Quantitative assessment of CYP11B1 and CYP11B2 expression in aldosterone-producing adenomas

F Fallo, V Pezzi, L Barzon, P Mulatero, F Veglio, N Sonino and J M Mathis

Department of Medical and Surgical Sciences, Division of Endocrinology, University of Padua, Via Ospedale 105, 35128 Padua, Italy, Department of Pharmaco-Biology, University of Calabria, Arcavacata di Rende, Italy, Department of Histology, Microbiology and Medical Biotechnologies, University of Padua, Padua, Italy, Department of Medicine and Experimental Oncology, University of Turin, Turin, Italy and Department of Cellular Biology and Anatomy, LSU Health Sciences Center, Shreveport, Louisiana, USA

(Correspondence should be addressed to F Fallo; Email: francesco.fallo@unipd.it)

Abstract

Background: The presence and pathophysiological role of CYP11B1 (11β-hydroxylase) gene in the zona glomerulosa of human adrenal cortex is still controversial.

Methods: In order to specifically quantify CYP11B1, CYP11B2 (aldosterone synthase) and CYP17 (17α-hydroxylase) mRNA levels, we developed a real-time RT-PCR assay and examined the expression in a series of adrenal tissues, including six normal adrenals from patients adrenalectomized for renal cancer and twelve aldosterone-producing adenomas (APA) from patients with primary aldosteronism.

Results: CYP11B1 mRNA levels were clearly detected in normal adrenals, which comprised both zona glomerulosa and fasciculata/reticularis cells, but were also measured at a lower range (P < 0.05) in APA. The levels of CYP11B2 mRNA were lower (P < 0.005) in normal adrenals than in APA. In patients with APA, CYP11B2 and CYP11B1 mRNA levels were not correlated either with basal aldosterone or with the change from basal aldosterone in response to posture or to dexamethasone. No correlation between CYP11B1 mRNA or CYP11B2 mRNA and the percentage of zona fasciculata-like cells was observed in APA.

Conclusions: Real-time RT-PCR can be reliably used to quantify CYP11B1 and CYP11B2 mRNA levels in adrenal tissues. Expression of both genes in hyperfunctioning zona glomerulosa suggests an additional formation of corticosterone via 11β-hydroxylase, providing further substrate for aldosterone biosynthesis. CYP11B1 and CYP11B2 mRNA levels in APA are not related to the in vivo secretory activity of glomerulosa cells, where post-transcriptional factors might ultimately regulate aldosterone production.
intensive (11). In addition, levels of CYP11B1 and CYP11B2 transcripts in APA were correlated with both the cell histotype of tumors and aldosterone response to dynamic tests in vivo.

**Materials and methods**

**Patients**

Twelve patients (eight women and four men, aged 31 – 64 years) with APA were studied at our centres (Table 1). All were hypertensive and had hypokalemia of varying degree, compared with the normal patients. Plasma renin activity (PRA) was suppressed and unresponsive to stimuli such as upright posture and captopril administration, and the plasma aldosterone (pmol/l)/PRA ratio (ng/ml/h) was greater than 1385 (i.e. 50 expressing aldosterone as ng/dl). The differential diagnosis between APA and hyperaldosteronism due to bilateral idiopathic adrenal hyperplasia was made by computerized axial tomography, adrenal scintiscan with 75Se-methyl-nor-cholesterol after dexamethasone suppression, and/or aldosterone/cortisol ratio measurements in adrenal venous blood (12). Glucocorticoid-remediable aldosteronism associated with adrenal tumors was excluded by either negative long PCR or Southern blotting for the chimeric gene in leukocyte DNA, as previously described (13). The subjects were on a diet containing 120 – 150 mmol sodium and 60 mmol potassium daily for 2 weeks, and all medications, including spironolactone, were withdrawn for at least 2 weeks before the diagnostic tests. None of the patients had any other disease. Specifically, the patients with APA underwent: (a) postural test for PRA and plasma aldosterone defined by the hormone response, after overnight recumbency, to 2 h upright position from 0800 to 1000 h; and (b) supine plasma aldosterone change at 0800 h after short-term dexamethasone administration trial (0.5 mg 6-hourly for 4 days), with plasma cortisol suppression, i.e. < 138 mmol/l, as index of the dexamethasone effect. All patients underwent unilateral adrenalectomy, and the diagnosis of adenoma was surgically confirmed. After surgery, all APA patients showed normalization of serum potassium and hormone levels, with restoration of a normal aldosterone response to upright posture. Blood pressure normalization or satisfactory control by low-dose conventional anti-hypertensive drugs paralleled restoration of electrolyte and hormonal pattern. Six adrenal glands were obtained from patients undergoing unilateral expanded nephrectomy for kidney cancer and used as controls (Table 1); none of these patients (four men and two women, aged 47 – 66 years) had clinical symptoms of adrenal dysfunction. All patients gave informed consent before participating in this study, which was approved by the local Ethics Committees.

**Tissues**

After removal of adrenal tissue from patients who underwent unilateral adrenalectomy for APA or nephrectomy for renal cancer, a portion was frozen.

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**Table 1** Details of patients with APA and normal adrenals.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/sex</th>
<th>BP (mmHg)</th>
<th>K (mmol/l)</th>
<th>Sup/Upr PRA (ng/ml per h)</th>
<th>Sup/Upr ALDO (pmol/l)</th>
<th>Post-dex ALDO (pmol/l)</th>
<th>Tumor size (cm)</th>
<th>ZF-like cells (%)</th>
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<td>APA</td>
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**Normal**

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<th>Patient no.</th>
<th>Age/sex</th>
<th>BP (mmHg)</th>
<th>K (mmol/l)</th>
<th>Sup/Upr PRA (ng/ml per h)</th>
<th>Sup/Upr ALDO (pmol/l)</th>
<th>Post-dex ALDO (pmol/l)</th>
<th>Tumor size (cm)</th>
<th>ZF-like cells (%)</th>
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<td><strong>Means ± S.E.M.</strong></td>
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BP, blood-pressure; Sup/Upr PRA, supine/upright plasma renin activity; Sup/Upr ALDO, supine/upright plasma aldosterone; Post-dex ALDO, aldosterone levels after dexamethasone; ZF, zona fasciculata.
immediately in liquid nitrogen and stored at −80°C until processing. The adrenal cortex was separated visually from adrenal medulla, while it was not possible to obtain an adequate separation of the outer zone, i.e. the glomerulosa part, from the remaining tissue of normal adrenal specimen. The central portion of each APA was studied. The adenos weighed between 2.8 and 15.7 g.

**Hormone assays**

Plasma aldosterone and PRA were determined by radioimmunounassay (RIA), with kits purchased from Sorin Biomedical Diagnostics (Vercelli, Italy). The intra- and interassay coefficients of variation (CV) for aldosterone were 7.9% and 9.6% respectively; the normal range is 138–550 nmol/l. Intra- and interassay CV values were 4.1 and 5.0% respectively; the normal range is 0.4–3.0 ng/ml per h supine and 1.5–6 ng/ml per h upright. Plasma cortisol was measured using an RIA kit from Diagnostic Products (Los Angeles, CA, USA). The intra- and interassay CV values for PRA were 7.9% and 9.6% respectively; the normal range is 55–330 pmol/l supine and 140–830 pmol/l upright. The intra- and interassay CV values for aldosterone sequence from +341 to +403 were isolated by PCR, by real-time RT-PCR. The primers and probes, and real-time PCR The primers and probes for the CYP11B1, CYP11B2, and CYP17 genes were determined with the assistance of the computer program Primer Express (Perkin Elmer Applied Biosystems), which selected the theoretically optimized sequences for this system. Primer pairs were selected so that they were located on different exons to prevent amplification from any contaminating genomic DNA. The same forward amplification primer 5'-GGCAGAGGCAGAGATGCTG-3' for both the CYP11B1 and CYP11B2 genes and different reverse primers 5'-TCTTGGTATGTCTCCACCTG-3' for CYP11B1 and 5'-CTTGATTTAGTGCTCCACCCAGG-3' for CYP11B2 were respectively used. The sequence of probes for CYP11B1 and CYP11B2 were 5'-TGGCTGACCATGTGCTAGAAACACCT-3' and 5'-CTGCCACAGCTGCTGAAGCCT-3' respectively. The primers and probes for CYP17 analysis were: forward 5'-TCTCTGGGCGGCCCT-3'; reverse 5'-AGGGATACCTTACGGTGT-3'; probe 5'-TGCACTCTCAGACATCGGTTC-3'. Probes were labeled with a reporter fluorescent dye EAM (6-carboxyfluorescein) at the 5' end and a quencher-fluorescent dye TAMRA (6-carboxy-tetramethylrhodamine) at the 3' end. PCR reactions were performed using the ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems) in a total volume of 50 μl reaction mixture containing 5 μl cDNA template, 25 μl TaqMan Universal PCR Master Mix (Perkin Elmer Applied Biosystems), 0.1 μmol/l probe and 0.1 μmol/l of each primer. Negative controls contained water instead of first-strand cDNA. The PCR conditions were established as follows: after incubation at
50°C for 2 min and denaturing at 95°C for 10 min. 45 cycles were performed at 95°C for 15 s, at 60°C for 60 s. To quantify transcripts of the genes precisely, we monitored 18S ribosomal RNA (18S) levels as the quantitative control and each sample was normalized on the basis of its 18S content. The 18S quantification was performed using a TaqMan Ribosomal RNA Control Reagent kit (Perkin Elmer Applied Biosystems) and the method of PCR was followed using the manufacturer’s protocol. Briefly, 50 µl reaction mixture containing 5 µl cDNA template, 25 µl TaqMan Universal PCR Master Mix, 50 nmol/l specific primer and 200 nmol/l probe (VIC™) was amplified by the program as follows: after incubation at 50°C for 2 min and denaturing at 95°C for 10 min. 40 cycles were performed at 95°C for 15 s and at 60°C for 1 min. The standard curves for CYP11B1 and CYP11B2 are shown in Fig. 1 and were generated using serially diluted solutions (21.75–0.002175 attomoles) of plasmid clones containing either the CYP11B1 cDNA (Fig. 1A) or the CYP11B2 cDNA (Fig. 1C) as templates. The standard curve for CYP17 was generated using serially diluted solutions (19.38–0.001938 attomoles) of pCYP17. The standard curve for 18S was generated using serially diluted solutions of standard cDNA derived from human mRNA included in the TaqMan Ribosomal RNA Control Reagent kit (Perkin Elmer Applied Biosystems). ABI Prism 7700 measured the ratio (defined as the normalized reporter signal Rn) between the fluorescence signal of reporter dye and the fluorescence of the passive reference dye of each sample in every cycle and calculated the ΔRn defined as Rn minus the baseline signals established in the first 15 cycles. The more template present at the beginning of the reaction, the fewer number of cycles it takes to reach a point at which the fluorescent signal is first recorded as statistically significant above background. This point is defined as Cx, and occurs during the exponential phase of amplification. The amount of target gene expression was calculated from the standard curves.

Figure 1. CYP11B1 and CYP11B2 standard curves by real-time RT-PCR. Samples containing five different concentrations (a, 21.75 attomoles; b, 2.175 attomoles; c, 0.2175 attomoles; d, 0.02175 attomoles; e, 0.002175 attomoles) of plasmid clones containing either CYP11B1 cDNA (A) or CYP11B2 cDNA (C) were subjected to real-time PCR. Cycle number is plotted vs change in normalized reporter signal (ΔRn). ΔRn increases during PCR as the CYP11B1 or the CYP11B2 PCR product copy number increases until the reaction reaches a plateau. Standard curves plotting log starting copy number of CYP11B1 cDNA (B) and CYP11B2 cDNA (D) versus threshold cycle (Cx). The parameter of Cx represents the fractional cycle number at which a significant increase in the fluorescence signal above a baseline signal can be first detected. Dots represent data obtained from standard curve point samples. Two replicates for each standard curve point sample were performed, but the data for only one are shown here.
for CYP11B1 (Fig. 1B) or the CYP11B2 (Fig. 1D). The amount of target gene expression was calculated from the standard curve plotting log starting copy number versus Ct. Quantitative normalization of cDNA in each sample was performed using expression of the 18S as an internal control. CYP11B1, CYP11B2 and CYP17 mRNA levels are shown as attomoles/μg total RNA. Three different real-time PCR assays were conducted in duplicate for each sample and a mean value was used for calculation of the mRNA levels. The variability of Ct values between duplicates within the same run was no more than 3% while the CV values between different runs were no more than 5%. Tests of sensitivity revealed that our method can reliably detect samples as low as 0.001 attomoles mRNA/μg total RNA. Three different real-time PCR assays were conducted for CYP11B1 at different concentrations of pCYP11B1 as template and a second reaction in which the primers and probes used to detect CYP11B1 were added as serial dilutions of pCYP11B1 as template. In both experiments no fluorescence was detected, demonstrating that non-cross-reactivity was produced in both reactions.

Statistics

Data are expressed as means ± S.E.M. Intergroup differences in means were tested using unpaired Student’s t-test, and the least square method was used for correlations. When there was evidence of abnormal value distribution, non-parametric tests were used for testing differences in means (Wilcoxon signed rank) and for correlations (Spearman rank correlation). A P < 0.05 was considered significant.

Results

The mRNA levels of CYP11B1, CYP11B2 and CYP17 from APA and normal adrenals are shown in Table 2 and Fig. 2. Levels of CYP11B1 mRNA were clearly detected in normal adrenals, which comprised both zona glomerulosa and fasciculata/reticularis cells, but were also detected at a lower range in APA tissues (3.78 ± 4.17 vs 4.04 ± 0.59 attomoles/μg total RNA, P < 0.05). The levels of CYP11B2 mRNA were lower in normal adrenals than in APA (0.044 ± 0.014 vs 0.344 ± 0.056 attomoles/μg total RNA, P < 0.005). No correlation between CYP11B2 mRNA level and APA size was found (Z, i.e., correlation coefficient = −0.15, P = 0.88). In patients with APA, the levels of CYP11B1 mRNA (Z = −0.55, P = 0.57 supine; Z = 1.07, P = 0.28 upright) and CYP11B2 mRNA (Z = −0.16, P = 0.87 supine, Z = −0.89, P = 0.37 upright) were not correlated with basal aldosterone.

In addition, there was no correlation between CYP11B1 mRNA (Z = 1.70, P = 0.09; Z = −1.04, P = 0.29) as well as CYP11B2 mRNA (Z = 1.58, P = 1.1; Z = 0.69, P = 0.48) and the change from basal aldosterone to dexamethasone administration (mean change +127 ± 99 pmol/l) or to dexamethasone administration (mean change −159 ± 119 pmol/l) respectively. No correlation between CYP11B1 mRNA (Z = 2.21,
$P = 0.12$) or CYP11B2 mRNA ($Z = 0.23, P = 0.81$) and the percentage of zona fasciculata-like cells was observed in APA.

CYP17 mRNA levels were similar in normal adrenals and in APA ($492.67 \pm 108.56$ vs $373.90 \pm 60.19$ attomoles/µg total RNA, $P =$ not significant).

**Discussion**

Functional zonation of the human adrenal cortex, i.e. the ability of each zone to differentially produce aldosterone and cortisol, relies on the zone-specific expression of CYP11B1 and CYP11B2 isozymes. Zonal expression of the isozymes results from transcriptional regulation of their coding genes (6, 15). The high similarity of the two isozymes has hampered the production of specific antibodies for their immunohistochemical detection in adrenal tissues (16), and measurement of enzyme activities in different types of adrenal cells (17–19) does not distinguish between 11β-hydroxylation due to CYP11B1 or CYP11B2 genes. In our study, we have shown a large amount of CYP11B1 transcript in the whole normal adrenal, which included both zona glomerulosa and the much larger zona fasciculata/reticularis.

In agreement with previous reports using conventional RT-PCR (3) and in situ hybridization (8, 9), our technique has shown the presence of CYP11B1 transcript in the zona glomerulosa-derived cells of APA, and in addition allowed its absolute quantification. The competitive RT-PCR analysis employed in a previous study (7) could not provide accurate measurements of CYP11B1 mRNA; e.g. stable differences in amplification efficiency between target and competitor would remain undetected (20). Furthermore, in our method, the Ct value used for quantitation is measured during a period when the PCR amplification is still in the log phase of amplicon accumulation. This circumvents many of the problems associated with quantitation in the plateau stage of a PCR amplification. Expression of CYP11B1 in hyperfunctioning zona glomerulosa suggests a formation of corticosterone via 11β-hydroxylase, additional to that driven by aldosterone synthase, which may be used as substrate for the increased aldosterone biosynthesis. In vitro studies on CYP11B2 transfected Chinese hamster COS-7 cells (21) and rat Leydig cells (22) show a better efficiency to metabolize DOC rather than corticosterone as exogenous substrate. However, in human APA, where high amounts of the corticosterone derivative 18-OHB are also secreted (23), the combination of abundant substrates available (i.e. DOC, corticosterone, 18-OHB) would reasonably contribute to the overproduction of aldosterone. The demonstration in APA of CYP17, encoding the 17α-hydroxylase enzyme required for cortisol but not for aldosterone biosynthesis, further supports the concept that cortisol can be produced by aldosteronoma cells, as shown in previous studies (9, 24–27).

At variance with CYP11B1, CYP11B2 mRNA levels were very low in normal adrenals. This does not appear
to be due to sample damage during processing, since CYP11B1 and CYP17 mRNA were measurable in the same specimens, but probably reflects anatomical zone differences. As we measured CYP11B2 expression in the mixed extract of zona glomerulosa and fasciculata/reticularis, we cannot discuss its presence outside the zona glomerulosa. However, studies reporting lack of in vitro release of aldosterone from human zona fasciculata cells (9), as well as barely distinguishable expression of CYP11B2 by in situ hybridization in the same zone (8), do not support this possibility. As expected (3, 7, 28), the levels of CYP11B2 mRNA were consistently higher in APA. The aldosterone-producing tumors are commonly believed to originate from zona glomerulosa; however, most adenoma cells have the histological appearance of fasciculata cells and only a minority are small and compact, similar to normal glomerulosa cells. Several studies have shown distinct biochemical characteristics of patients with primary aldosteronism due to APA, based on different aldosterone responses to upright posture (7, 29–31) and dexamethasone administration (12, 13, 32–34). Responsiveness to endogenous angiotensin II and ACTH inhibition may reflect a different prevalence of zona fasciculata-like cells within the tumor (30, 35). No relationship was found in our patients with APA between CYP11B1 and CYP11B2 transcript levels and aldosterone secretion, either basal or in response to dynamic testing. In this regard, lack of data on related protein concentrations in our study or in response to dynamic testing. In this regard, lack of data on related protein concentrations in our study could also be the small number of tumors examined, post-transcriptional factors might ultimately regulate aldosterone production, determining the various functional features of APA. The lack of correlation between the percentage of fasciculata-like cells and the mRNA levels of either CYP11B1 or CYP11B2 seems to exclude the possibility that expression of these genes in the tumors defines cell phenotype in APA.

In conclusion, real-time RT-PCR can be reliably used to quantify CYP11B1 and CYP11B2 mRNA levels in adrenal tissues. Expression of CYP11B1 in APA suggests an additional formation of corticosterone via 11β-hydroxylase. The mRNA levels of CYP11B1 and CYP11B2 in APA are not related to the in vitro secretory activity of glomerulosa cells, where post-transcriptional factors might ultimately regulate aldosterone production.

References


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