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Role of Ketoconazole Treatment in Urinary-Free Cortisol-to-Cortisone and Tetrahydrocortisol-to-Tetrahydrocortisone Ratios in Nonectopic Cushing's Syndrome

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We hypothesized that in nonectopic Cushing syndrome there is an insufficient activity of type II (renal) 11β-hydroxysteroid dehydrogenase (11β-HSD2) that is related to cortisol excess, rather than to corticotropin (adrenocorticotropic hormone [ACTH]) levels. We measured plasma ACTH and urinary-free cortisol (UFF), urinary-free cortisone (UFE), tetrahydrocortisol (UTHF), and tetrahydrocortisone (UTHE) in 24-h urine samples of 24 healthy subjects and 15 patients diagnosed with nonectopic Cushing syndrome. Then, in the group of patients, a new 24-h urine sample was collected after treatment with 800 mg daily of ketoconazole. The UFF/UFE and UTHF/UTHE ratios were calculated as an estimation of 11β-HSD2 activity. The patients had an increase in both the UFF/UFE (19.95 ± 10.3 vs 5.78 ± 4.72 nmol/24 h; $p < 0.0001$) and UTHF/UTHE ratios (5.36 ± 5.23 vs 1.39 ± 0.95 nmol/24 h; $p < 0.001$). Both UFF/UFE and UTHF/UTHE ratios decreased after ketoconazole treatment (19.95 ± 10.3 vs 12.2 ± 6.9 nmol/24 h; $p < 0.005$; and 5.36 ± 5.23 vs 1.62 vs 1.21 nmol/24 h; $p < 0.001$, respectively). The control subjects had a significant relationship between UFF and UFE ($r = 0.70$, $p < 0.0001$), and between UTHF and UTHE ($r = 0.75$, $p < 0.0001$) that did not exist in the patient group. After ketoconazole treatment, the decrease in cortisol excretion in the patient group allowed a positive and significant relation between UFF and UFE ($r = 0.64$, $p < 0.01$) and between UTHF and UTHE ($r = 0.56$, $p < 0.05$) to appear. There was not any significant relationship between either UFF/UFE or UTHF/UTHE ratios and plasma levels of ACTH.

Key Words: Cushing's syndrome; cortisol; cortisone; 11β-hydroxysteriod dehydrogenase; corticotropin; apparent mineralocorticoid excess.

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Introduction

The mineralocorticoid receptor (MR) is nonselective in vitro and binds cortisol and aldosterone with nearly similar affinity *(1,2)*. Aldosterone selectivity in renal MRs is mainly owing to type II 11β-hydroxysteroid dehydrogenase (11β-HSD2). This enzyme protects tubular MRs from cortisol by converting cortisol to inactive urinary-free cortisone (UFE) *(3,4)*. This mechanism allows selective access of aldosterone to renal MR, despite a 100-fold molar excess of circulating free glucocorticoids. Therefore, both congenital abnormalities of 11β-HSD2 (the syndrome of apparent mineralocorticoid excess) and acquired inhibition of the enzyme by liquorice or carbenexolone ingestion result in cortisol acting as a potent mineralocorticoid *(5)*.

In ectopic Cushing syndrome with high adrenocorticotropic hormone (ACTH) levels, the ratios of urinary-free cortisol (UFF) to UFE and tetrahydrocortisol (UTHF) to tetrahydrocortisone (UTHE) is increased, suggesting inhibition of renal 11β-HSD2 by ACTH or by ACTH-dependent steroids *(6–8)*. In humans, a marked increase in the cortisone/UFE ratio in urine or plasma during ACTH infusion, but not following hydrocortisone infusion *(6,9)*, has been observed. This leads to the conclusion that ACTH inhibits renal 11β-HSD2. However, Ulick et al. *(7)* favor saturation of the enzyme by cortisol excess *per se* as the cause of the high urinary-free cortisol (UFF)/UFE ratio in high ACTH states.

The measurement of UFE and the UFF/UFE ratio or the ratio between their metabolites UTHF and UTHE is a significant advance in the assessment of 11β-HSD2 activity in vivo *(10)*. In the present study, we measured UFF, UFE, UTHF, and UTHE in normal subjects and in patients with Cushing syndrome at baseline and after lowering cortisol synthesis with ketoconazole. We hypothesized that healthy subjects must have a positive and significant relation between UFF and UFE and between UTHF and UTHE excretion. The cortisol excess in patients with Cushing syndrome might cause an enzyme overload, and in this case, the reported relation would disappear. Finally, if the supposed impaired activity of the 11β-HSD2 is related to cortisol excess, when lowering cortisol synthesis using ketoconazole, the decreased

Table 1
UFF, UFE, UTHF, and UTHE
in Healthy Subjects and Patients with Cushing Syndrome[a]

	Control subjects (n = 24)	Cushing syndrome (n = 15)
UFF (nmol/24 h)	17.9 ± 15.8	145.5 ± 79.9[b]
UFE (nmol/24 h)	3.1 ± 2.5	7.3 ± 3.6[c]
Urinary THF (nmol/24 h)	311.2 ± 147.5	4970 ± 4015[d]
Urinary THE (nmol/24 h)	432.2 ± 302.2	927.6 ± 668.8[e]
UFF/UFE ratio (nmol/24 h)	5.78 ± 4.72	19.95 ± 10.3[b]
THF/THE ratio (nmol/24 h)	1.39 ± 0.95	5.36 ± 5.23[d]

[a] Values are means ± SD.
[b] $p < 0.0001$.
[c] $p < 0.0005$.
[d] $p < 0.001$.
[e] $p < 0.05$.

UFF excretion might allow a positive and significant relationship between UFF and UFE and between UTHF and UTHE to appear. Moreover, this would be independent of the pituitary (high plasma concentrations of ACTH) or adrenal origin (subnormal values of plasma ACTH) of the disease. Additionally, we investigated the relation among UFF/UFE and UTHF/UTHE ratios and plasma concentrations of ACTH and blood pressure (BP).

Results

The mean values of UFF, UFE, UTHF, and UTHE in control subjects and patients, as well as UFF/UFE and UTHF/UTHE ratios (estimations of 11β-HSD2 activity), are shown in Table 1. As expected, the excretion of all studied steroids was higher in patients than in control subjects. In particular, UFF/UFE and UTHF/UTHE ratios were higher in the patient group.

In the patient group, all the studied steroids significantly decreased after ketoconazole treatment (baseline vs postketoconazole: UFF, 145.5 ± 79.9 vs 45.4 ± 24.5 nmol/24 h, $p < 0.0001$; UFE, 7.3 ± 3.6 vs 3.7 ± 1.6 nmol/24 h, $p < 0.001$; UTHF, 4970 ± 4015 vs 805 ± 667 nmol/24 h, $p < 0.01$; and UTHE, 927.7 ± 668.8 vs 495.6 ± 308.3 nmol/24 h, $p < 0.05$). Moreover, UFF/UFE and UTHF/UTHE ratios significantly decreased (baseline vs postketoconazole, respectively: 19.95 ± 10.3 vs 12.2 ± 6.9 nmol/24 h, $p < 0.005$; 5.36 ± 5.23 vs 1.62 ± 1.21 nmol/24 h, $p < 0.001$). Mean values of ACTH did not significantly change (baseline vs postketoconazole: 9.35 ± 6.5 vs 9.74 ± 8.2 pmol/L). However, systolic, diastolic, and mean BP significantly decreased, respectively (baseline vs postketoconazole: 152.7 ± 15.8 vs 117.1 ± 11.7 mmHg, $p < 0.0001$; 103.1 ± 28.6 vs 72.7 ± 13.3 mmHg, $p < 0.0001$; and 119.2 ± 24.3 vs 87.2 ± 12.8, $p < 0.0001$).

Figures 1A and 2A, respectively, illustrate that healthy subjects had a highly significant relationship between UFF and UFE, and between UTHF and UTHE. As observed in

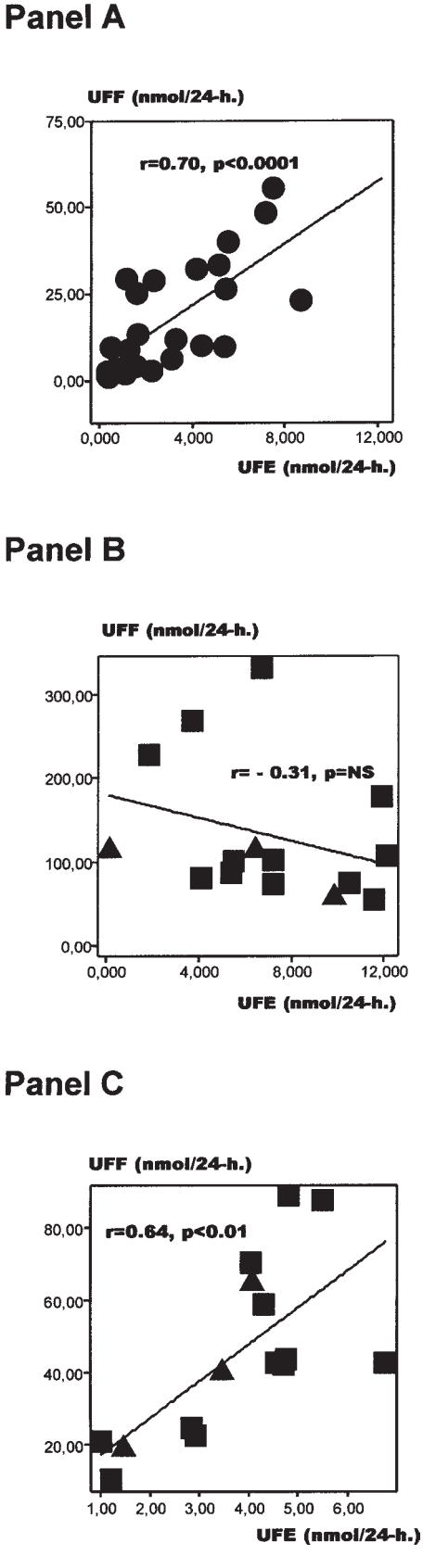

Fig 1. Relation between UFF and UFE. (**A**) Healthy subjects; (**B**) patients with Cushing syndrome at high plasma cortisol concentrations; (**C**) patients with Cushing syndrome at low plasma cortisol concentrations achieved after treatment with ketoconazole. Origin of disease: △ = adrenal; □ = pituitary.

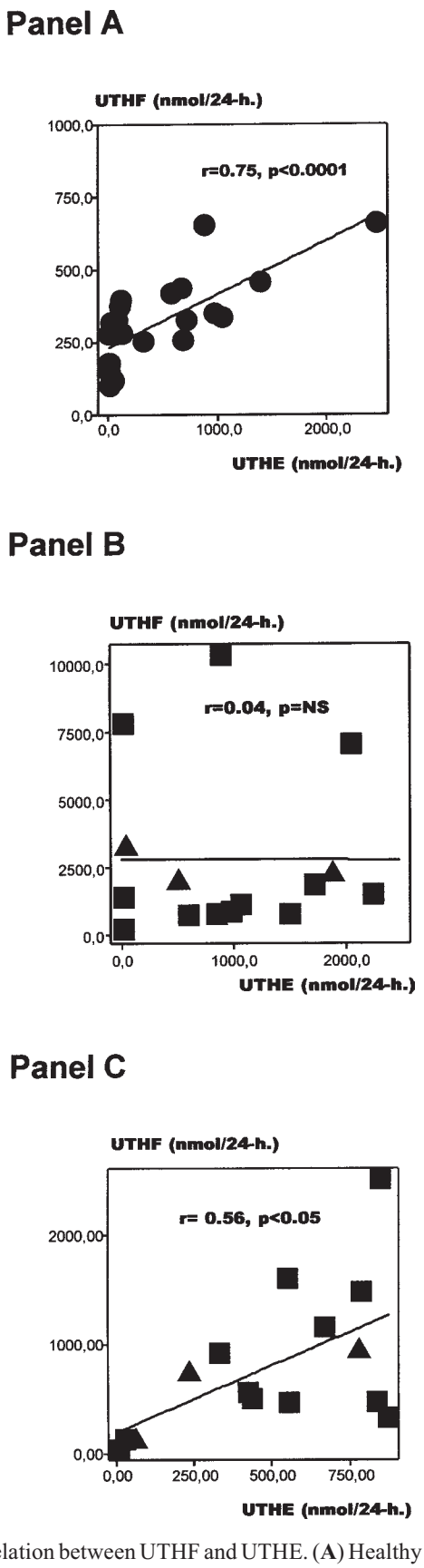

Fig. 2. Relation between UTHF and UTHE. (**A**) Healthy subjects; (**B**) patients with Cushing syndrome at high plasma cortisol concentrations; (**C**) patients with Cushing syndrome at low plasma cortisol concentrations achieved after treatment with ketoconazole. Origin of disease: △ = adrenal; □ = pituitary.

Figs. 1B and 2B, this relation did not exist in the patients with Cushing syndrome. The range of variation in UFF in the patients and control subjects was, respectively, 71.9–332.9 and 1.16–55.4 nmol/24 h. However, the range of variation in UFE in the patients and control subjects was, respectively, 0.1–12.5 and 0.3–8.7 nmol/24 h. This indicates an overlap between values of UFE in control subjects and patients. In this respect, there were 10 patients with Cushing syndrome having UFE values lower than the highest value of the control subjects despite no one having lower levels of UFF than the highest of the control subjects. When lowering levels of cortisol synthesis using ketoconazole, a marked decrease in UFF excretion allowed a significant relation between UFF and UFE and between UTHF and UTHE to appear (Figs. 1C and 2C).

Finally, there was not any relationship between 11β-HSD2 activity (estimated as UFF/UFE and UTHF/UTHE ratios) and plasma ACTH values ($r = 0.22$ and $r = 0.11$, respectively; p = NS). However, there was a positive and significant relation among the mentioned ratios and mean arterial BP ($r = 0.50$ and $r = 0.48$, respectively; $p < 0.05$).

Discussion

In ectopic Cushing syndrome with high plasma levels of ACTH, an impaired activity of the 11β-HSD2 has been described *(5–8)*. This might lead to the conclusion that ACTH inhibits renal 11β-HSD2. In this respect, it has been reported that rat adrenal cells treated with ACTH show an inhibition of 11β-HSD2 activity *(11)*. However, Diederich et al. *(12)*, working with slices prepared from human kidneys and measuring the conversion in vitro of cortisol to cortisone, did not find any effect of incubation with increasing concentrations of ACTH on 11β-HSD2 activity. Moreover, patients with adrenal Cushing syndrome (with subnormal plasma values of ACTH) have mineralocorticoid manifestations similar to that observed in 11β-HSD2 deficiency states, which cannot be explained by the action of ACTH. Finally, a deficient activity of the enzyme has also been reported in human hypertension *(13–18)*, preeclampsia *(19)*, spontaneously hypertensive rats *(20)*, Lyon hypertensive rats *(21)*, and Dahl salt-sensitive rats *(22)*, and in these conditions, the mechanism of inactivation should be other than ACTH.

In the present study, we observed that patients with nonectopic Cushing syndrome had significantly higher values of UFF/UFE and UTHF/UTHE ratios than healthy control subjects, thus suggesting an insufficient activity of renal 11β-HSD2 (Table 1). We hypothesized that this apparently decreased activity of 11β-HSD2 is related to cortisol excess rather than to ACTH. In this respect, we have observed that the studied healthy subjects showed a strongly significant relation between UFF and UFE, and between UTHF and UTHE (Figs. 1A and 2A). This indicates that more cortisol implies more cortisone excretion and, therefore, an efficient activity of renal 11β-HSD2. However, in the group of patients

with Cushing syndrome this relation was lost (Figs. 1B and 2B), and this seems to be independent of the adrenal or pituitary origin of the disease. Although the mean value of UFE was significantly higher in the patient group (Table 1), there was an overlap between values in both groups. In this respect, the ranges of variation of UFE in patients and control subjects were, respectively, 0.1–12.5 and 0.3–8.7 nmol/24 h. Consequently, it seems that there were a number of patients showing values of UFE inappropriate to that of cortisol. Those patients might have an enzyme overload, which might be responsible for the loss of relation between UFF and UFE, and between UTHF and UTHE, which was present in healthy subjects (Figs. 1A and 2A).

An alternative approach for testing the hypothesis that the mechanism of the insufficient activity of the 11β-HSD2 in Cushing syndrome is related to cortisol excess might simply be to reduce this excess. In this respect, the antifungal drug ketoconazole inhibits the adrenal production of cortisol and has proved to reduce the mineralocorticoid manifestations of patients with Cushing syndrome *(23,24)*. In this study, the patients in the Cushing syndrome group took four daily doses of 200 mg of ketoconazole for 1 wk, and then a 24-h urine sample was again collected. After ketoconazole treatment, mean values of cortisol significantly decreased, and as is shown in Figs. 1C and 2C, the impaired relation between UFF and UFE and between UTHF and UTHE was restored, resembling that observed in healthy subjects. In this situation, mean values of ACTH were not significantly different from those at baseline. Moreover, there was not any significant relationship, at baseline, between either plasma concentrations of ACTH and UFF/UFE ratio or between plasma ACTH and UTHF/UTHE ratio ($r = 0.22$ and $r = 0.11$, respectively). However, there was a significant relation among these ratios and mean BP ($r = 0.50$ and $r = 0.48$ respectively; $p < 0.05$).

All these facts taken together lead us to conclude that the studied patients with adrenal or pituitary Cushing syndrome, apparently have a deficient activity of renal 11β-HSD2, which is related to cortisol excess rather than to corticotropin levels. After ketoconazole treatment, the decrease in cortisol synthesis apparently allowed a more efficient activity of the enzyme and restored the impaired relation between UFF and UFE, and between UTHF and UTHE, then resembling that observed in healthy subjects. Moreover, this supposed impaired activity of the enzyme might influence the mechanism of the high BP observed in the syndrome, since there was a significant relationship between baseline UFF/UFE and UTHF/UTHE ratios with mean BP, and since after ketoconazole treatment, BP significantly decreased. Finally, although an apparently impaired activity of the enzyme has been previously reported in Cushing syndrome, the reason for this defective activity was unclear, and a substrate saturation or inhibition by ACTH *per se* or ACTH-dependent steroids was suggested. To date, the present study is the first to analyze UFF/UFE and UTHF/UTHE ratios at high (pre-ketoconazole) and at low (post-ketoconazole) plasma cortisol concentrations in the same group of patients with Cushing syndrome. Our results showed that the reason for the impaired activity is directly related to cortisol excess rather than to ACTH or ACTH-dependent steroids.

Materials and Methods

Subjects and Procedure

The study was conducted in accordance with the guidelines proposed in The Declaration of Helsinki and was approved by the ethical committee of our hospital.

Twenty-four healthy subjects and 15 patients diagnosed with Cushing syndrome (pituitary/adrenal origin: 12/3) participated in the study. Patients and control subjects were similar regarding age and gender (32.2 ± 10.4 vs 31.3 ± 5.8 yr and male/female ratio of 2/13 and 3/21 respectively; p = not significant for both comparisons).

Subjects with a history of alcohol abuse or having any cause of pseudo–Cushing syndrome were not included in the study. Seven days before obtaining the baseline sample, and during the treatment period (in the patient group), all subjects were advised not to consume alcohol or liquorice, use oral or topical steroids, or take drugs affecting BP.

The diagnosis of Cushing syndrome was made by measuring cortisol concentrations in 24-h urine samples (higher than 150 μg/24 h in two different measurements) in subjects with clinical signs and symptoms of the disease. Plasma and urine cortisol were measured by a competitive chemiluminiscent immunoassay (Ciba-Corning ACSTM). The etiologic diagnosis was based on plasma corticotropin values (measured using commercial IRMA kits; Nichol Institute Diagnostic) and nuclear magnetic resonance imaging or computed tomography of the adrenal or pituitary glands, according to the levels of corticotropin. A desmopressin test and an 8-mg dexamethasone suppression test were performed in all patients. Details of the patients with Cushing syndrome are given in Table 2.

After subjects were included in the study, all drugs affecting BP were discontinued for at least 7 d. BP was measured on three occasions after the subjects had rested for 10 min, and the average of the readings was obtained. The mean BP was calculated as follows and expressed in millimeters of mercury: [(2 × diastolic BP) + (systolic BP)]/3. A 24-h urine sample was collected for measurement of UFF, UFE, and UTHF and UTHE (as described later) in both control subjects and patients with Cushing syndrome.

Next, the patients took four daily doses of 200 mg of ketoconazole for 1 wk. BP was again measured, and a new 24-h urine sample was collected and processed for measurement of the mentioned steroids.

UFF, UFE, UTHF, and UTHE Measurements

Samples

Twenty-four-hour urine specimens were collected by the patients. During the collection interval, the urine was stored

Table 2
Details from Patients with Cushing Syndrome[a]

Patient no.	Age (yr)	Sex	Diagnosis	UC (μg/24 h)	PC (mmol/L)	ACTH (pmol/L)
1	27	F	Pituitary MA	756	938	11.88
2	27	F	Pituitary MA	1034	1082	19.58
3	40	F	Adrenal A	307	803	—
4	22	F	Pituitary MA	456	726	5.94
5	24	F	Pituitary MA	414	941	7.92
6	21	F	Pituitary MA	1121	877	17.16
7	31	M	Pituitary MA	1523	977	20.68
8	33	M	Pituitary MA	401	640	7.04
9	36	F	Adrenal MH	417	621	—
10	30	F	Pituitary MA	589	726	9.02
11	56	F	Pituitary MA	301	679	8.58
12	19	F	Pituitary MA	405	739	12.32
13	39	F	Adrenal A	388	764	—
14	47	F	Pituitary MA	379	640	8.36
15	31	F	Pituitary MA	523	767	11.88

[a]UC, urinary cortisol; PC, plasma cortisol; A, adenoma; MA, microadenoma; MH, macronodular hyperplasia. —, nonappreciable. The expressed values of UC were obtained by standard competitive chemiluminiscent immunoassay.

at 4ºC without preservative. Aliquots of about 10 mL were frozen until analysis.

Analytical Procedures

Before analysis, the samples were filtered and 35 μmol/L of internal standard (fludrocortisone) was added to 2 mL of urine (Merck, Darmstadt, FRG). The mobile phase consisted of two components: A (*n*-hexane) and B (*n*-hexane/isopropanol) (75/25) using a gradient from 15 to 100% of B. A flow rate of 1.3 mL/min for 60 min was achieved using a Hitachi L-6200 pump (Merck, Hitachi, Tokyo, Japan).

The absorbance of the column eluent was monitored in a UV-VIS Hitachi L-6200 detector (Merck) at 250 nm. For UTHF and UTHE, the wavelength was 220 nm.

The concentrations were calculated from peak areas of both internal standard and the different analyzed compounds in an Integrator SP-4290 (Spectra-Physics, San José, CA).

Statistical Analyses

The relationship among variables was evaluated using single linear regression analysis. Kolmogorow test was used to estimate the normality of the samples. For comparing means, the student's *t*-test for independent or dependent samples was used, and when abnormally distributed, the Mann Whitney U or the Wilcoxon test (respectively) was used. The SPSS 10.0 statistical software was utilized. All values are expressed as mean ± SD.

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