Congenital deficiency of 11β-hydroxysteroid dehydrogenase (apparent mineralocorticoid excess syndrome): Diagnostic value of urinary free cortisol and cortisone

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ABSTRACT. The syndrome of apparent mineralocorticoid excess (AME) is an inherited form of hypertension. This disorder results from an inability of the enzyme 11β-hydroxysteroid dehydrogenase (11β-OHSD) to inactivate cortisol to cortisone. The diagnosis of AME is usually based on an elevated ratio of cortisol to cortisone reduced metabolites in the urine [tetrahydrocortisol plus allotetrahydrocortisol to tetrahydrocortisone (THF+alloTHF/THE)]. The principal site of "A" ring reduction is the liver, but AME arises from mutation in the gene encoding 11β-OHSD2 in the kidney. We used a gas chromatographic/mass spectrometric method to measure the urinary free cortisol (UFF) and free cortisone (UFE) in 24 patients affected by the two variants of AME [19 with the classical form (type I) and 5 with the mild form called AME type II] in order to provide a more reproducible *in vivo* measure of the renal enzymatic activity. Type I patients were divided into two groups: children under 12 and adults. UFF levels (μg/24 h) did not differ between under-12 controls and AME type I children (mean±SD, 9±4 and 15±12, respectively), but was significantly higher in affected adults compared to controls: (62±32 *vs* 29±8, $p<0.01$). No differences were found between adult controls and AME type II patients (29±8 and 37.0±14, respectively). UFE was undetectable in 63% of AME type I and significantly lower in AME type II ($p<0.05$). As a consequence UFF/UFE ratio was significantly higher in AME type I patients both in children and adults compared to controls (AME children: 5.1±2.6; normal children: 0.43±0.2, $p<0.01$; AME type I adults: 17.7±19.6; normal adults: 0.54±0.3 $p<0.01$). For AME type II, where UFE was detectable in every case, the UFF/UFE ratio was significantly higher than adult controls (2.75±1.5 *vs* 0.54±0.3, $p<0.01$). In conclusion, our study indicates that UFE and UFF/UFE ratio are sensitive markers of 11β-OHSD2, directly reflecting the activity of the renal isozyme and readily identifying patients with AME. The presence of an altered UFF/UFE ratio in both types of AME, although with different degree of severity, calls for re-evaluation and the classification of AME as a single disorder.

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INTRODUCTION

Apparent mineralocorticoid excess syndrome (AME) is characterized by clinical features suggesting excessive production of a mineralocorticoid-like substance with hypertension, plasma volume expansion, hypokalemic alkalosis and a suppressed renin-angiotensin-aldosterone system (1, 2). Experimental and biochemical evidence indicates that affected patients have congenital deficiency of 11β-hydroxysteroid dehydrogenase (11β-OHSD) (3, 4). This enzyme interconverts cortisol (F) to its inactive metabolite cortisone (E). Since F, but not E, is a potent agonist of epithelial type I mineralocorticoid receptors, it has been proposed that impaired metabolism exposes the kidney to an excess of F which can then act as a potent mineralocorticoid (5, 6). The diagnosis of AME has been based on an abnormally high ratio

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of the two combined F urinary reduced metabolites tetrahydrocortisol+5α-tetrahydrocortisol (THF+alloTHF) to the reduced E metabolite tetrahydrocortisone (THE); nevertheless Ulick *et al.* reported an AME variant identified as type II AME, with the same clinical features as the original syndrome except for a near normal (THF+alloTHF)/THE ratio (7).

Two different 11β-OHSD isozymes were described. 11β-OHSD1 is mainly localized to glucocorticoid target tissues such as liver, lung, gonad, adipose tissue and skin (8-10). 11β-OHSD2 is predominantly expressed, together with the mineralocorticoid receptors, in the renal distal tubules and collecting ducts (11), in the distal colon, in the parotid gland and also in the placenta where it protects the fetus from excessive amounts of maternal F (12, 13).

Recently, mutations in the kidney 11β-OHSD2 enzyme have been characterized in patients affected by AME type I and II (14, 15). The less severe biochemical feature in type II patients compared to type I appear to be explained on the basis of mutations which result in some residual functional enzymatic activity.

The "net" *in vivo* conversion of F to E involves both isoforms in tissues expressing these enzymes. As AME is a disorder of the "renal" 11β-OHSD2 enzyme, a direct measure of the ratio of urinary free cortisol/cortisone fractions (UFF/UFE) should better reflect 11β-OHSD2 isozyme activity with respect to the ratio of liver reduced metabolites (THF+alloTHF)/THE. We used a Gas Chromatographic/Mass Spectometric (GC/MS) method to measure UFF and UFE in patients affected by the two variants of AME in order to provide a more reproducible *in vivo* measure of renal enzymatic activity.

METHODS

We analyzed 24-h urine collections of 24 patients suspected of AME referred to our laboratory from different countries. Five samples were from patients affected by AME type II, the clinical details of which have been reported (16, 17). Control groups include 33 adults (17 males and 16 females) aged 24.8±2.1 years and 12 children (6 males and 6 females) under 12 (mean age 9.5±1.7).

Shackleton's method was used to measure THF, allo-THF and THE (18). To measure "free" steroids, we used a recently described GC/MS method (19) suitable for inconjugated F and its 3-oxo-4-ene metabolites E, 18-OHF, 6β-OHF, 20α and 20β-DHF and 18-OXOF. We used stable isotope-labeled internal standards for E and F to provide a high degree of accuracy (trideutero-cortisone and tetradeutero-cortisol, respectively). Following addition of a deuterated internal standard cocktail composed of d_3 E (0.12 μg), d_4 F (0.18 μg), d_2 6β-hydroxycortisol (0.8 μg) and d_2 18-hydroxycortisol (0.4 μg) to 5 ml of urine, we carried out steroid extraction on a Sep-pak C18 cartridge. We added 200 ng of stigmasterol and cholesteryl-butyrate to this extract as external standard and prepared Methyloxime-trimethylsilyl ethers according to established procedures (18). Following derivatization, excess reagents were removed by lipidex chromatography. We analyzed samples in a 15-m DB1 capillary column housed in a Hewlett-Packard 5790 mass spectrometer. Hormone levels were measured by monitoring selected ions (SIM) for analytes and internal standards (m/z 531 and 534 for E, 605 and 609 for F). Relative peaks are automatically determined and a report sheet is generated giving μg/24 h of the individual compound. Validation of the method was performed by dividing the area under the curve of increasing analyte amount by the ratio between increasing amounts of the analyte and fixed concentration of the internal standard. We obtained an r=0.998 for F and an r=0.999 for E.

We analyzed all our samples according to the main parameters used to determine F metabolism in AME:

a) F/E metabolite ratio (THF+alloTHF/THE) as a measurement of "global" 11β-OHSD activity (11β-OHSD1+11β-OHSD2) as discussed above;
b) UFF/UFE ratio as a marker for the activity of 11β-OHSD2;
c) cortisol turnover quotient (THF+alloTHF+THE)/UFF represents a non-invasive method to measure F metabolic clearance. It was calculated by dividing the sum of the three major F tetra-hydroderivates by urinary UFF in a 24/h collection as described elsewhere (16, 20);
d) F "A" ring reduction constant (THF+alloTHF/UFF) represents the irreversible conversion of F to "A" ring reduced metabolites THF and alloTHF (20);
e) A-ring reduced/A-ring intact ratio (THF+alloTHF+THE)/(UFF+UFE) represents a more complete index of the total 11β-OHSD activity (11β-OHSD1+11β-OHSD2), including reduced and free fractions of E;
f) 5α/5β-tetrametabolite ratio (alloTHF/THF) shows the relative preponderance of the two main pathways of F metabolism, 5α *vs* 5β reduction.

Results are expressed as mean±SD; data were analyzed by the non-parametric Mann-Withney U test and a $p<0.05$ was considered significant.

RESULTS

UFF, UFE and UFF/UFE ratio (Table 1)

We separated type I patients into two groups: children under 12 and adults.

UFF level (μg/24 h) did not differ between under-12 controls and AME type I children (under-12 controls: 9±4; AME I: 15±12), but was significantly higher in affected adults compared to controls: 62±32 *vs* 29±8, $p<0.01$). No differences were found between adult controls and AME type 2 patients (29±8 and 37±14, respectively).

The most striking difference between AME patients and controls was the UFE value; in fact, it was undetectable in 63% (12/19) of AME type I cases and significantly lower in AME type II (adult controls: 54±22 μg/24 h; under-12 controls: 26±13; AME type II: 17±10, $p<0.05$). For calculation purpose, we arbitrarily considered the UFE value equal to the method detection limit (2 μg/24 h) in those patients where it was undetectable. The mean UFF/UFE ratio was significantly higher in AME type I patients both in children and adults compared to controls (AME children: 5.1±2.6; normal children: 0.43±0.2, $p<0.01$; AME type I adults: 17.7±19.6; normal adults: 0.54±0.3, $p<0.01$). For AME type II, where UFE was detectable in every case, the UFF/UFE ratio was significantly higher than adult controls (2.75±1.5 *vs* 0.54±0.3, $p<0.01$). Although the UFF/UFE value between AME type I and II was clearly different (17.7±19.6 *vs* 2.75±1.5), p was above the limit of significance ($p<0.06$).

Table 1 - *Urinary free cortisol (UFF) and cortisone (UFE), UFF/UFE and (THF+alloTHF)/THE ratio in patients with apparent mineralocorticoid excess (AME).*

Subject	Age	Sex	UFF	UFE	UFF/UFE	(THF+αTHF)/THE
AME type I children						
A	-	M	8	2	4	19.4
B	3	F	15	2	7.5	48.0
C	2	F	6	2	3	17.7
D	11	M	20	2	10	9.1
E	10	M	11	2	5.5	59
F	4	F	44	11	4.0	6.8
G	-	F	8	5	1.6	5.2
H	-	M	9	2	4.5	8.5
I	-	M	4	2	2	13.5
J	6	M	25	3	8.3	31.2
K	4	M	12	2	6.0	15.8
Mean±SD			15±12	3.18±2.7	5.1±2.6	21.9±17.6
Adults						
L	28	M	39	2	19.5	6.7
M	42	M	42	2	21	54.6
N	26	F	59	18	3.3	7.8
O	13	M	99	35	2.8	6.7
P	14	M	57	22	2.6	8.9
Q	28	M	122	2	61	25.4
R	31	M	45	2	22.5	46.7
S	13	F	30	5	6.0	7.0
Mean±SD	24.3±10.3		62±32	11±12.5	17.7±19.6	20.4±19.7
AME type II						
T	15	F	40	8.0	5.0	1.9
U	36	F	42	29	1.4	2.5
V	32	F	29	22	1.3	3.0
W	33	M	17	5.0	3.4	4.0
Y	32	F	57	22	2.6	2.5
Mean±SD	29.6±8.3		37±14	17±10	2.7±1.5	2.8±0.7
Controls						
Adults	34.8±2.1		29±8	54±22	0.54±0.3	1.35±0.3
Children	9.5±1.7		9±4	26±13	0.43±0.2	0.79±0.2

AlloTHF: allotetrahydrocortisol; THE: tetrahydrocortisone; THF: tetrahydrocortisol.

In patients with detectable UFE we analyzed the UFF/UFE *vs* the (THF+alloTHF)/THE ratio. A strong correlation was observed between the ratios (r=0.76 and $p<0.02$). However, neither the (THF+-alloTHF)/THE nor the UFF/UFE ratio correlated with total F metabolites (tetra-hydroderivative plus cortols and cortolones).

(THF+αTHF)/THE ratio, cortisol turnover quotient, cortisol A-ring reduction constant, A-ring reduced/A-ring intact ratio and 5α/5β metabolite ratio (Tables 1 and 2)

(THF+alloTHF/THE) ratio was significantly increased in AME type I patients, both in children and adults compared to control groups (under-12 controls: 0.79±0.2; AME children: 21.9±17.6; adult controls: 1.35±0.3; AME adults: 20.4±19.7, $p<0.01$).
This ratio was also higher in AME type II patients (2.8±0.7) compared to adult controls ($p<0.01$). Even if the absolute values between AME type I and II patients were grossly different (20.4±19.7 *vs* 2.8±0.7) we did not find a clear statistical difference ($p<0.07$).
In order to study other metabolic parameters and to compare homogenous groups, only AME type I patients over 12 were included in the calculations.
The excretion of F three major metabolites (THF, alloTHF and THE) usually decreased in both variants and, as a consequence of the normal or high excretion of UFF, F turnover quotient was markedly diminished (controls: 446±64; type I: 54±33, $p<0.01$; type II: 57±28, $p<0.03$).
F A-ring reduction constant appears to be similar in AME type I and in type II (48.5±29 *vs* 45.5±23, respectively) but it is significantly lower compared to normal subjects (256±37; AME type I, $p<0.01$, and AME type II, $p<0.05$).
We obtained similar results in AME type I and type II for A-ring reduced/A-ring intact ratio, (45.4±26 and 38.8±16, respectively), both lower than controls (66.3±31; $p<0.03$, each one).
The 5α/5β metabolite ratio appeared to be the same for type II and controls (0.66±0.28 *vs* 0.96±0.56, respectively) while for type I the ratio was higher (2.34±0.8, $p<0.01$).

DISCUSSION

The pathophysiology of apparent mineralocorticoid excess syndrome has now been satisfactorily explained in terms of its clinical, biochemical and genetic basis. An inability of the renal 11β-OHSD2 enzyme to inactivate F to E is the cause of sodium retention, plasma volume expansion, renin and aldosterone suppression and hypertension (6). Clinical data show an improvement of the disorder after inhibition of the endogenous F secretion by means of dexamethasone treatment (21, 22). No defects in the gene encoding 11β-OHSD1 in the liver were found in AME patients (23); in contrast, mutations were found in the renal 11β-OHSD2 isoform in AME type I (17, 24). Recently, a point mutation in exon 5 of 11β-OHSD2 gene in AME type II patients and a genetic defect resulting in mild low-renin hypertension have been found (15, 25), also explaining the decreased activity of the enzyme and the elevated UFF/UFE ratio. Evidence also suggests that in AME not only there is reduced 11β-OHSD activity, but also reduction in 5β-reductase activity and/or generalized steroid A-ring reduction (16, 20, 26).
In this paper we analyze in more detail the biochemical aspects of the syndrome. In the light of our results, the decision to assign the individual patients to AME type I and type II group appears to be rather arbitrary. The only possible basis was the (THF+alloTHF)/THE ratio, but it is evident that some patients (M, Q and R) have a highly significant different UFF/UFE and (THF+alloTHF)/THE ratio when compared to the other 10 adult AME type I and type II patients (Fig. 1). At the beginning of the study, we decided to consider type II the patients already described to have this variant (7, 17) (samples kindly referred to us by Dr. R. Tedde and Dr. A. Pala, University of Sassari, Italy).
The diagnosis of AME is usually based on a high ratio of F to E reduced metabolites in the urine (THF+alloTHF/THE) but, since the reduction of "A" ring takes place mainly in the liver, the (THF+alloTHF)/THE ratio reflects the liver set-point of F to E conversion but does not directly reflect the activity of the renal 11β-OHSD2 enzyme. We have

Table 2 - *Main biochemical parameters in apparent mineralocorticoid excess (AME).*

Subjects	F turnover quotient	A-ring reduction constant	A-ring red./intact ratio	5α/5β ratio
Adult controls	446±64	256±37	66.3±31	0.96±0.56
AME type I (adults)	54±33**	48.5±29**	45.4±26*	2.34±0.8**°°
AME type II	57±28*	45.5±23*	38.8±16*	0.66±0.28

*$p<0.05$ *vs* controls; **$p<0.01$ *vs* controls; °°$p<0.01$ *vs* controls and AME type 2.

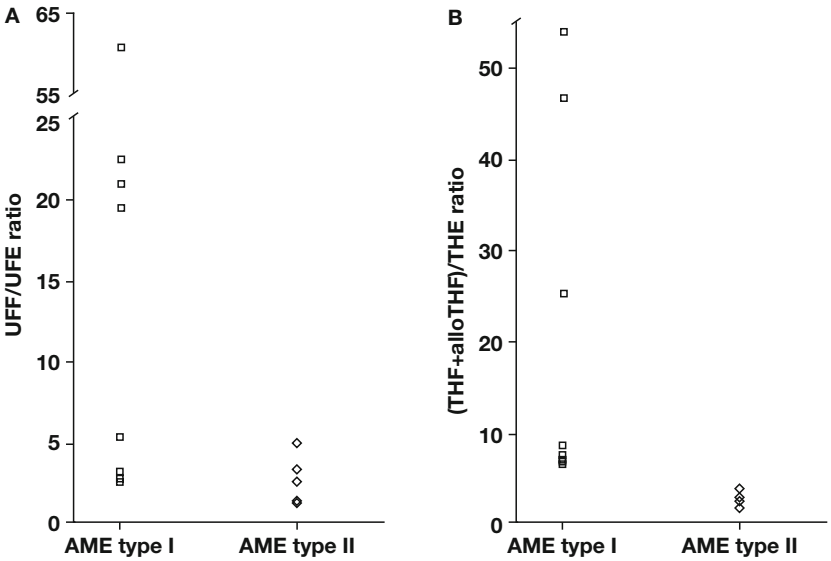

Fig. 1 - *UFF/UFE ratio (A) and (THF+alloTHF)/THE ratio (B) in AME type I and AME type II patients. AlloTHF: allotetrahydrocortisol; AME: apparent mineralocorticoid excess; THE: tetrahydrocortisone; THF: tetrahydrocortisol; UFE: urinary free cortisone; UFF: urinary free cortisol.*

used UFE as a new and more accurate marker of peripheral metabolism of cortisol. We measured the UFF/UFE ratio in a large group of AME type I patients and five type II patients. UFE level was lower in both groups although with different degrees of severity. Both groups had normal or elevated UFF level and, as a consequence, the ratio UFF/UFE was higher than controls.

Considering the (THF+alloTHF)/THE ratio, we observed significantly higher values in both AME forms compared to controls. In the original paper describing AME type II (7) and in the subsequent works (16, 20) Ulick *et al*. found no differences between controls and patients. This result may reflect the relatively small number of controls and patients selected and the fact that patients and controls came from different laboratories. The presence of altered (THF+alloTHF)/THE and UFF/UFE ratios in both type of AME, although with different degree of severity, and the wide overlap in UFF/UFE ratios between individual type I and type II patients (Fig. 1) call for a reclassification of AME as a single disorder. The recent finding of a point mutation in the 11β-OHSD2 gene in an AME type II family, similar to those demonstrated in AME type I patients, would confirm this theory (15). Interestingly, unlike other 11β-OHSD2 mutations, in AME type II the mutant enzyme retains some activity, in keeping with the reported correlation between genotype and phenotype in this condition (27).

In our study, THE was always detectable in both AME type I and II patients, although 12 subjects had undetectable UFE in AME type I variant (detection limit for both methods is 2 μg/24 h). This suggests that UFE may be more sensitive than THE in the diagnosis of AME.

Is 11β-OHSD deficiency a frequent disorder? About 80% of hypertensive patients are labeled as having "essential hypertension". Within the broad group of patients with low-renin hypertension there may be individuals who have an undiagnosed form of 11β-OHSD deficiency. Our preliminary study in a non-selected normotensive and hypertensive population in Sardinia (Italy) has identified a heterozygote state for AME type 2 mutation in about 1:40 individuals, suggesting that other homozygous affected subjects will exist (A. Li *et al*. unpublished observation). Hypertension has a prevalence of 15% in Western countries, so a low-cost, rapid screening method is required. GC/MS is a time-consuming and expensive methodology to measure reduced metabolites and it is not suitable to screen a large number of hypertensives; on the other hand, radioimmunoassay methods for free F and E measurement in urine are now available. The study confirms the relative preponderance of α with respect to β reduced metabolites in the type I variant (26), but the significance of this minor difference in steric conformation of F metabolites is uncertain. The other parameters considered (cortisol turnover quotient, F A-ring reduction constant and A-ring reduced/A-ring intact ratio) appeared similar in AME variants and different from controls. This suggests that the severity of F metabolic derangement as seen in AME type I has a detrimental effect on 5-α reductase, rather than a co-existing defect in that enzyme *per se*.

In conclusion, our study indicates that UFE and the UFF/UFE ratio are sensitive markers of 11β-OHSD2, directly reflecting the activity of the renal isozyme. The UFF/UFE ratio readily identifies patients with AME. Together with the other biochemical parameters considered in the study, the UFF/UFE ratio demonstrates that the syndrome is a continuum of biochemical and clinical abnomalities and that a single classification that would take into account all 11β-OHSD defective activities (both reductase and oxidative) could better reflect the real nature of AME.

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