CLINICAL STUDY

Absence of D147E mutation of CYP11B2 gene in hypertensive patients with increased corticosterone and aldosterone production

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Abstract

Objective: 11β-Hydroxylase and aldosterone synthase are two highly homologous genes involved in different forms of human hypertension and in different animal models of hypertension. It has been shown that the conservative substitution D147E in the human CYP11B2 gene results in an increased production of corticosterone and aldosterone *in vitro*. A gene conversion between the CYP11B1 and CYP11B2 genes could be responsible for such a substitution.
Methods: In this study we investigated the presence of the mutation D147E of CYP11B2 in a group of 128 patients with primary aldosteronism, 68 patients with essential hypertension and increased corticosterone production and in 48 normal volunteers.
Results and conclusions: We did not identify any patient carrying this mutation, indicating that if it exists it is very rare and so has no relevance in determining the increased steroid excretion seen in some subtypes of human hypertension.

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Introduction

11β-Hydroxylase (*CYP11B1*) and aldosterone synthase (*CYP11B2*) are two highly homologous genes lying in tandem on chromosome 8 (1). Their coding sequences are 95% identical (2). 11β-Hydroxylase is expressed in the zona fasciculata-reticularis of the adrenal cortex where it catalyses the conversion of 11-deoxycortisol to cortisol and deoxycorticosterone (DOC) to corticosterone. Aldosterone synthase is exclusively expressed in the zona glomerulosa (3, 4) where it catalyses the conversion of DOC to corticosterone and further converts corticosterone to 18-hydroxycorticosterone (18OHB), which in turn is converted to aldosterone.

Recently amino acid residues 288 and 320 of CYP11B2 have been shown to confer the two additional activities in position C18 to the enzyme (5, 6). Chimeric genes originating from the fusion between *CYP11B1* and *CYP11B2* are responsible for the rare, dominantly inherited form of hypertension named glucocorticoid remediable aldosteronism (GRA) (7, 8), whereas the two genes do not appear to be involved in the familial aldosteronism not suppressible by dexamethasone (9, 10). Polymorphic variants of CYP11B2 have been shown to be associated with essential hypertension (EH) and

idiopathic hyperaldosteronism (IHA) (11, 12). In the Dahl salt-resistant (SR) rat, mutations in the CYP11B1 and CYP11B2 genes result in decreased production of 18-hydroxy-deoxycorticosterone (18OHDOC) and increased production of aldosterone (13–15). The introduction of these mutations into the human *CYP11B2* resulted in an increased production of corticosterone and aldosterone *in vitro* (16). A recent study by Fisher *et al.* (17) highlighted the role of the D147E mutation in human aldosterone synthase *in vitro*. In particular, the substitution of the Asp147 of *CYP11B2* with the corresponding 11β-hydroxylase-specific glutamate residue caused a marked increase of corticosterone production and a smaller increase of aldosterone production. In a previous study, it was reported that patients with EH have a mildly increased production of corticosterone that, in combination with a slightly deficient 11β-hydroxysteroid dehydrogenase, could play a pathogenetic role in the blood pressure increase (18). Therefore, the D147E mutation could be potentially involved in EH and in primary aldosteronism (PA).

We investigated the presence of the D147E mutation in CYP11B2 in a group of patients with PA, in patients with EH and increased corticosterone production, and in normal volunteers.

Table 1 Clinical and biochemical parameters of populations studied. Values are means ± S.D.

Parameter	EH	APA	IHA	Control subjects
Sample size (n)	68	38	90	48
Age (years)	50.9 ± 9.4	51.1 ± 7.2	50.3 ± 8.5	52.2 ± 7.9
Gender (M/F)	34/34	20/18	47/43	24/24
Systolic BP (mmHg)	150.0 ± 8.3	203.9 ± 24.1	193.9 ± 27.9	119.0 ± 10.1
Diastolic BP (mmHg)	101.0 ± 6.3	115.1 ± 11.1	117.2 ± 16.1	74.0 ± 8.9
Supine PRA (ng/ml per hour)	0.87 ± 0.09	0.13 ± 0.07	0.16 ± 0.10	2.07 ± 0.17
Upright PRA (ng/ml per hour)	–	0.17 ± 0.10	0.28 ± 0.18	–
Supine aldosterone (ng/dl)	8.2 ± 0.6	39.8 ± 12.7	24.7 ± 8.8	6.8 ± 0.6
Upright aldosterone (ng/dl)	–	47.1 ± 16.2	39.8 ± 15.3	–
Plasma cortisol (nmol/l)	281.0 ± 20.7	284.3 ± 33.2	288.1 ± 22.8	272.0 ± 17.3
Corticosterone metabolites (μg/24 h)[a]	578.8 ± 41.7	–	–	335.8 ± 18.8

[a] Corticosterone metabolites = THB + alloTHB + THA.

Methods

Study population

The patients recruited for this study were Caucasians from northern Italy and Sardinia. The study was approved by the local committees and all subjects gave informed consent. We studied 38 patients with aldosterone-producing adenoma (APA), 90 patients with IHA, 140 patients with EH and 30 normal volunteers. The criteria used for the differential diagnosis of the different forms of PA and for EH were described previously (12, 19, 20). Patients with other forms of secondary hypertension were excluded and also those with diabetes and renal diseases. Patients were consuming a daily diet containing 120–150 mmol sodium and 60 mmol potassium for at least 2 weeks and had been off medication for at least 1 month. Control subjects were matched for age, sex and body mass index. Female patients were either in the follicular phase or post-menopausal and none was on oral contraceptive therapy. GRA was excluded by amplification of the DNA extracted from leucocytes by the long-PCR technique as described previously (6, 19).

Hormonal measurements

Plasma aldosterone and plasma renin activity (PRA) were determined by RIA using kits purchased from Sorin Biomedical Diagnostics (Vercelli, Italy). The intra- and inter-assay coefficients of variation (CVs) for aldosterone were 7.9 and 9.6% respectively. The intra- and inter-assay CVs for PRA were 5.4 and 9.1% respectively. Patients were left in a recumbent position for 1 h before supine samples were collected and for 2 h in an upright position before upright samples were collected. Plasma cortisol was measured using a specific RIA kit (INCSTAR; Sorin Biomedical) from blood samples collected at 0800 h.

The sum of urinary corticosterone metabolites tetrahydro-corticosterone (THB), allo-tetrahydro-corticosterone (alloTHB) and 11-dehydro-tetrahydrocorticosterone (THA) was used as an index of corticosterone generation rate. Procedures for the extraction, purification and quantitation of THB, alloTHB and THA have been described in detail elsewhere (21). Briefly, steroid metabolite conjugates were extracted from aliquots of 24 h urine collections and hydrolysed using a preparation of β-glucuronidase. Methyloxime trimethylsilyl ether derivatives were synthesized and the resulting mixture analysed by gas chromatography–mass spectrometry.

PCR amplification and genotyping of CYP11B2

Genomic DNA was prepared from peripheral blood leucocytes with microspin columns (Qiamp blood kit; Qiagen, Valencia, CA, USA). Analysis of the D147E mutation was performed by first amplifying this region by PCR using gene-specific primers (sense: 5′-TGG GGC TGG ACC TTC CCG CAT-3′; antisense: 5′-TTC CAG AGC AGG TTC CTG GG-3′). The 50 μl reaction was subjected to 35 cycles of 94 °C for 1 min, 65 °C for 1 min and 72 °C for 2 min with an additional 5 s each cycle, followed by a final extension at 72 °C for 7 min. This reaction allows the amplification of a 2.8 kb fragment that includes exons 1–3 of *CYP11B2*. The amplified DNA samples were subjected to electrophoresis in a 1% agarose gel and transferred to nylon membranes (GENE Screen Plus; DuPont-New England Nuclear, Ontario, Canada) that were subsequently hybridized with oligonucleotides that had been radioactively labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The membranes were incubated with the appropriate radiolabelled allele-specific oligonucleotide (D147: 5′-CCAGATGTGCTGTCGCC; E147: 5′-CCAGA-AGTGCTGTCGCC-3′) in a solution containing 0.9 mol/l NaCl, 0.09 mol/l Na citrate, 1% SDS and 1×Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone and 1% BSA fraction V) for 4 h at 42 °C. The final stringent washes before autoradiography were conducted in 0.9 mol/l NaCl, 0.09 mol/l Na citrate and 0.5% SDS at 54 °C. As a positive control we used a CYP11B2 cDNA carrying the mutation D147E

introduced by PCR mutagenesis. Results were compared using Student's *t*-test.

Results

Clinical and hormonal parameters of the different subgroups of patients studied are summarized in Table 1.

The urinary excretion rates of THB, alloTHB and THA were significantly higher ($P < 0.01$) in the group of patients with EH compared with the control group.

We did not find any allele carrying the E147 residue in the CYP11B2 gene in any of our samples of hypertensive patients and normal volunteers. All the PCR products transferred to the nylon membranes were hybridized with the end-labelled oligonucleotide specific for the D147 allele. In a sample of ten patients with IHA and ten with EH presenting with an increased corticosterone secretion, we also sequenced exon 3 of CYP11B2 as previously described (22), confirming the absence of the mutation D147E as shown by the hybridization studies (Fig. 1).

In all groups of patients we found a high frequency of conversion between the CYP11B1 and CYP11B2 genes, but never involving the sequence coding for amino acid residue 147 (6, 12).

Discussion

11β-Hydroxylase and aldosterone synthase catalyse the terminal steps in cortisol and aldosterone biosynthesis in human adrenal cortex. Both enzymes are able to perform 11β-hydroxylation of the substrate, but only aldosterone synthase is able to further catalyse 18-hydroxylation and 18-oxidation of the substrate corticosterone. Single amino acids are responsible for the additional activities in position C18 displayed by aldosterone synthase: Gly288 confers the 18-hydroxylase activity to the enzyme and Ala320 confers 18-oxidase activity (5, 6). Therefore, single amino acid substitutions can determine important changes in the enzyme activity.

It has been shown by *in vitro* studies that mutations in the human *CYP11B2* gene corresponding to the mutations observed in the same gene in the Dahl SR strain of rat caused an increased production of corticosterone and aldosterone (16). However, such mutations have never been described in human hypertensive patients (our unpublished observations). A recent study by Fisher *et al.* (17) showed that the conservative substitution of Asp147 in *CYP11B2* by the corresponding residue coded by *CYP11B1* results in an enzyme which catalyses increased production of corticosterone and aldosterone. A gene conversion between the *CYP11B1* and *CYP11B2* genes could be responsible for this substitution *in vivo*. This kind of recombination is a frequent event between this type of highly homologous gene (6, 11, 23, 24). Gene conversions between 21α-hydroxylase (CYP21A) and a highly homologous pseudogene carrying deleterious mutations (CYP21B) are responsible for a high percentage of 21α-hydroxylase deficiencies (25).

Mutations of the type described in the work of Fisher *et al.* could have a relevance in human hypertension and in particular in patients with an increased production of corticosterone and aldosterone. It has previously been noted that plasma corticosterone concentration tends to be slightly raised in young adults with a familial predisposition to hypertension (26). Moreover, corticosterone production rate from isolated adrenocortical cells is higher in tissues from SHR rats, a model for EH, than from WKY rats (27). Also, the hypertensive strain of the Milan rat has higher plasma corticosterone concentrations (28). Corticosterone displays 20% of the potency of cortisol and circulates at concentrations 1–2% of those of cortisol, but 20–40 times higher than aldosterone. In this respect, the contribution of an increased secretion of corticosterone would be expected to play a marginal role in hypertension. However, corticosterone binds 11β-hydroxysteroid dehydrogenase with greater affinity than cortisol (29); an impaired activity of the enzyme has been demonstrated in EH patients (21). For these reasons an increased secretion of corticosterone in EH patients could play a role in the pathogenesis of the disease.

Thus, one would expect to find the described mutation in patients with increased corticosterone secretion previously reported by Soro *et al.* (18) or in patients with PA.
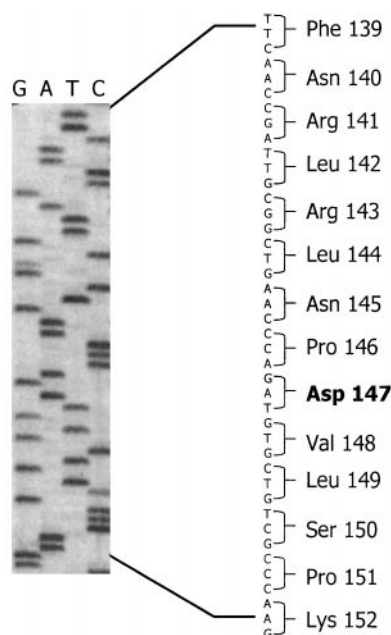

Figure 1 Sequence of exon 3 of CYP11B2 from a patient with PA.

However, we did not find the D147E mutation in a large sample of patients with IHA, aldosterone-producing adenomas and in EH patients with an increased production of corticosterone.

This work demonstrates that if the D147E mutation exists it is very rare and so is not an important determinant in the increased steroid excretion seen in some subtypes of human hypertension.

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