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The Effect of Long-term Glucocorticoid Therapy on Glucocorticoid Receptor Content and on Steroid Response to ACTH

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The effect of long-term glucocorticoid therapy for systemic diseases on glucocorticoid receptor (GR) content and on basal and ACTH-stimulated levels of plasma and salivary cortisol, 17α-hydroxyprogesterone, androstenedione, 11β-hydroxyandrostenedione, DHEA, its sulfate and sex hormone-binding globulin (SHBG), as well as on basal levels of aldosterone, was investigated in a group of 24 children treated with prednisone for at least 8 months. The therapy was interrupted 24 h before the ACTH test and before plasma and saliva sampling. The control group consisted of 21 healthy children of corresponding age and sex. The patients were divided into two subgroups with normal and subnormal basal cortisolemia, they also differed in their response to ACTH. The GR levels in patient groups were indistinguishable from those found in controls. No correlation was found between GR content and basal levels of the above steroids or their response to ACTH. The best markers, apart from basal cortisolemia, for evaluation of the degree of suppression of adrenal function appeared to be the response of salivary (but not of plasma) cortisol and 17α-hydroxyprogesterone to ACTH. Surprisingly, significantly lower levels of SHBG levels, which rose markedly after ACTH, were found in all the patients.

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INTRODUCTION

Since the introduction of glucocorticoids into the therapy of non-endocrine diseases it has become clear that these steroids considerably affect endogenous adrenal [1, 2] and gonadal [3] steroidogenesis, as well as blunt the responsiveness of the steroidogenic glands to central stimuli [4].

A compromise has been sought between the undoubted benefit of glucocorticoid treatment and its undesirable side-effects on endocrine balance. Various treatment regimens have been suggested in order to sustain regeneration of the hypothalamo–pituitary–adrenal axis (HPA). The beneficial effect of an alternate glucocorticoid therapy has been repeatedly demonstrated [5, 6]. The patients differed according to whether hypothalamo–pituitary function (as reflected by ACTH secretion) or adrenal production was recovered first, indicating that there may be different degrees of affect on HPA function [7].

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Later, with a better understanding of the molecular mechanism of steroid hormone action, the question emerged of the possible up- or down-regulation of glucocorticoid receptors (GR) by their cognate ligands [8]. While a convincing body of experimental evidence was obtained to show that glucocorticoids do, indeed, down-regulate their own receptors, the clinical studies addressing this issue were not so unequivocal. Thus, down-regulation of GR by both natural and synthetic glucocorticoids was demonstrated in cultured cells and in experimental animals [9], and, more recently, following the disclosure and cloning of the GR gene, on the level of m-RNA of this gene, as well [10–12].

Contradictory data concerning the eventual down-regulation of GR in humans under various pathophysiological conditions have recently been summarized [9]. In their study Pardes *et al.* [9] demonstrated the down-regulation of GR in mononuclear lymphocytes in 9 adult patients receiving glucocorticoid therapy for systemic diseases, but not in patients with Cushing's Syndrome.

The problem of the impact of long-term glucocorticoid therapy on endocrine balance is of particular

importance for children suffering from systemic diseases (e.g. rheumatoid arthritis, bronchial asthma, various chronic nephropathies or systemic lupus erythematosus), who are condemned to lifelong treatment. In this study with children receiving long-term prednisone therapy for the above-mentioned systemic diseases we attempted to show how this treatment influences adrenal steroid production, its responsiveness to ACTH and the GR content. As concerns the latter, the following questions were addressed: Does the long-term glucocorticoid therapy influence the levels of peripheral receptors? Do GR levels correlate with basal levels of the relevant adrenal steroids? Is there a relationship between GR content and responsiveness of the adrenal cortex to ACTH stimulus? How does long-term glucocorticoid treatment affect some of the regulatory mechanisms involved in steroid biosynthesis?

Apart from GR and selected steroids of predominantly adrenal origin, sex hormone-binding globulin (SHBG) was also followed up, with regard to its possible role as one of the links between hormonal steroids and diabetic risk factors [13].

EXPERIMENTAL

Patients

In total, 24 children and adolescents, 15 females aged 3 to 20 (mean 12.0) years and 9 boys aged 6 to 16 (mean 11.0) years were investigated. This group was composed of 16 subjects with juvenile chronic arthritis (the polyarticular form), 4 children with chronic nephrotic syndrome, 2 patients with bronchial asthma, 1 girl with systemic lupus erythematosus and 1 girl with chronic hepatitis. The basic clinical data of the patients is shown in Table 1. The children received orally 0.04–1.4 mg/kg per day of prednisone for 8–204 months (see Table 1). The therapy was interrupted 24 h before the ACTH test and blood and saliva sampling.

Controls

The control group consisted of 21 healthy children, 10 girls aged 6 to 17 (mean 10.9) years and 11 boys aged 7 to 17 (mean 11.8) years. None of them had a medical history of glucocorticoid therapy. In each case, the parents gave their informed consent to the test.

ACTH test and sample collection

The children received 0.25 mg of Synacthen (Ciba, Switzerland) i.v. at 8 a.m. Peripheral venous blood was collected before (sample 0), and 30 and 60 min after the injection. At time 0, blood was taken directly into a sterile solution of heparine in saline (1 vol per 5 vol of blood) and used immediately for separation of lymphocytes for determination of glucocorticoid receptor content. Plasma was stored frozen in aliquots at $-20°C$ until analyzed.

Saliva was collected in disposable plastic tubes with funnels at the same times as blood, and was stored as

Table 1. Basic clinical data on the patients together with individual values of GR content and basal cortisol levels

Patient No	Sex	Age	Diagnosis	Last dosage (mg/kg, day)	Duration of treatment (months)	GR (sites/cell)	Cortisol (nmol/l)	Group[a]
1	M	8	Nephrotic Sy.	1.40	32	5041	38 0	I
2	F	14	Nephrotic Sy.	0.58	97	4531	276	II
3	F	16	Bronchial asthma	0 17	41	6821	233	II
4	M	16	Bronchial asthma	0.22	65	16107	17.3	I
5	F	16	JCA	0.27	43	5444	103	I
6	M	9	Nephrotic Sy	0 22	19	10356	311	II
7	F	9	Nephrotic Sy.	0.40	43	6544	347	II
8	F	7	JCA	0 90	52	4098	241	II
9	F	10	JCA	0.18	48	5880	301	II
10	F	20	JCA	0 14	204	6119	288	II
11	F	9	Chronic hepatitis	0.60	60	6203	112	I
12	M	11	JCA	0.25	120	6959	299	II
13	F	3	JCA	0 43	8	5684	185	II
14	M	6	JCA	0 46	35	6033	363	II
15	M	13	JCA	0.36	36	7358	333	II
16	M	12	JCA	1.00	108	12449	115	I
17	M	15	JCA	0 16	63	—	587	II
18	F	12	SLE	0.23	8	5236	89 1	I
19	F	8	JCA	0.23	72	7577	387	II
20	F	8	JCA	0.05	48	7459	300	II
21	F	16	JCA	0 13	84	6512	334	II
22	F	15	JCA	0 05	96	7008	33.5	I
23	F	17	JCA	0 04	48	7702	106	I
24	M	9	JCA	0 12	38	11660	107	I

JCA, juvenile chronic arthritis, SLE, systemic lupus erythematosus.

[a]Groups I and II, patients with basal cortisolemia below and within the range of normal levels (135–605 nmol/l), respectively

above. Prior to analysis the samples were thawed, centrifuged and the clear supernatant was taken for the assay.

Analytical methods

All steroid hormones were determined by radioimmunoassay using antisera prepared in the authors' laboratory. Plasma and salivary cortisol were determined by the non-extraction method of Rolleri *et al.* [14] modified by Bičíková *et al.* [15]. Androstenedione and 11β-hydroxyandrostenedione were determined by the method of Putz *et al.* [16, 17], 17α-hydroxyprogesterone according to Hubl *et al.* [18] but using ^{3}H-labeled tracer [19], aldosterone according to the method of Jovett *et al.* [20] modified by Putz *et al.* [21], and dehydroepiandrosterone and its sulfate by the method of Dvořák *et al.* [22]. Sex hormone-binding globulin (SHBG) was measured by a non-competitive immunoradiometric assay using commercial kits purchased from Orion Diagnostics (Finland).

GRs in peripheral lymphocytes were measured by the single-point assay based on the determination of specific dexamethasone binding, requiring no more than 10 ml of fresh blood [23].

Statistical analysis

Analysis of variance was used for statistical evaluation of the differences between the groups of subjects, using Scheffe's method of contrasts. The significance of the effect of ACTH on the levels of selected hormones was determined by using both paired and unpaired Student's t-tests. Linear regression and correlation were calculated by the method of least squares.

RESULTS

The effect of long-term prednisone therapy on the basal hormone levels and on GR content

The mean basal cortisol level $\pm$SD in all the patients was 233 ± 137 nmol/l in comparison with 338 ± 119 nmol in the healthy children, i.e. still within the range of normal values reported by the authors' laboratory (135–605 nmol/l).

The patients were divided into two subgroups with basal cortisolemia below the range of normal levels (Group I) and within it (Group II). The statistical evaluation of the data showed that belonging to one of these groups did not depend on the treatment regime, i.e. on the dose or duration of the therapy.

The mean basal levels $\pm$SD of GRs in peripheral lymphocytes, of plasma and salivary cortisol, and of other hormones of interest including SHBG in the groups of patients and controls are summarized in Table 2, along with the statistical evaluation of the differences between the groups.

Individual values of basal cortisolemia and of GR are shown in Table 1, along with relevant clinical data.

No significant differences were found between the groups in the glucocorticoid receptors. Basal levels of

Table 2. Mean basal levels $\pm$ SD and range of selected hormones and of GRs in peripheral lymphocytes in children receiving long-term prednisone therapy and in healthy controls, along with statistical evaluation of the differences between the groups.

Parameter (unit)	Group I	(*n*)	Group II	(*n*)	Controls	(*n*)	Significance of differences I/C	II/C	I/II
GR (Sites/cell)	8483 ± 3883 (5041 – 16107)	(9)	6566 ± 1494 (4098 – 10356)	(14)	7397 ± 1132 (4560 – 9701)	(21)	NS	NS	NS
Plasma cortisol (nmol/l)	80.1 ± 38.9 (17.3 – 115)	(9)	319 ± 90.6 (185 – 587)	(15)	338 ± 119 (157 – 592)	(21)	**	NS	**
Salivary cortisol (nmol/l)	7.31 ± 4.80 (2.35 – 17.0)	(9)	19.2 ± 11 2 (5 01 – 49 0)	(15)	14.5 ± 4.67 (9.75 – 22.8)	(9)	NS	NS	*
17α-OH-P (nmol/l)	1.51 ± 0 82 (0 22 – 2 90)	(8)	3.36 + 2.06 (0.70 – 13.3)	(15)	4.06 ± 1.32 (2.00 – 6.11)	(15)	**	NS	NS
ADION (nmol/l)	1.20 ± 0 73 (0.34 – 2.16)	(9)	1.57 ± 1.13 (0.10 – 3.40)	(15)	2.45 ± 1.96 (0.10 – 6.27)	(12)	NS	NS	NS
11β-OH-ADION (nmol/l)	5.81 ± 4.77 (0.73 – 13.7)	(6)	7.96 ± 5.06 (1 88 – 16 1)	(10)	5 71 ± 2.65 (2.38 – 10.2)	(9)	NS	NS	NS
Aldosterone (nmol/l)	0.141 ± 0.102 (0 01 – 0.22)	(9)	0.188 ± 0.106 (0.077 – 0.39)	(13)	0 124 ± 0.114 (0 031 – 0.35)	(17)	NS	NS	NS
DHEA-S (μmol/l)	0 73 ± 0.91 (0.049 – 2.40)	(9)	0.87 ± 1 01 (0.026 – 3.10)	(15)	1 75 ± 2.25 (0.12 – 5.10)	(12)	NS	NS	NS
DHEA (nmol/l)	13.0 ± 12.1 (1.15 – 30.2)	(8)	14.6 ± 15.4 (2.00 – 64.0)	(15)	45 7 ± 28 7 (11 7 – 80.7)	(5)	*	*	NS
SHBG (nmol/l)	17.6 ± 6.03 (7 52 – 24.9)	(7)	29.8 ± 10.8 (13.8 – 42.7)	(12)	56 0 ± 29.3 (16.6 – 108)	(19)	**	*	NS

Group I, patients with subnormal basal cortisolemia, II, patients with normal cortisolemia, controls (C), healthy subjects.

*Significant at 95% level, **significant at 99% level; NS, not significant

GRs, glucocorticoid receptors; 17α-OH-P, 17α-hydroxyprogesterone; ADION, androstenedione, 11β-OH-ADION, 11β-hydroxyandrostenedione; DHEA-S, dehydroepiandrosterone sulphate; DHEA, unconjugated dehydroepiandrosterone; SHBG, sex hormone-binding globulin.

Table 3. Significance of the differences between basal and maximal ACTH stimulated levels of 5 hormonal steroids and SHBG in patients and controls

	Group I		Group II		Controls	
Hormone	Single	Paired	Single	Paired	Single	Paired
Plasma cortisol	0 0128	0 0397	0.0001	0.0000	0.0000	0.0000
Salivary cortisol	NS	NS	0.0072	0.0041	0.0000	0.0001
17α-Hydroxyprogesterone	NS	NS	0 0036	0.0016	0.0001	0.0006
Androstenedione	NS	NS	0.0475	NS	NS	0 0298
11β-Hydroxyandrostenedione	NS	NS	NS	0.0108	0.0003	0.0005
SHBG	0 0034	0.0003	0.0101	0.0018	NS	NS

P values obtained from single and paired *t*-test are given. NS, not significant

17α-hydroxyprogesterone significantly lower than in the control subjects were only found in Group I. Decreased levels of dehydroepiandrosterone, but not of its sulfate, were found in both groups of patients. Surprisingly, plasma levels of SHBG significantly lower than in the controls were also found in both groups of patients.

The effect of long-term prednisone therapy on responsiveness to ACTH

Altogether, only plasma cortisol was increased significantly after ACTH in all the groups, as confirmed by both simple and paired *t*-tests (see Table 3). Salivary cortisol, plasma 17α-hydroxyprogesterone, androstenedione and its 11β-hydroxyderivative were significantly elevated in controls and in patients with normal basal cortisolemia (Group II), but not in the children with subnormal basal cortisol levels (Group I). No significant differences were found between basal and ACTH stimulated levels of dehydroepiandrosterone and its sulfate in all the groups.

On the other hand, a significant rise of SHBG after ACTH was found in both groups of patients, with the maximum reached as early as 30 min after ACTH administration.

The effect of ACTH on the rise of cortisol, 17α-hydroxyprogesterone and SHBG is shown in Figs 1–3.

The values of the maximum rise of plasma levels of cortisol, androstenedione and 11β-hydroxyandrostenedione after ACTH did not differ significantly between the groups, contrary to salivary cortisol, where significantly lowered values were found in the patients (Table 4). Significantly lower values of plasma 17α-hydroxyprogesterone were found in Group I only.

Correlation analysis

To find out whether any relationship existed between GR content and the hormones under study, the values of GR in both groups of patients and in controls were correlated with basal, as well as with ACTH stimulated levels of the above hormones and SHBG. No significant correlation was found in any group, nor did GR correlate with the response to ACTH, as expressed by

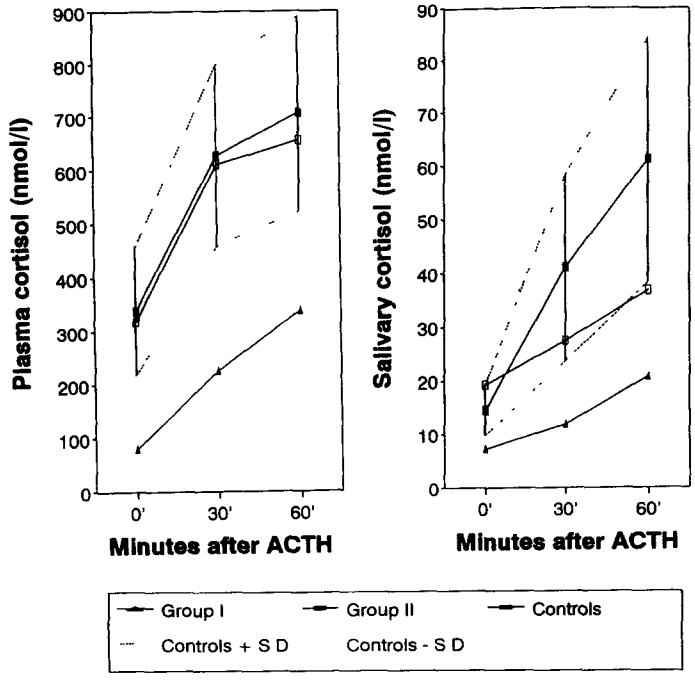

Fig. 1. Mean plasma and salivary cortisol levels before and after ACTH stimulation in patients and controls. Group I, patients with subnormal basal cortisolemia; Group II, patients with normal basal cortisolemia.

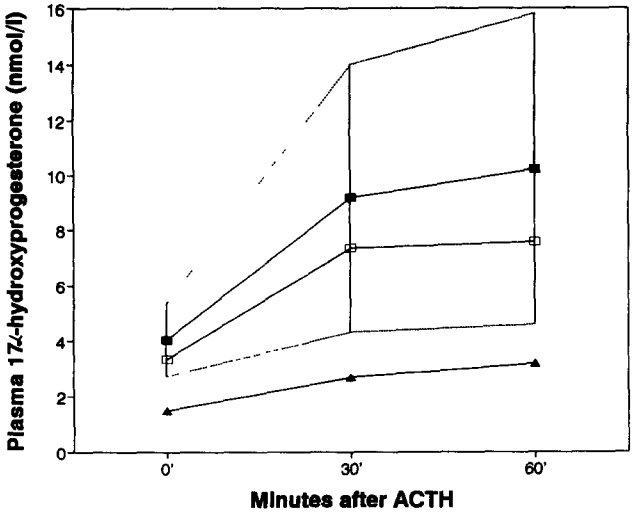

Fig. 2. Mean plasma 17α-hydroxyprogesterone levels before and after ACTH stimulation in patients and controls. The symbols and groups are the same as in Fig. 1.

the maximum increments of the ACTH-responsive hormones.

Further, the corresponding basal and maximum ACTH-stimulated levels of steroid hormones studied and of SHBG in each group were correlated with each other. An extract of the results is given in Table 5, showing the correlation coefficients for the selected pairs of hormones, where, at least in one instance, a correlation at a 99% level of significance or higher does occur.

No correlation was found between either basal or stimulated levels of the above mentioned steroid hormones, on the one hand, and aldosterone, dehydroepiandrosterone, its sulfate and SHBG, on the other. Basal levels of DHEA correlated with its sulfate in Group I only.

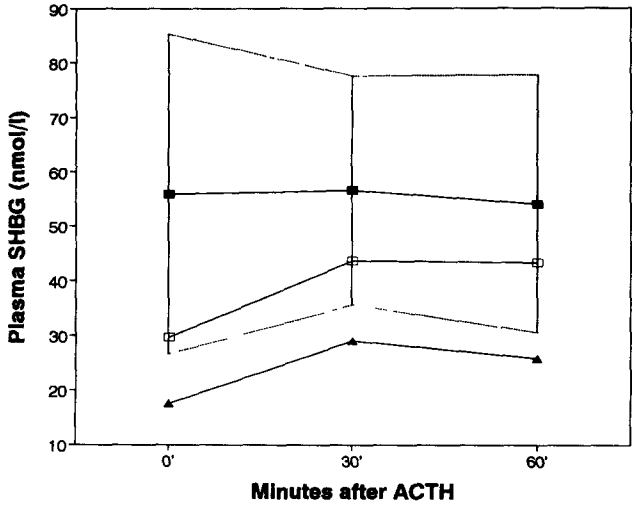

Fig. 3. Mean plasma SHBG levels before and after ACTH administration in patients and controls. The symbols and groups are the same as in Fig. 1.

Nor was any correlation obtained between the above data and either the last dose or duration of the prednisone treatment.

DISCUSSION

The decrease of GR content as early as after 1 week of treatment with various glucocorticoids, which still persisted 1 week after discontinuation of the therapy, was first reported in healthy volunteers by Schlechte *et al.* [24]. In a similar experiment, Shipman *et al.* [25] demonstrated an even more rapid fall of GR in normal subjects given exogenous dexamethasone. More recently, levels of GR slightly but nevertheless significantly lower than in the control group were found in adult patients ($n = 9$) receiving prolonged glucocorticoid therapy for systemic diseases [9].

Our results on children with systemic diseases treated for a long time with prednisone (mean duration of the therapy was 52 months, the shortest duration was 8 months) did not confirm the findings mentioned above. GR levels in these patients were indistinguishable from those found in healthy children of the same age, regardless of the severity of suppression of the adrenal function as demonstrated by basal cortisol production.

No relationship was revealed between GR content and production of the steroids studied or their responsiveness to ACTH. These findings do not support the concept of simple down-regulation of GR by corticoids and indicate that "GR control in humans may be a more complex phenomenon than that expected from animal and cell-culture studies" (Pardes *et al.* [9]).

The fact that years of therapy with glucocorticoids did not irreversibly affect the binding capacity of GR in the periphery may be considered encouraging, though it should be kept in mind that GR binding need not reflect the ability to respond to glucocorticoid treatment, as demonstrated by the indistinguishable GR content in corticoid-sensitive and -resistant patients with bronchial asthma [26].

From these results it is evident that other parameters should be followed to assess the degree of affect of the HPA function. The first marker of the extent of adrenal suppression appeared to be basal cortisolemia, according to which the patients could be divided into two subgroups.

Children with subnormal cortisolemia (Group I) also had significantly lower levels of 17α-hydroxyprogesterone and DHEA (see Table 2) and they differed considerably in their response to ACTH. Although in all but one patient of this group a distinct rise of *plasma* cortisol occurred by more than 100%, their *salivary cortisol* and 17α-hydroxyprogesterone responses were low and insignificant (see Figs 1 and 2 and Tables 3 and 4).

On the whole, closer mutual correlation of the adrenal steroids studied was found after ACTH stimulation, being more apparent in Group I patients (Table 5).

Table 4. Maximal rise (mean ± SD and range) of plasma and salivary cortisol, androstenedione, 11β-hydroxyandrostenedione, 17α-hydroxyprogesterone (in nmol/l) and SHBG after ACTH in patients and controls along with statistical evaluation of the differences between the groups

Hormone	Group I	Group II	Controls	Significance of differences I/C	II/C	I/II
Plasma cortisol	248 ± 323 (0 – 818)	345 ± 205 (101 – 766)	369 ± 135 (132 – 609)	NS	NS	NS
Salivary cortisol	14.5 ± 29.4 (0 – 82.8)	16.7 ± 19.5 (0 – 53.9)	47 2 ± 17 5 (20 2 – 74 0)	*	*	NS
17α-OH-P	1 77 ± 2.67 (0 – 7.80)	4 48 ± 4 11 (0.20 – 13.7)	6.59 ± 5.45 (1 3 – 10 1)	**	NS	NS
ADION	1.15 ± 1.69 (0 – 4.33)	1 05 ± 1 41 (0 – 5 07)	1.52 ± 1 53 (0 – 6.90)	NS	NS	NS
11β-OH-ADION	8.54 ± 7 96 (0 – 22 5)	5.92 ± 2.90 (0 – 10 5)	8 83 ± 4 98 (3 90 – 15.6)	NS	NS	NS
SHBG	10.3 ± 3 13 (5.80 – 14.3)	14 2 ± 11.2 (0 – 40 8)	0 71 ± 4 67 (−8.4 – 11.0)	NS	**	NS

*Significant at 95% level; **significant at 99% level; NS, not significant.
For abbreviations see legend to Table 2.

A new and surprising finding was the significantly lowered levels of SHBG in the patients when compared with healthy subjects. In all the patients, in contrast to the controls, an increase of SHBG occurred after ACTH administration, with the maximum at the 30th min (Fig. 3). This rise was highly significant as confirmed by both a simple and paired t-test (Table 3).

So far, controversial results were obtained concerning the effect of glucocorticoids and ACTH on SHBG. Both a decrease [27, 28] and an increase [29] of this protein following dexamethasone were reported. No effect or decrease of SHBG was described after ACTH administration to healthy children, depending on whether a short- or long-test protocol was used [30]. This was not the case for our patients receiving long-term glucocorticoid therapy, where markedly suppressed endogenous ACTH production may be anticipated. Our results suggest the possibility of a direct stimulatory effect of ACTH on SHBG, not mediated by glucocorticoids, and they require further elucidation.

CONCLUSION

The following conclusions may be drawn from this study:

—Not even long-term glucocorticoid therapy seems to affect the levels of GR, as measured by a binding assay.

Table 5. Correlation between selected basal and maximal ACTH stimulated hormone levels in patients and controls. Coefficients of correlation (r) for corresponding number of pairs (n) are given

Correlated hormone pairs		Group I r	(n)	Group II r	(n)	Controls r	(n)
Plasma/salivary cortisol	Basal	NS		NS		NS	
	ACTH	0.908***	(9)	0.853***	(14)	0.912***	(9)
Plasma cortisol/ 17α-OH-P	Basal	NS		NS		NS	
	ACTH	0 952***	(8)	NS		0.503*	(15)
Plasma cortisol/ ADION	Basal	NS		NS		NS	
	ACTH	0 950***	(8)	NS		NS	
Salivary cortisol/ 17α-OH-P	Basal	NS		NS		NS	
	ACTH	0.974***	(8)	NS		NS	
Salivary cortisol/ ADION	Basal	NS		NS		NS	
	ACTH	0 898**	(8)	NS		0 923*	(5)
Salivary cortisol/ 11β-OH-ADION	Basal	NS		0.901***	(9)	NS	
	ACTH	0 736*	(8)	0 955***	(8)	NS	
17α-OH-P/ ADION	Basal	NS		NS		NS	
	ACTH	0 932***	(8)	NS		NS	
ADION/ 11β-OH-ADION	Basal	NS		NS		NS	
	ACTH	0.971**	(5)	0 844*	(6)	0 880*	(6)
DHEA-S/DHEA	Basal	0 968**	(6)	NS		0 889*	*(5)

*, **, ***Significant at 95, 99 and 99 9% level, respectively
For abbreviations see Table 2.

—The GR binding does not correlate with basal levels or the ACTH response of the main adrenal steroids.

—The best markers of the degree of suppression of the adrenal steroid production appeared to be basal cortisolemia, the response of salivary (but not of plasma) cortisol and the response of plasma 17α-hydroxyprogesterone, respectively, to ACTH.

—Significantly decreased basal levels of SHBG, which were elevated after ACTH, were found in the patients under long-term glucocorticoid therapy in contrast to the controls. This suggests the possibility of a direct effect of ACTH on SHBG.

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