Apparent activities of 21-hydroxylase, 17α-hydroxylase and 17,20-lyase are impaired in adrenal incidentalomas

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Abstract

Objective: An increased response of 17-hydroxyprogesterone to ACTH stimulation has been observed in adrenal incidentaloma and linked to an impairment of either 21-hydroxylase or of 11β-hydroxylase activity. To analyse this question further, we investigated the steroidogenic pathways in a series of 17 adrenal incidentalomas.

Design and Patients: 17 patients (7 women, 10 men; mean age, 62 ± 12 years) with non-histologically analyzed adrenal incidentalomas were prospectively evaluated.

Methods: The following variables were investigated: 24-h urinary methanephrines and free cortisol excretion; plasma levels of ACTH and dehydroepiandrosterone; overnight dexamethasone suppression test; 1–24 ACTH stimulation test with measurement of: cortisol, 11-deoxycortisol, 17-hydroxyprogesterone, aldosterone, 11-deoxycortosterone, progesterone, 17-hydroxypregnenolone, Δ4-androstenedione, dehydroepiandrosterone and 21-deoxycortisol.

Results: Discordant features of subclinical hypercorticism were noted in one case. No patient had dehydroepiandrosterone sulfate levels in the normal range for his or her age. Peak 17-hydroxyprogesterone and peak 21-deoxycortisol disclosed impairment of 21-hydroxylase in 11 and 10 cases respectively. An increased 11-deoxycortisol/cortisol ratio identified reduced activity of 11β-hydroxylase in 11 patients. Eight patients displayed features of mild 17,20-lyase impairment, which was related to 21-hydroxylase dysfunction. Whereas only 2 patients showed no enzyme modification, 9 displayed alterations of at least two pathways.

Conclusion: In our hands, a combination of enzyme dysfunction was frequently observed. Shared biochemical mechanisms could explain combined 17,20-lyase and 21-hydroxylase alterations, whereas coexistence of 21-hydroxylase (particularly when based on peak 21-deoxycortisol) and 11β-hydroxylase is more puzzling.
Subjects and methods

Patients

Seventeen patients (7 women, 10 men; mean age, 62 ± 12 years; range, 38–88 years) with asymptomatic incidentally discovered adrenal tumors were prospectively evaluated. Ultrasound or CT scans of the abdomen set up for extraterritorial complaints discovered the adrenal tumors. These patients were recruited during the period 1997–1998 and were either diagnosed in our department or in other departments of our university hospital. When diagnosis was made during an ultrasound procedure, patients were subsequently studied by CT scan with intravenous contrast medium. The non-adrenocortical nature of the mass was eventually proven for three patients (paraganglioma, pheochromocytoma, and metastatic neoplasm). Thus, these subjects were not included in this series. Investigations were performed according to the Helsinki Declaration and had been reviewed by an ethical committee.

Endocrine assessment

Adrenal function, as standard laboratory blood tests, was assessed using routine techniques. The following parameters were obtained: (i) 24-h urinary methanephrine excretion, (ii) 24-h urinary free cortisol excretion (85–280 nmol/24 h), (iii) baseline serum cortisol and ACTH at 0800 h and 2300 h, (iv) aldosterone and active renin in the supine position and after remaining standing for 2 h, (v) serum DHEA-S at 0800 h (female: 0.3–9 μmol/l, male: 0.3–12 μmol/l), (vi) overnight low dose dexamethasone test (1 mg, orally, at 2400 h and measurement of serum cortisol at 0800 h on the following morning), (vii) ACTH stimulation test (i.v. bolus of 250 μg tetracosactide) started between 0800 h and 0900 h with blood samples taken at 0 and 60 min for measurement of the following hormones: progesterone, 17-hydroxypregnenolone (17-OHG), 17-OHP, Δ4-androstenedione (Δ4-A), dehydroepiandrosterone (DHEA), 11-deoxycorticosterone (DOC), aldosterone, 11-deoxy cortisol (S), cortisol (F), and 21-DF.

Catecholamine metabolites levels were determined by high-pressure liquid chromatography. Plasma ACTH was determined with an immunoradiometric assay (Nichols, Avon, UK). Active renin (Sanofi-Pasteur, Marnes la Coquette, France), aldosterone (Immunotech, Marseille, France), DHEA-S (Immunotech), Δ4-A (Immunotech), 17-OHP (Chiron, Cergy-Pontoise, France) and free urinary F (Chiron) were measured by RIA. Progesterone and F levels were determined by chemiluminescent techniques (Chiron). DOC, 21-DF, DHEA, and 17-OHG were measured as previously published (21) by RIA, after plasma extraction and chromatography on celite microcolumns. The inter-assay coefficients of variation (CV) were between 4–8% for the commercial techniques, and between 4.4 and 9.6% for the other steroids (DOC, 21-DF, DHEA, 17-OHG).

An adequate dexamethasone suppression test was demonstrated when morning F fell below 100 nmol/l (36 μg/l). Impaired 21-hydroxylase activity was assumed when the poststimulation 17-OHP level exceeded 15 nmol/l (22). Poststimulated 21-DF levels over 2.2 nmol/l were taken as indicative of 21-hydroxylase impairment (20, 21). A post-stimulative S/F ratio above 0.12 was taken as indicative of an altered 11β-hydroxylase activity; this cut-off value was determined from previously published studies (12, 23–25). Similarly, it was assumed that 17,20-lyase was impaired when the 17-OHGS/DHEA ratio and the 17-OHP/Δ4-A ratio after stimulation were above 3.0 (26–30).

Statistical analysis

Non parametric methods were preferred because criteria of the so-called normal distribution were not satisfied. Therefore, Wilcoxon or Mann-Whitney rank sums test, and Spearman correlation methods were used as appropriate. The level of statistical significance was set at P < 0.05. Data are expressed as means ± 1 s.d.

Results

Clinical data and routine laboratories results

Pertinent clinical data for the patients and CT scan features of their adrenal masses are depicted in Table 1. The size of the mass averaged 27.1 mm (15–60 mm). Whereas these features did not trigger the imaging procedure, overweight (body mass index (BMI) > 27 kg/m²), high blood pressure (antihypertensive treatment) and diabetes (oral antidiabetic agents or insulin treatment) were present, respectively, in 47, 42 and 42% of the cases, and as compared with the general population clearly overrated. The largest diameter and the CT scan density of the adrenal mass were not correlated with any of the measured variables.

Urinary excretion of methanephrenes and free F were in the normal ranges for all the subjects. Serum F after 1 mg dexamethasone was below 100 nmol/l in all but one patient in whom this increased level (370 nmol/l) was the sole evidence of pre-clinical Cushing’s syndrome (patient 15). Considering the results of Orentreich et al. (31), it appeared that the DHEA-S level scored low for age for every patient. The plasma DHEA-S level was not correlated with age, whereas it was negatively correlated with aldosterone (P = 0.031), 17-OHP (P = 0.043), 21-DF (P = 0.037), and DOC (P = 0.037) increments after stimulation. No pertinent correlation was identified for the post-dexamethasone F level.

Investigation of apparent steroidogenic enzymes activities

21-Hydroxylase The potency of 21-hydroxylase was examined by at least three variables: the post-ACTH level of 17-OHP and of 21-DF, and the ratio of 17-OHP/S.
As shown in Table 2, peak 17-OHP levels were above the normal range (15 nmol/l) in 11 patients and above 30 nmol/l (i.e. levels observed in non-classical form of 21-hydroxylase deficiency) in 5 cases. The figure for 21-DF was similar (Table 2), with only one patient (patient 16) under the upper limit of normal for our laboratory (1.5 nmol/l) and 10 subjects with peak 21-DF levels above the cut-off of 2.2 nmol/l, a level that has been shown to differentiate heterozygotes for 21-hydroxylase deficiency from non-affected subjects.

In contrast and as shown in Fig. 1, only 2 subjects displayed 17-OHP/S ratios compatible with impaired 21-hydroxylase activity (i.e. above 4) after 1–24 ACTH stimulation. This discrepancy was readily explained by a concurrent increase of plasma S concentration, thus minimizing increased peak 17-OHP levels.

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As shown in Table 3, comparison of clinical and hormonal data of patients defined by an apparent impaired activity of 21-hydroxylase versus patients with other altered tests did not disclose any differences apart from the identifying variables (peak 17-OHP, and peak 21-DF).

Peak 17-OHP was positively correlated with peak 21-DF ($P=0.009$). Peak 17-OHP and peak 21-DF were negatively correlated with BMI ($P=0.011$ and $P=0.02$ respectively) and supine aldosterone ($P=0.021$ and $P=0.034$ respectively). Finally, peak 21-DF was also correlated to post-dexamethasone F levels ($P=0.045$).

11β-Hydroxylase After stimulation, S levels were elevated in 12 instances, and were frankly increased (above 20 nmol/l) in 4 of these subjects (Table 2). An impaired apparent activity of 11β-hydroxylase, as defined by an S/F ratio above 0.012, was observed for 11 patients (Fig. 1). When subjects with an impaired activity of 11β-hydroxylase were compared with the other patients (Table 3), the only significant difference (besides baseline and peak S levels) was the largest diameter of the adrenal mass ($P=0.025$). Aside from a positive relation between peak S levels and the size of the

| Table 1 Clinical data of patients with adrenal incidentaloma. Average results are expressed as means ± 1 s.d. and range. |
|---|---|---|---|---|---|---|---|
| Patient | Sex | Age (years) | Sex | Size (mm) | Density (Hounsfield unit) | BMI (kg/m²) | High blood pressure† | Diabetes† |
| 1 | M | 73 | Left | 60 | – | 34.7 | No | No |
| 2 | M | 72 | Bilateral | 15/20 | – | 28.1 | Yes | Yes |
| 3 | M | 52 | Right | 20 | 5 | 29.8 | Yes | Yes |
| 4 | M | 64 | Right | 36 | 10 | 23.8 | No | No |
| 5 | M | 67 | Left | 30 | 36 | 29.1 | Yes | Yes |
| 6 | F | 49 | Left | 15 | – | 26.1 | Yes | No |
| 7 | F | 72 | Right | 30 | 0 | 22.2 | Yes | No |
| 8 | F | 38 | Left | 15 | –8 | 27.1 | No | No |
| 9 | F | 65 | Bilateral | 15/15 | – | 20.1 | Yes | No |
| 10 | F | 88 | Right | 28 | 20 | 17.1 | No | Yes |
| 11 | F | 58 | Bilateral | 35/28 | 2 | 21.9 | No | No |
| 12 | M | 66 | Left | 40 | –13 | 26.5 | Yes | Yes |
| 13 | M | 55 | Bilateral | 20/15 | – | 27.4 | No | No |
| 14 | M | 62 | Left | 23 | 25 | 25.1 | Yes | Yes |
| 15 | F | 47 | Bilateral | 35/25 | – | 33.5 | No | No |
| 16 | M | 58 | Bilateral | 15/12 | – | 32.1 | Yes | No |
| 17 | M | 67 | Left | 23 | 57 | 28.2 | Yes | No |
| 10 | M | 62 | Right | 27.1 ± 11.8* | 13.4 ± 21.4 | 26.6 ± 4.7 | 9 Yes | 7 Yes |
| 7 | F (38–88) | Left | (15–60) | (15–60) | (17–34.7) | 52.9% | 42.2% |
| 6 Bilateral | 21.4 | 26.6 | 4.7 | 9 Yes | 7 Yes |
| 8 | No | 10 No |

* The largest mass was considered in the case of bilateral masses; † high blood pressure and diabetes were defined by the medical history and the therapeutic regimen of the patient.

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mass \((P = 0.027)\), no pertinent correlation was identified for peak \(S\) level or \(S/F\) ratio.

**3β-Hydroxysteroid dehydrogenase** The apparent activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) did not appear to be altered as judged by the DHEA/D4-A and 17-OHG/17-OHP ratios, which were all below the upper normal limit (Fig. 1).

**17α-Hydroxylase/17,20-lyase** Normal baseline progesterone levels and absence of increased progesterone/17-OHP ratios after stimulation (Fig. 1) were against an impairment of 17α-hydroxylase activity. Apart from low DHEA-S levels, as previously mentioned, baseline DHEA and D4-A levels were found to be within normal limits for all our patients. After ACTH stimulation there was an increase in these two variables, 178 ± 67% and 162 ± 92% respectively. In the basal state, increased 17-OHG/DHEA (i.e. > 1.3) and/or increased 17-OHP/D4-A (i.e. > 1.5) ratios were found to be slightly altered in 8 patients. After ACTH stimulation, an apparent impaired activity of 17,20-lyase identified by an abnormal ratio of 17-OHG/DHEA, or of 17-OHP/D4-A (> 3.0), both in the basal condition and after stimulation, was observed in 8 cases. These so-called deficient patients when compared with the other patients differed for the following parameters: supine aldosterone \((P = 0.027)\), post-dexamethasone \(F\) level \((P = 0.036)\), peak 21-DF \((P = 0.012)\) and baseline DHEA \((P = 0.041)\) levels. Although DHEA and D4-A appeared to be correlated at
Table 3: Comparison of patients with and without apparent impairment of steroid enzymes activities. Results are means ± s.d.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Normal</th>
<th>Impaired</th>
<th>Normal</th>
<th>Impaired</th>
<th>Normal</th>
<th>Impaired</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59.3 ± 10.1</td>
<td>63.4 ± 12.9</td>
<td>57.0 ± 13.2</td>
<td>62.4 ± 12.1</td>
<td>58.4 ± 11.4</td>
<td>65.9 ± 11.7</td>
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<tr>
<td>Largest diameter (mm)</td>
<td>29.7 ± 16.9</td>
<td>25.6 ± 8.7</td>
<td>17.0 ± 4.0</td>
<td>&lt;0.05</td>
<td>24.0 ± 9.2</td>
<td>30.5 ± 14.1</td>
</tr>
<tr>
<td>DHEA-S (μmol/l)</td>
<td>1.15 ± 0.7</td>
<td>1.00 ± 0.7</td>
<td>1.35 ± 0.07</td>
<td>&lt;0.05</td>
<td>1.06 ± 0.78</td>
<td>1.04 ± 0.72</td>
</tr>
<tr>
<td>21-Hydroxylase</td>
<td>253 ± 77</td>
<td>142 ± 112</td>
<td>208 ± 105</td>
<td>126 ± 105</td>
<td>236 ± 113</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>11-Hydroxylase</td>
<td>58 ± 8</td>
<td>92 ± 100</td>
<td>41 ± 20</td>
<td>108 ± 107</td>
<td>52 ± 21</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>17,20-Lyase</td>
<td>4.7 ± 1.2</td>
<td>4.6 ± 3.1</td>
<td>4.7 ± 1.6</td>
<td>5.0 ± 2.8</td>
<td>3.7 ± 1.5</td>
<td>5.6 ± 3.2</td>
</tr>
<tr>
<td>F post DXM (nmol/l)</td>
<td>13.5 ± 0.9</td>
<td>&lt;0.01</td>
<td>24.3 ± 11.1</td>
<td>26.9 ± 16.0</td>
<td>17.7 ± 6.2</td>
<td>32.9 ± 16.4</td>
</tr>
<tr>
<td>Peak 17-OHP (nmol/l)</td>
<td>0.5 ± 0.2</td>
<td>0.7 ± 0.3</td>
<td>0.63 ± 0.27</td>
<td>0.64 ± 0.29</td>
<td>0.52 ± 0.14</td>
<td>0.79 ± 0.32</td>
</tr>
<tr>
<td>Peak 21-DF (nmol/l)</td>
<td>20.0 ± 0.9</td>
<td>&lt;0.05</td>
<td>3.8 ± 2.9</td>
<td>9.0 ± 8.9</td>
<td>2.7 ± 1.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Baseline 17-OHP (nmol/l)</td>
<td>14.0 ± 3.5</td>
<td>11.4 ± 4.3</td>
<td>7 ± 0.1</td>
<td>&lt;0.01</td>
<td>13.8 ± 3.4</td>
<td>12.4 ± 9.5</td>
</tr>
<tr>
<td>Peak S (nmol/l)</td>
<td>16.7 ± 6.5</td>
<td>15.0 ± 9.2</td>
<td>7 ± 0.1</td>
<td>&lt;0.01</td>
<td>18.5 ± 7.6</td>
<td>14.5 ± 7.2</td>
</tr>
<tr>
<td>Peak DHEA (nmol/l)</td>
<td>5.9 ± 5.2</td>
<td>3.8 ± 3.2</td>
<td>5.8 ± 4.9</td>
<td>4.5 ± 4.3</td>
<td>6.9 ± 4.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Baseline DHEA-S (nmol/l)</td>
<td>14.6 ± 13.6</td>
<td>18.0 ± 10.8</td>
<td>12.8 ± 11.1</td>
<td>18.8 ± 7.6</td>
<td>17.8 ± 12.5</td>
<td>7.8 ± 4.0</td>
</tr>
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Post DXM, cortisol after 1 mg dexamethasone test. Peak, level measured 60 min after ACTH injection.

Relation between the various enzymatic alterations

When these 17 patients were analyzed as a group it appeared that 6 showed evidence as a group. It is evident that the hallmarks of the various enzymatic activities that were observed in our series are in agreement with published literature. The main result of this study rests on the complex of increased plasma 17-OHP response to ACTH stimulation. With the exception of DHEA-S that was observed, with no patients displaying the hallmarks of the various enzymatic activities. No relation could be drawn between 21-hydroxylase impairment or between 17,20-lyase and 11β-hydroxylase activities. No relation was identified by the positive relation between baseline 17-OHP/17-AH and the peak 21-DF levels (P=0.016).

Discussion

Whereas many features of our series are in agreement with published literature, some observations are quite surprising. It was identified by the positive relation between post-17-OHP/17-AH and the peak 21-DF levels (P=0.016). This series is remarkable for the very low level of adrenal autonomy observed, that is generally described with only one patient out of the 17 showing evidence of hypercortisolism. In many earlier studies (3–11), the prevalence of increased plasma 17-OHP response to ACTH stimulation has been pointed out to be between 30–70%. This finding has been pointed out to be much lower than the 15% that is generally observed, with only one patient out of the 17 showing evidence of hypercortisolism. In many earlier studies (3–11), the prevalence of increased plasma 17-OHP response to ACTH stimulation has been pointed out to be between 30–70%.
Altered steroidogenesis in adrenal incidentalomas

Wide range may reflect recruitment biases, or differences in laboratory techniques and, most likely, the use of non-homogenous criteria to define an abnormal 17-OHP response to ACTH stimulation. In agreement with these findings (3–11), the peak 17-OHP level was shown to be above 15 nmol/l in 11 patients (65%) and was frankly elevated (i.e. above 30 nmol/l) in 5 subjects (29%). Conversely, only one subject displayed a peak 21-DF value in the normal range whereas ten had levels above 2.2 nmol/l, a cut off that has been shown to separate normal subjects from heterozygous or homozygous carriers of 21-hydroxylase deficiency (20, 21). Although peak 17-OHP and peak 21-DF provide comparable results, we suggest that for individual patients peak 21-DF determination is better given its greater specificity (20, 21). This study did not identify predictive parameters indicating an impaired 21-hydroxylase activity. Overall, the meaning of these hormonal findings is equivocal. Indeed, they may indicate decreased activity of the steroid 21-hydroxylase enzyme, or alternatively they may simply parallel the increased volume of adrenal tissue. The substantial decrease, and moreover the return to normal values, of peak 17-OHP after surgical ablation that was observed in selected cases (5–7) and in a more comprehensive series (11) argue for the tumoral mass hypothesis or for some specific intra-tumoral abnormalities (i.e. functional impairment of the enzyme activity or a somatic mutation restricted to the adenoma). However, the reality of a true enzyme defect in a subset of patients could not be rejected for the following reasons: case reports (35–38) and prospective studies underlined the frequency of adenomatous lesions in subjects with steroid enzyme deficiencies (39); persistently elevated peak 17-OHP after adrenal surgery (6, 7, 11) or postsurgical adrenal insufficiency (40) despite absence of pre-surgical subclinical hypercortisolism’s features; high levels of 21-DF in 4 cases from this study; evidence for true germlinal mutation (41); and discovery of similar endocrine abnormalities in close relatives (patient 4, data not shown). As recently questioned by Reincke et al. (12), an alteration in 11β-hydroxylase could also explain the hormonal features observed in adrenal incidentalomas. Peak S levels were, unexpectedly, in the normal range or even slightly elevated, hence explaining the low frequency of increased 17-OHP/S ratios after ACTH stimulation. Similarly, post-stimulation S/F ratios were consistent with a decreased activity of 11β-hydroxylase in 11 instances. However, subjects with an increased S/F ratio had no lower F values or higher DOC levels than subjects whose S/F ratio scored in the normal range, thus casting some doubt on the relevance of these increased S/F ratios. A combined alteration of 21-hydroxylase and 11β-hydroxylase was observed in 7 cases. Apart from anecdotal records with evidence for an actual deficiency of these two enzymes (42), such findings have already been observed for patients with hyperandrogenism (23, 24, 43). The increased peak 21-DF levels displayed by 7 patients with high post-stimulation S/F ratios is difficult to reconcile with adrenal steroidogenesis. Actually: (i) 21-hydroxylation and 11β-hydroxylation have, in contrast to 21-hydroxylase and 17,20-lyase, very distinct molecular mechanisms (44, 45), hence the alteration of these two enzymes could not come from some shared biochemical step or participant; (ii) 21-DF synthesis requires 11β-hydroxylation, thus 21-DF should come from a tissue retaining some steroid 11β-hydroxylase activity. Therefore, the observed 11β-hydroxylase activity must come from an extra-adrenal source (i.e. the liver (46)) or, alternatively, from a distinct adrenal location other than the one responsible for 11β-hydroxylase deficiency (i.e. 11β-hydroxylase deficiency in the adrenal mass, but 11β-hydroxylase activity still present in adjacent and contralateral adrenal normal tissue). Given the very low level of DHEA-S that was observed it was tempting to search for clues for decreased activity of 17,20-lyase (19) or, alternatively, for increased activity of 3β-HSD (47). Precursor/product ratios for this latter enzyme did not argue for an alteration of its activity. Conversely, an apparent decreased activity of 17,20-lyase was identified in some patients whose 17-OHG/DHEA or 17-OHP/Δ4-A ratios were elevated. However: (i) these ratios were only slightly abnormal and were correlated to hallmarks of 21-hydroxylase activity; (ii) DHEA and Δ4-A levels were independent of the 17,20-lyase activity and not related to DHEA-S concentrations. Therefore, alterations of the 17,20-lyase precursor/product ratio do not shed light on the very low DHEA-S values that were noted in this series. Nevertheless, these findings recalling the observation of Peterson et al. (48) could be explained by some sort of piling up all along the F synthesis pathway or by some mechanisms shared by 21-hydroxylase and 17,20-lyase. For instance, these two microsomal steroid enzymes use the same electron donor (either P450 oxidoreductase or cytochrome b5) (44, 45). Thus, an impairment of this step could result in some alteration of both pathways.

The present data suggest that alterations of the steroidogenic pathways in adrenal incidentalomas, which are reminiscent of what has been noted in malignant adrenocortical neoplasms (49, 50), are probably much more complex than it was initially envisioned. The great frequency with which impaired activity of the steroidogenic enzymes were identified, particularly in combination, is an important finding of our study. Molecular approaches using sequence analysis of these three steroid hydroxylase genes will be necessary to distinguish between true germlinal or somatic mutations of these genes from functional abnormalities (41). Alternatively, in vitro studies with cells originating from adrenal incidentalomas may uncover some mechanism leading both to neoplastic transformation and to dysfunction of the steroidogenic pathways.
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