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Aldosterone synthase deficiency caused by a homozygous L451F mutation in the CYP11B2 gene

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Abstract

Isolated hypoaldosteronism is a rare cause of salt wasting in infancy and may be life-threatening, especially in the newborn infant. In a 3 wk-old-boy with hyponatremia and hyperkalemia a GC–MS steroid profile on a spot urinary sample showed no 18-oxygenated steroid metabolites indicative for aldosterone synthase deficiency type I. Sequence analysis of the *CYP11B2* gene revealed that the patient was homozygous for a novel missense mutation (L451F) caused by a T to C transition at position c.1351 in exon 8, whereas each non-symptomatic parent possessed only one mutated allele. The mutant cDNA was transiently expressed in a human cell line, HCT116 $p53^{-/-}$, and activity of the expressed protein optimized by co-expression of different adrenodoxin species, showing complete aldosterone deficiency with 11-deoxycorticosterone or corticosterone as substrates. The L451F mutation is the first mutation found located immediately adjacent to the highly conserved heme-binding C450 of the cytochrome P450. Computer modeling shows that replacement of leucine by phenylalanine leads to a steric effect in the immediate vicinity of the heme thereby preventing the activity of CYP11B2. Thus, by combining highly sensitive hormone detection in a spot urine sample with expression of the mutated cDNA in cell culture the phenotype of the patient can be correlated with a particular molecular defect.

Keywords: Aldosterone synthase deficiency (CMO I); CYP11B2; Steroid hydroxylation; Cell culture; GC–MS

Introduction

Aldosterone is the principal mineralocorticoid hormone in humans and is synthesized in a three-step reaction by aldosterone synthase (CYP11B2) in the adrenal *zona glomerulosa* [1]. The conversion of deoxycorticosterone (DOC) to aldosterone requires two hydroxylations at position 11β to form corticosterone (B), and at position 18 to form 18-hydroxycorticosterone (18-OH-B), and finally an oxidation at position 18. The gene encoding for CYP11B2 is on chromosome 8 in tandem with the highly homologous 11β-hydroxylase (CYP11B1) approximately 40 kb distant. The *CYP11B2* gene contains 9 exons spread over 7 kb [2,3]. Aldosterone synthase is synthesized as a preprotein of 503 amino acids, including a 24-residue N-terminal mitochondrial targeting sequence, which after translocation into the mitochondrial matrix is cleaved off to yield the mature enzyme. The molecular mass of CYP11B2 is 48.5 kDa, and the protein is bound to the inner mitochondrial membrane by an as yet undefined mechanism [4].

Aldosterone synthase deficiency is usually expressed in infancy as life-threatening electrolyte imbalance caused by mutations in the *CYP11B2* gene. Affected children typically display failure to thrive, vomiting and severe dehydration; the biochemical features are hyperkalemia, hyponatremia and metabolic acidosis with elevated plasma renin activity and low or undetectable aldosterone. Ther-

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apy with fludrocortisone corrects these alterations and permits adequate further growth and development [5,6].

Two types of aldosterone synthase deficiency can be differentiated. Type I CMO deficiency (corticosterone methyl oxidase type I—CMO I) is associated with low to normal levels of 18-OH-corticosterone, low levels of plasma aldosterone and very low to undetectable levels of urinary tetrahydroaldosterone. In contrast, type II CMO deficiency (corticosterone methyl oxidase type II—CMO II) is characterized by high levels of 18-OH-corticosterone, low levels of plasma aldosterone and subnormal to occasionally low normal levels of urinary tetrahydroaldosterone [7]. Both types of aldosterone synthase deficiency are caused by mutations in the aldosterone synthase gene [8]. CMO I patients, with low levels of plasma 18-OH-corticosterone and aldosterone, always have mutations that either completely inactivate CYP11B2 [9–12] or significantly impair its activity [13].

In this study, we describe a new mutation in the *CYP11B2* gene from a CMO I deficient patient and analyzed the mutant CYP11B2 activity on steroid biosynthesis. *In vitro* transfection studies showed that hydroxylation and oxidation by CYP11B2 of DOC or B was abolished in the mutant (L451F) protein. Three-dimensional molecular modeling of the mutated enzyme showed a steric effect caused by the increased volume of the changed amino acid next to the heme-binding cysteine in position 450.

Materials and methods

Case report

An apparently normal male infant of Turkish parents started to vomit repeatedly from the age of 2 wk onwards. Its birth weight was 3.170 kg, and length 50 cm. At the age of 3 wk, the child was admitted to a pediatric hospital weighing 3.200 kg. The serum sodium was 122 mM/L (normal range, 136–145 mM/L) and the serum potassium 7.1 mM/L (normal range, 3.5–4.5 mM/L). A GC–MS (gas chromatography–mass spectrometry) steroid profile of a spot urinary sample [14] showed the typical steroid profile of aldosterone synthase deficiency I. When treatment with fludrocortisone was started the patient recovered and his further development has been uneventful since then.

Chemicals

The expression vector pSVL was purchased from Pharmacia Biotech Inc. (Freiburg, Germany) and oligonucleotides synthesized at BioTez (Berlin, Germany). Cell culture media, pyruvate, glutamine, fetal bovine serum and antibiotics were from Sigma (Taufkirchen, Germany). Dimethylsulfoxide, 11-deoxycorticosterone, corticosterone, 18-hydroxycorticosterone and aldosterone were from Sigma (Taufkirchen, Germany) and [^{14}C]11-deoxycorticosterone and [^{3}H]11-corticosterone from DuPont NEN (Boston, USA). HPTLC silica gel plates and solvents were from Merck (Darmstadt, Germany).

Gas chromatography–mass spectrometry (GC–MS)

Urinary steroids were profiled on GC–MS with selected ion monitoring as described previously [14]. In brief, steroids were enzymatically hydrolyzed, solid-phase extracted and derivatized for GC–MS analysis. Free and conjugated urinary steroids were extracted on Sep-Pak C18 cartridges (Waters Associates, Milford, MA), and conjugates enzymatically hydrolyzed (type I powdered Helix pomatia, Sigma–Aldrich Corp., St. Louis, MO). Hydrolyzed steroids were recovered by Sep-Pak extraction, and known amounts of three internal standards (5α-androstane-3α,17α-diol, stigmasterol and cholesteryl butyrate) were added to a portion of each extract before formation of methyloxime-trimethylsilyl ethers. GC was performed in an Optima-1 fused silica column (Macherey-Nagel, Dueren, Germany) with helium as carrier at a flow rate of 1 ml/min. The chromatograph (Agilent 6890 series GC, Agilent 7683 Series Injector, Agilent Technologies, Waldbronn, Germany) was directly interfaced to a mass selective detector (Agilent 5973N MSD, Agilent Technologies) operated in the selected ion monitoring mode. Injections were made into an 80 °C (2 min) GC oven with the temperature then increased by 20 °C/min to 190 °C (1 min), to separate steroids it was then increased by 2.5 °C/min to 272 °C. Values for the excretion of individual steroids were determined by measuring selected ion peak areas against the internal standards.

PCR and sequencing

The CYP11B2 gene was selectively amplified in two segments (exons 1–6, 4.5 kb, using primers E16F and E16R shown in Table 1; exons 3–9, 3.7 kb, using primers E39F and E39R shown in Table 1). Briefly, 50 μl reactions contained 200 ng genomic DNA, 50 pmol of each primer, 200 μM of each deoxy-NTP, 2.5 U BIO-X-ACT DNA polymerase (Bioline company). Thermal cycler (PT-100, MJ Research Inc.) was set to 95 °C for 5 min; 60 °C for 1 min, followed by 30 cycles of 5 min at 68 °C, 30 s at 95 °C and 30 s at 60 °C, with final extension for 10 min, at 68 °C. The PCR product was purified from the agarose gel by using the NucleoSpin kit (Macherey-Nagel). All exons and intron/exon boundaries were sequenced using the primers E1–E9 shown in Table 1. The sequences were analyzed with the program CLUSTALW 1.8.

Site-directed mutagenesis

Mutations in the human CYP11B2 cDNA were generated in the vector pSVLh11B2 by site-directed mutagenesis using the Quik-Change Site-Directed Mutagenesis Kit (Stratagene Ltd, Cambridge, UK) according to manufacturer's instruction and using mutagenic primers E8MF and E8MR listed in Table 1. The *CYP11B2* sequence of the cell culture expression construct pSVLh11B2 used as wild type corresponds to that published by Kawamoto et al. [15] with one variation at position 249, where we found Ser instead of Arg, as described by Mornet et al. [2]. The 50 μl reaction contained 50 ng pSVL-11B2, 1× Pfu reaction buffer, 125 ng of each complementary primer pairs, 200 μM of each deoxy-NTPs

Table 1
Oligonucleotides

	Forward	Reverse	Sequences (5′ → 3′)
E16F	X		ACCAGACTTCTCCTTCATCTACCTT
E16R		X	GAGCGTCATCAGCAACGGAAACGCT
E39F	X		TCAGCACCTGTGGGCAGAAGCTAC AG
E39R		X	CCCGGATCCAGGCCCTGCCAGCAA GAT
E8MF	X		CATGCGCCAGTGCTTCGGGCGGCG CCTG
E8MR		X	CAGGCGCCGCCCGAAGCACTGGCG CATG
E1		X	GGGAATGGCAGTGCTGAGTG
E2		X	CCTGCTCCCAGCTCTCAGCT
E3	X		TGCAGGCCGATTCCCCTTGG
E4	X		GTGGGGAGGCAGCCAGGAGG
E5	X		AGGAGGAGGACACTGAAGGATG
E6	X		GGTGTCCCGGGGGCTGAGTC
E7/E8	X		TAGGAAGGGTGCAGAGAG
E9		X	TGACTCAGGAAGCTGTGC