n = 5), and 14 normal subjects (NS, 7 F and 7 M; age 54.6 ± 3.4 years) were studied. Patients with adrenal incidentalomas were further subdivided into two groups: 1) with SCS (n = 22; 14 F and 8 M; age 58.1 ± 1.2 years); 2) with non-functioning adrenal adenomas (NF; n = 11; 7 F and 4 M; age 55.9 ± 2.9 years). The diagnosis of SCS was based on a post 1-mg-DST cortisol level [1.8 µg/dl (50 nmol/l) combined with an abnormal result in at least one of the following tests: (1) post-low dose dexamethasone suppression test (LDDST) cortisol levels [1.8 µg/dl (50 nmol/l); (2) absence of cortisol rhythm [midnight

serum cortisol [7.5 µg/dl (220 nmol/)]; (3) low ACTH levels [<5 pg/ml (1.1 pmol/l)]; (4) high UFC [[100 µg/24 h (275 mmol/ 24 h)], in the absence of clinical signs or symptoms of cortisol excess.

The diagnosis of CS as well as the differentiation between ACTH-dependent and ACTH-independent was based on International Guidelines [11]. The patient with ACTH- dependent CS underwent pituitary MRI that detected a micro-adenoma in all cases.

All the subjects gave their informed consent to partici- pate in the study, which had been approved by the local ethical committee, in agreement with the Declaration of Helsinki.

All the subjects underwent 1-mg DST (1 mg dexameth asone administered orally at 2300 hours, and blood sample drawing on the following morning at 0800 hours for deter mination of serum cortisol concentration) and alprazolam (ALP, 1 mg administered orally at 2200 hours) + 1-mg DST.

Serum cortisol levels (1 µg/dl 9 27.7 = 1 nmol/l; nor- mality, range 6.2–19.4 µg/dl) were measured in duplicate by competitive electrochemiluminescence immunoassay on cobas e601 instrument (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim, Germany), with the sensitivity of the assay that was 0.01 µg/dl and the intra- and inter-assay coefficients of variations that ranged from 3.0 to 5.7 % and from 2.4 to 6.2 %, respectively.

Plasma ACTH levels (1 pg/ml 9 0.22 = 1 pmol/l; nor- mality, range 5–46 pg/ml) were measured in duplicate by Immulite 2000 (Siemens Healthcare Diagnostics Inc., Llanberis, Gwynedd LL55 4EL, UnitedKingdom), with the sensitivity of the assay that was 5 pg/ml, and the inter-and intra-assay coefficients of variation that ranged from 6.1 to 10 % and from 6.7 to 9.5 %, respectively.

Urinary free cortisol levels (UFC, 1 µg/24 h 9 2.75 =1 nmol/d; normality, range 10–90 lg/24 h) were measured in duplicate by micro particle-based chemiluminescence auto- mated on Architect i2000 analyzer without extraction (Abbott Diagnostics, Abbott Park, IL, USA), with the sensitivity of the assay that was 1.0 µg/24 h and the inter-and intra-assay coefficients of variation that ranged from 4.2 to 8.9 % and from 4.7 to 11.5 %, respectively.

Statistical analysis

The data were expressed as mean ± SEM.

All statistical analyses were performed using Statistical Package for the Social Science (SPSS 19.0 for Windows: SPSS Inc., 1989–2005, Chicago IL, USA) [30]. A value of $P < 0.05$ was considered to be significant.

The statistical analysis was carried out by using non- parametric Mann–Whitney test for the comparison between groups and the Wilcoxon test for the comparison between two testing sessions in the same group of patients.

Correlations between cortisol levels after ALP + 1-mg DST and midnight serum cortisol, ACTH levels and UFC in SCS patients were carried out by using the Pearson correlation coefficient and multiple linear regression anal- ysis among the above variables was then performed.

Results

Cortisol levels (mean ± SEM) after 1-mg DST were lower ($P = 0.012$) in SCS (3.9 ± 0.3 µlg/dl) than in overt CS (10.4 ± 1.9 µg/dl), while they were higher ($P = 0.0005$) than those in NF (1.1 ± 0.1 lg/dl) and NS (1.5 ± 0.1 µg/dl). Considering individual cases, 1-mg DST inhibited cortisol levels <3 µg/dl in 8 SCS patients (36 %, cases 1, 3, 5, 12, 13, 14, 15, 20) (Table 1; Figs. 1, 2).

After ALP pre-treatment cortisol levels further decreased ($P = 0.04$) in SCS (3.0 ± 0.3 µg/dl), but neither in CS (9.3 ± 1.3 µg/dl) nor in NF (1.3 ± 0.1 µg/dl) and in NS (1.3 ± 0.1 µg/dl). In SCS, cortisol levels after ALP ? 1-mg DST persisted lower ($P = 0.0005$) than those in CS, but higher ($P = 0.0005$) than those in NF and NS (Table 1; Fig. 1).

Considering individual cases, cortisol levels after ALP + 1-mg DST were [3 µg/dl in all CS patients, while ALP did not further inhibit post DST cortisol levels in none of NF and NS subjects. On the contrary, ALP pre-treatment further reduced cortisol levels <3 lg/dl in 11 SCS patients (50 %, cases 4, 5, 7, 8, 9, 10, 12, 15, 17, 18, 20) and <1.8 lg/dl in 5 SCS patients (23 %, cases 4, 7, 8, 15, 18) (Table 1; Fig. 2).

In SCS a significant positive correlation between corti- sol levels after ALP + 1-mg DST and midnight serum cortisol levels was found ($r = 0.44$, $P = 0.04$), while no significant correlation between cortisol levels after ALP + 1-mg DST and ACTH levels or UFC was found.

At the multiple regression analysis, in SCS midnight serum cortisol was the best predictor of cortisol levels after ALP + 1-mg DST ($b = 0.48$, $P = 0.02$).

Discussion

The results of this study show that ALP, a benzodiazepine activating GABA-ergic receptors, amplified the cortisol inhibition exerted by 1-mg DST in patients with subclinical Cushing's syndrome, while it did not modify the cortisol levels in those with overt Cushing's syndrome.

The inhibitory effect of GABA/BDZ receptor activation on HPA activity has previously been demonstrated in both animals and humans. Among the various BDZ commonly used in clinical practice, ALP showed so far the most remarkable inhibitory effect on the HPA axis, being evi- dent on both basal and stimulated-ACTH and cortisol secretion, at least in physiological conditions [15, 16]. In fact, ALP has been shown to significantly reduce basal corticotroph and adrenal secretion either in animals [15] or in humans [16, 24, 31]. On the other hand, ALP has been shown to significantly blunt the HPA response to several stimulations; in particular, while in animals it has been demonstrated to inhibit the corticotroph response to insulin- induced hypoglicemia and serotonin-stimulated CRH secre tion [15], in humans it significantly decreases the ACTH/ cortisol response to metyrapone and insulin-induced hypo- glycemia, the most potent stimulations of corticotroph secretion [24, 25].

About mechanisms of action, either CRH- or AVP- mediated central actions has been hypothesized. In particular, ALP has been demonstrated to act at hypothalamic level, via inhibition of CRH release, being able to inhibit the ACTH and cortisol response to CRH-mediated stimuli, to AVP but not to exogenous CRH [17–20, 24]. On the other hand, other studies indicate that ALP could act via AVP-mediated mechanisms [27], although some authors did not find any significant effect of ALP on AVP release in humans [23]. It has also been proposed that ALP may influence HPA axis by acting at supra-hypothalamic level, as it has been shown to counteract the stimulatory effects of substances which reduce the GC-mediated negative feed-back by acting at supra-hypothalamic level [24, 26]. Moreover, although BDZ failed to modify CRH-induced ACTH secretion from rat pituitary [15], the existence of GABA/BDZ receptors in the pituitary gland suggested a direct modulator role of GABA and/or BDZ on cortico- troph secretion induced by ACTH secretagogues other than CRH.

Our present results showing that ALP partially amplifies the cortisol inhibition exerted by 1 mg DST in patients with subclinical hypercortisolism suggest that GABA/BDZ system still modulates cortisol secretion in these patients, indicating that this condition is not characterized by a complete ACTH independence of cortisol secretion. Con versely, in patients with overt Cushing's syndrome, both pituitary ACTH-secreting

and cortisol-secreting adrenal adenomas, ALP did not modify cortisol response to 1 mg DST, confirming previous data in literature demonstrating that these patients are not sensitive to the inhibitory effects of GABA/BZD [22, 27].

In this context, we hypothesized that ALP could be more effective in subclinical Cushing's syndrome patients with non- suppressed ACTH levels, suggesting in these patients a more clear ACTH dependence of cortisol secretion, possibly reflecting a somewhat functional HPA hyperactivation and not a true autonomous cortisol secretion. Unfortunately, we did not find a significant correlation between basal ACTH levels and suppression of cortisol after ALP + DST. This may simply reflect the small number of subjects enrolled in our study, although the hypothesis that in these patients the cortisol inhibiting effect of GABA/BZD does occur independently of ACTH levels cannot be excluded.

Our present results showing a positive correlation between cortisol levels after ALP + 1-mg DST and mid-night serum cortisol levels in SCS are in agreement with previous reports showing an individual set point of sensitivity to the mechanisms controlling HPA activity [32], again suggesting a somewhat functional HPA hyperactivation and not a true autonomous cortisol secretion in these patients; moreover, similarly to what reported in patients with overt Cushing's syndrome [11], both these hormonal alterations may be the earliest and most sensitive markers of endoge- nous glucocorticoid excess in these patients.

Finally, a peripheral effect of ALP on adrenal gland can be hypothesized. In fact, ALP has been demonstrated to act, in the adrenal gland, through PBR, structurally and functionally different to GABA receptors, that play a critical role in steroidogenetic processes involved in the synthesis of both neurosteroids and peripheral steroid hormones [28, 29]. Although PBR activation by BDZ has been demonstrated to stimulate steroidogenesis in the adrenal gland by some authors, other studies showed an inhibitory effect of BDZ on ACTH-induced steroidogenesis in ani- mals [28, 29, 33]. The inhibitory effect on adrenal secretion induced by the activation of PBR system could explain the ALP effect on cortisol response to DST in our subclinical hypercortisolemic patients, in agreement with previous data showing that ALP significantly blunts the adrenal response to low ACTH doses in humans [23]. Conversely, the absence of modulation by ALP on cortisol secretion in patients with overt Cushing's syndrome would suggest that PBR system is unable to overcome the strong stimulatory effect induced by either autonomous adrenal adenoma or exaggerated ACTH stimulation on cortisol release.

Although the clinical usefulness of ALP to increase the sensitivity of 1-mg DST in patients with adrenal inciden- talomas remains to be clarified, it's noteworthy that with the cortisol cut-off of 1.8 μg/dl (50 nmol/l), about 23 % of patients classified as affected by SCS using 1-mg DST would be considered normal after ALP pre-treatment. Moreover, by using the cutoff of 3.0 lg/dl (83 nmol/l), that represents the best compromise between sensitivity and specificity accordingly to the opinion of some expert [4], 50 % of patients classified as affected by SCS by using 1-mg DST would be considered normal after ALP pre-treatment.

Our choice of screening for SCS with a lower cut-off than those recommended for adrenal incidentalomas by international guidelines [8, 12, 14] could have led us to a decrease in specificity and, therefore, to more false diag- nosis of SCS, but it was needed for the comparison with CS patients, accordingly to The Endocrine Society Clinical Guidelines [11]. On the other hand, this was the cut-off recommended by the French Society of Endocrinology in patients with adrenal incidentalomas [13].

Recognizing the limitation of any single test of the HPA axis activity, ALP ? 1-mg DST might become a new tool for the diagnosis of SCS in patients with adrenal inciden- talomas with not concordant results provided that patients with suppression of cortisol levels after ALP ? 1 mg DST below \1.8 lg/dl show a different progression of the dis- ease, in term of hormonal, clinical and morphological parameters during a long-term follow up compared with the non-suppressing patients. On the other hand, the doubt remains as to whether the patients defined as SCS are really hypercortisolemic and whether the ALP ? 1-mg DST is really capable of distinguishing SCS from NF amongst

patients with adrenal incidentaloma.

In conclusion, this study shows that ALP amplifies the cortisol inhibition exerted by 1-mg DST in patients with subclinical hypercortisolism but not in those with overt hypercortisolism. A not complete ACTH independence of cortisol secretion and/or a peripheral adrenal inhibiting GABA/BZD effect is suggested in subclinical Cushing's syndrome patients. The clinical usefulness of ALP to increase the sensitivity of 1-mg

DST for the diagnosis of subclinical Cushing's syndrome in patients with adrenal incidentalomas as well as to predict different clinical out- comes remains to be clarified.

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Conflict of interest The authors declare that they have no conflict of interest.

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Declaration of interest

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Table 1 Anthropometric, clinical and hormonal data of patients with subclinical Cushing's syndrome

Case (n)	Sex	Age (years)	Adrenal imaging	ACTH (pg/ml)	UFC (lg/ 24 h)	Cortisol midnight (lg/dl)	Cortisol LDDST (lg/dl)	Cortisol 1-mg DST (lg/dl)	Cortisol ALP ? 1-mg DST (lg/dl)
1	F	63	L, 30 mm	10	66	8.7	3.5	2.8	3.3
2	F	62	R, 40 mm	15	72	14.6	9.6	5.6	6.0
3	F	59	R, 23 mm	5	23	2.6	3.1	2.3	3.8
4	F	55	R, 30 mm	11	32	5.5	3.6	4.2	1.7
5	F	57	L, 30 mm	18	136	6.0	4.8	2.6	2.7
6	F	51	R, 28 mm	5	57	8.0	3.1	3.1	4.2
7	F	59	R, 12 mm	19	33	6.0	2.6	4.8	1.5
8	M	63	R, 25 mm	7	63	4.2	3.3	4.4	1.7
9	M	64	R, 30 mm	9	34	5.1	5.4	3.8	2.3
10	F	58	L, 29 mm	9	30	5.0	2.4	3.4	2.6
11	M	59	R, 25 mm	19	36	3.3	6.3	7.0	3.4
12	F	61	L, 12 mm	16	38	7.0	2.7	2.4	2.7
13	M	59	R, 15 mm	11	30	4.3	3.2	2.8	4.5
14	M	59	R, 15 mm	12	30	4.3	3.2	2.8	3.6
15	F	67	R, 12 mm	11	79	4.8	3.6	2.9	1.7
16	F	58	R, 40 mm	13	35	3.6	5.0	3.8	5.0
17	M	57	R, 28 mm	10	54	4.2	3.1	4.6	2.7
18	M	53	L, 30 mm	7	91	3.1	4.5	5.3	1.7
19	F	42	R, 24 mm	18	37	6.3	2.7	6.0	5.1
20	F	49	L, 10 mm	15	45	3.7	3.6	2.5	2.8
21	M	67	R, 36 mm	18	22	5.7	4.0	4.4	4.0
22	F	56	L, 30 mm	19	128	4.7	4.2	4.5	3.1
Mean		58.1		14.1	53.3	5.5	4.0	3.9	3.0
SEM		1.2		1.5	3.7	0.5	0.3	0.3	0.3

R, right adrenal adenoma, L, left adrenal adenoma, ACTH (pg/ml; 1 pg/ml 9 0.22 = 1 pmol/l); UFC (lg/24 h; 1 lg/24 h 9 2.75 = 1 nmol/d); cortisol (lg/dl; 1 lg/dl 9 27.59 = 1 nmol/l); LDDST, low dose dexamethasone suppression test; 1-mg DST , 1 mg dexamethasone suppression test; ALP, alprazolam

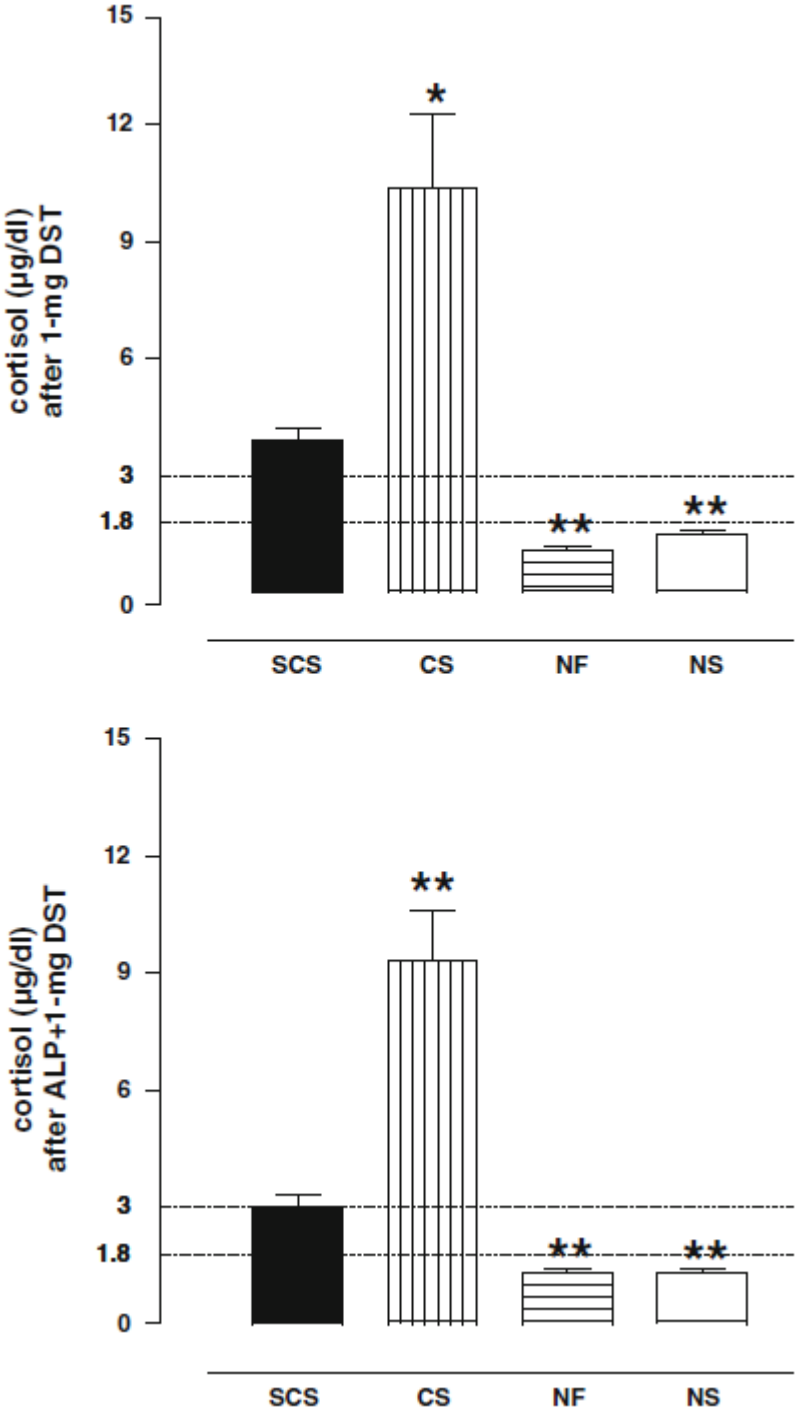

Fig. 1 Cortisol levels (mean ± SEM, 1 µg/dl × 27.59 = 1 nmol/l) after 1 mg dexamethasone suppression test (1-mg DST; *upper panel*) and alprazolam (ALP) + 1-mg DST (*lower panel*) in patients with subclinical Cushing's syndrome (*SCS*), overt Cushing's syndrome (*CS*), non-functioning adrenal adenoma (*NF*) and normal subjects (*NS*). $*P = 0.012$ (vs. SCS); $**P = 0.0005$ (vs. SCS)

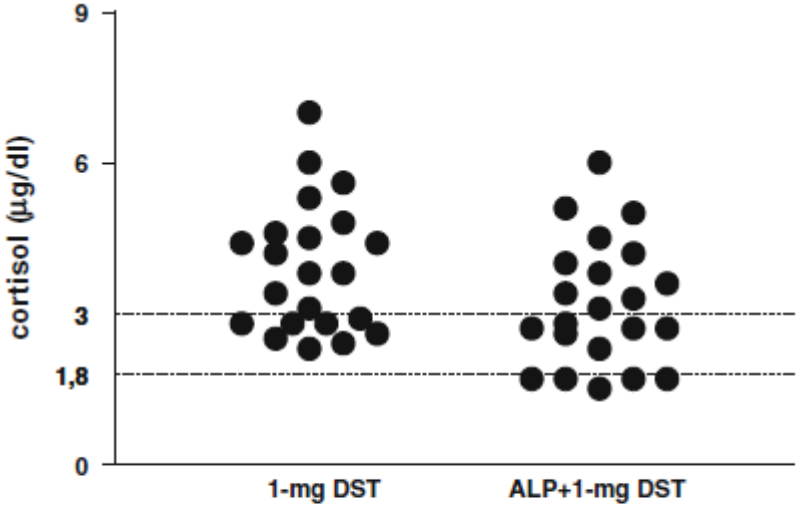

Fig. 2 Individual cortisol levels (1 µg/dl × 27.59 = 1 nmol/l) after 1 mg dexamethasone suppression test (1-mg DST) and after alprazolam (ALP) + 1-mg DST in patients with subclinical Cushing's syndrome