Novel P450c17 Mutation H373D Causing Combined 17α-Hydroxylase/17,20-Lyase Deficiency

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Context: Combined 17α-hydroxylase/17,20-lyase deficiency is a rare autosomal recessive form of congenital adrenal hyperplasia presenting with hypertension and sexual infantilism. This disorder is caused by defects in P450c17, encoded by the *CYP17A1* gene.

Objective: We describe a 14-yr-old female with clinical and hormonal features of 17α-hydroxylase/17,20-lyase deficiency and identify and characterize the activities of her *CYP17A1* mutations.

Methods: The coding regions of the *CYP17A1* gene were amplified by PCR and sequenced. Mutations were recreated in P450c17 cDNA expression vectors; activities in transfected COS-1 cells were assayed by conversion of radiolabeled precursor steroids. One mutant was also expressed in *Escherichia coli*, and the reduced adsorption spectrum was measured.

Results: The patient carried the previously described mutation R96W and the novel missense mutation H373D. Neither mutant had detectable activity when expressed in COS-1 cells. Membrane preparations from *E. coli* expressing the H373D mutant vector produced an absorption peak at 420 nm, whereas the wild-type produced a peak at 450 nm, suggesting that the H373D mutation interferes with protein folding.

Conclusion: The novel P450c17 mutation H373D abolished enzyme activity because of protein misfolding. These data indicate an important role for this residue in P450c17 activity. **(*J Clin Endocrinol Metab* 94: 3089–3092, 2009)**

P450c17 is the single microsomal cytochrome P450 enzyme that catalyzes both the 17α-hydroxylation required to produce the 17-hydroxy 21-carbon precursors of cortisol, 17-hydroxyprenenolone (17OH-Preg) and 17-hydroxyprogesterone (17OHP), and the 17,20-lyase activity needed to produce 19-carbon precursors of sex steroids (1, 2). P450c17 is encoded by a gene termed *CYP17A1*, consisting of eight exons (3) and located on chromosome 10q24.3 (4). *CYP17A1* mutations cause 17α-hydroxylase deficiency, a rare form of congenital adrenal hyperplasia characterized by sexual infantilism, 46,XY sex reversal, hypertension, and high ratios of C21 to C19 steroids (5). The lack of 17α-hydroxylase activity disrupts cortisol secretion, driving the compensatory overproduction of a glucocorticoid, corticosterone, and a mineralocorticoid, deoxycorticosterone, causing hypertension and hypokalemia. Rare patients may also have isolated 17,20-lyase deficiency, characterized by low C19 steroids with normal cortisol (6–8). We describe a novel missense mutation and functional characterization of the *CYP17A1* gene in a 14-yr-old girl with hypertension and sexual infantilism.

Subject and Methods

Case report

A Caucasian female was evaluated for hypertension and palpitations at the age of 7 yr. The mother's ethnicity is German-French Canadian, and the father's ethnicity is Scottish-Irish-Italian; there is no known consanguinity. Neither the 13-yr-old brother nor the patient's parents are hypertensive. At age 7, her random ambulatory blood pressure (BP) was

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Abbreviations: BP, Blood pressure; DHEA, dehydroepiandrosterone; 17OHP, 17-hydroxyprogesterone; 17OH-Preg, 17-hydroxyprenenolone.

118/84 mm Hg (95th/99th percentiles) with an unremarkable electrocardiogram. Further evaluation was delayed until age 11, when she was admitted for viral pneumonia with a BP of 149/101 mm Hg (>99th percentile), ranging as high as 157/107 mm Hg during hospitalization. Subsequent BPs ranged from 117/69 to 130/90 mm Hg; electrocardiogram and renal sonogram were normal. Her serum sodium was 142 mEq/liter, and the potassium was 3.3 mEq/liter; plasma renin activity was undetectable (<0.1 ng/ml · h), and aldosterone was elevated (20.9 ng/dl). Blood urea nitrogen and creatinine were normal. CT angiography of the abdomen showed no renal artery stenosis. Glucocorticoid-remediable aldosteronism was diagnosed, but *CYP11B1* sequencing was normal.

At age 11, she was referred to one of us (P.W.S.). Physical examination showed height 150.8 cm (+ 0.6 SD), weight 47.7 kg (+ 0.9 SD), and Tanner stage 1 without clitoromegaly or genital ambiguity. Ambulatory monitoring for 24 h revealed an average BP of 147/94 mm Hg. Cosyntropin stimulation (Table 1) was notable for extremely high corticosterone and 18-hydroxycorticosterone levels; slightly elevated pregnenolone, progesterone, and deoxycorticosterone; and low levels of 17OH-Preg, 17OHP, cortisol, 11-deoxycortisol, aldosterone, and dehydroepiandrosterone (DHEA). Plasma renin activity was undetectable. Karyotype was 46,XX. Estradiol was low at 0.23 ng/dl with elevated gonadotropins (FSH and LH, 63 and 25 mIU/ml, respectively). Ultrasonography revealed hypoplastic uterus and ovaries with normal-sized kidneys and adrenals. Her bone age was 7 10/12 yr at chronological age 14 4/12 yr. Sodium restriction was continued, and oral hydrocortisone (7.5 mg twice daily; 10 mg/m^2 · d) was started, resulting in a somewhat lower, but still elevated BP, averaging 134/89 mm Hg. Subsequent treatment with oral prednisolone (5 mg twice daily; equivalent to ~40 mg hydrocortisone; 26 mg/m^2 · d) improved BP into the normal range and suppressed corticosterone, whereas plasma renin activity became measurable. Low-dose transdermal estrogen replacement was started.

DNA sequencing

With informed consent, leukocyte genomic DNA was extracted, and all eight exons of the *CYP17A1* gene were amplified by PCR using previously described primers and conditions (9). The sizes of the PCR products were confirmed by electrophoresis in 1.5% agarose gel. The PCR products were treated with exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT; USP Corp., Cleveland, OH) and sequenced, and the data were analyzed using Sequencher version 4.2 (Gene Codes, Ann Arbor, MI).

Construction of CYP17A1 expression vectors

The R96W mutant was recreated in the human P450c17cDNA (2) by PCR-based, site-directed mutagenesis using primers 5′-AAGGACTTCTCTGGGTGGCCTCAAATGGCAA-3′ and 5′-TTGCCATTTGAGGCCACCCAGAGAAGTCCTT-3′ and cloned in pcDNA3 (Invitrogen, Carlsbad, CA). The H373D mutant was created similarly using 5′-CCTATGCTCATCCCCGACAAGGCCAACGTTG-3′ and 5′-CAACGTTGGCCTTGTCGGGGATGAGCATAGG-3′. The methylated parental wild-type cDNA was digested with 10 U *Dpn*I at 37 C for 180 min, and the remaining unmethylated mutagenized cDNA plasmid was used to transform *Escherichia coli* DH5α. The mutagenized cDNAs were verified by sequencing.

Transient transfection of COS-1 cells and assays for enzyme activities

COS-1 monkey kidney cells were grown in DMEM supplemented with 10% fetal calf serum and antibiotics at 37 C in a humidified 5% CO_2 incubator. Cells were seeded onto six-well plates (Falcon 3046; BD Biosciences, Lincoln Park, NJ) at approximately 50% confluence and transfected with 400 ng of pcDNA3-c17 plasmids using Effectene (QIAGEN, Valencia, CA). Cells incubated for 65 h after transfection were assayed for P450c17 activities. Cells were washed three times with serum-free DMEM and incubated with 0.5 ml medium containing labeled steroid; 17α-hydroxylase assays used 100,000 cpm [^{14}C]progesterone (PerkinElmer, Norwalk, CT) for 40 min, and 17,20-lyase assays used 100,000 cpm [^{3}H]17OH-Preg (American Radiolabeled Chemicals, St. Louis, MO) for 80 min. Medium was collected, and steroids were extracted and separated by thin-layer chromatography as described (10) and quantitated by phosphorimaging using Scion Image software (Scion Corp., Frederick, MD).

Bacterial expression of P450c17, preparation of membranes, and spectral studies

The pCWori-mod(His)4 expression plasmid containing the cDNA for human P450c17 with amino-terminal modifications that facilitate bacterial expression (11) was transformed into *E. coli* JM109. Ampicillin-resistant colonies were grown at 30 C to OD = 0.4, and P450c17 expression was induced with 0.4 mM isopropyl-1-thio-β-d-galactopyranoside at 28 C for 60 h. The bacteria were treated with lysozyme (0.5 mg/ml) and EDTA [0.1 mM (pH 8.0)] to generate spheroplasts. The spheroplasts were pelleted by centrifugation (5000 × *g* for 15 min); resuspended in 100 mM potassium phosphate (pH 7.6), 6 mM MgOAc, 0.1 mM dithiothreitol, 20% (vol/vol) glycerol, 1 mM phenylmethylsulfonylfluoride, and 0.1 mM DNase I; and disrupted by sonication (20 sec on/off cycles for 10 min at 40% power) (model 550; Fisher Scientific, Pittsburgh, PA). The lysate was cleared of cellular debris by centrifugation at 12,000 × *g* for 15 min, and then the membranes in the supernatant were pelleted at 150,000 × *g* for 90 min at 4 C. Membranes were re-

TABLE 1. Basal and 60-min post-cosyntropin adrenal steroid profile

Steroids	Basal	Post-cosyntropin	Reference values (basal, prepubertal female)	Reference values (stimulated, prepubertal female)
Cortisol (μg/dl)	<1	<1	3–21	20–31
Deoxycorticosterone	36	68	2–34	22–120
11-Deoxycortisol	<10	15	20–155	95–254
Corticosterone	13,900	37,300	70–1,860	2,520–5,650
18-Hydroxycorticosterone	423	894	2.4–10.5	95–289
Aldosterone	2.8	4.3	3–35	14–42
Pregnenolone	320	325	20–140	39–130
Progesterone	42	73	<10–26	48–171
17OH-Preg	42	45	10–186	70–656
17OHP	<10	<10	3–90	85–280
DHEA	70	75	31–345	69–322
Testosterone	4.3	4.9	<3–10	[a]

Values are in nanograms per deciliter, unless otherwise indicated. Reference values are from Esoterix Inc. (Calabasas Hills, CA), where the assays were performed.

[a] Testosterone is not significantly changed after ACTH.

suspended in 50 mM potassium phosphate (pH 7.4) and 20% (vol/vol) glycerol. An aliquot of membranes corresponding to 2 mg of protein was made up to 2 ml with the same buffer, and the mixture was divided equally between two cuvettes. Sodium dithionite 50 mg was added to each cuvette, and the baseline spectrum was recorded between 400 and 500 nm. CO was then bubbled through the sample cuvette for 2 min to obtain the reduced CO spectrum.

Results

Sequencing the *CYP17A1* gene from the patient's DNA showed compound heterozygosity for the mutations 458C>T in exon 1 (NCBI reference sequence NG_007955), changing arginine 96 to tryptophan (R96W); and 5001C>G in exon 6, changing histidine 373 to aspartic acid (H373D). Parental DNA was not available for investigation. The R96W mutation has been reported previously (12); the H373D mutation is novel.

To assess the activities of these mutations, we constructed vectors for their expression and transfected these into nonsteroidogenic COS-1 cells and assessed their 17α-hydroxylase and 17,20-lyase activities by examining the conversions of radiolabeled progesterone to 17OHP (hydroxylase) and 17OH-Preg to DHEA (lyase) (10, 13). Neither the R96W nor H373D mutants had detectable 17α-hydroxylase or 17,20-lyase activity, whereas the wild-type P450c17 showed robust activity (Fig. 1A).

A previous study suggested that a different mutant at position 373, H373L, was inactive because the mutant protein failed to bind the heme group found in all cytochrome P450 enzymes (9). Therefore, we prepared membrane fractions from *E. coli* expressing either wild-type or H373D P450c17 and assayed heme incorporation by CO-induced difference spectra (14). The wild-type P450c17 showed a characteristic peak at 449 nm upon addition of CO, whereas the H373D mutant vector produced a peak at 420 nm, typical of a denatured P450 (Fig. 1B). These results suggest that the H373D mutation causes a defect in protein folding.

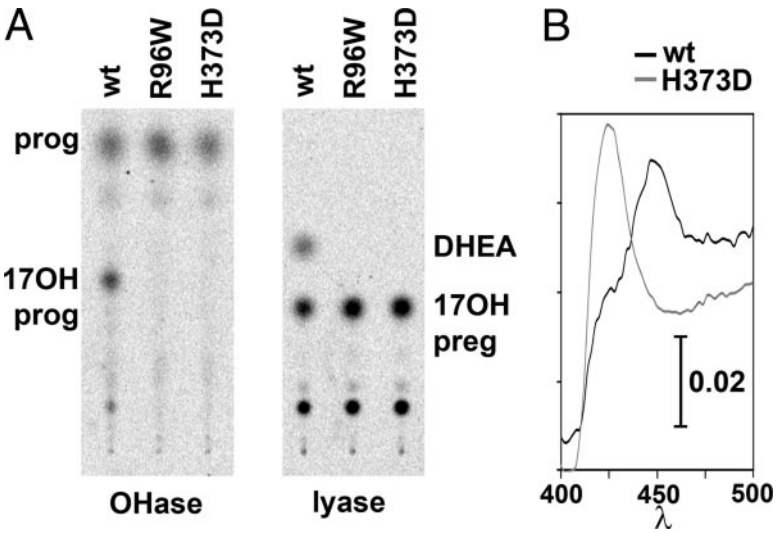

FIG. 1. Characterization of the P450c17 mutants. A, Autoradiogram of a thin layer chromatogram showing that wild-type (wt) P450c17 converts progesterone (Prog) to 17-hydroxyprogesterone (17OHprog) and 17α-hydroxypregnenolone (17OHpreg) to DHEA. The mutations R96W and H373D have no detectable 17α-hydroxylase (OHase) or 17,20-lyase (lyase) activity. B, CO-reduced difference spectra from *E. coli* membranes expressing wt P450c17 or H373D mutation. The wild-type protein has the expected peak at 450 nm, whereas the mutant peak is at 420 nm.

Discussion

P450c17 catalyzes 17α-hydroxylase and 17,20-lyase activities and hence is the qualitative regulator of steroidogenesis that determines the class of steroid made (15). The adrenal zona glomerulosa does not express P450c17 (16) and hence produces 17-deoxysteroids leading to aldosterone. The adrenal zona fasciculata expresses the 17α-hydroxylase activity but very little of the 17,20-lyase activity of P450c17, and hence produces 21-carbon, 17-hydroxysteroids, leading to cortisol. The adrenal reticularis, testicular Leydig cells, and ovarian theca cells express both the 17α-hydroxylase and 17,20-lyase activities of P450c17, and hence produce the 19-carbon 17-hydroxysteroid DHEA, the precursor of sex steroids. The discrimination between a cell having only hydroxylase activity or both hydroxylase and lyase activities is based on the abundance of the redox partner, P450 oxidoreductase, and the presence of two factors that facilitate the interaction of P450c17 and P450 oxidoreductase: the serine phosphorylation of P450c17 and the allosteric action of cytochrome b5 (17). Correspondingly, most P450c17 mutations causing isolated 17,20-lyase deficiency lie in the redox-partner binding site of P450c17 (6, 7, 18).

The R96W mutation has been reported in two French-Canadian siblings with 46,XY pseudohermaphroditism and combined 17α-hydroxylase/17,20-lyase deficiency (12). Structural modeling suggests that R96 lies within flanking strand 2 of β-sheet 1, and the guanidinium group of R96 appears to form hydrogen bonds with carbonyl groups of residues A113 and F114 (19). Removal of this positively charged group, which occurs in mutation R96W, appears to destabilize the protein by disrupting this interaction between the two domains, leading to complete inactivity of the enzyme (20). Although our patient's parental DNA was not available, we would speculate that the French-Canadian mother donated the R96W mutation.

The H373D mutation is novel, but the related H373L mutation has been described in two Japanese sisters with hypertension and sexual infantilism (9). The H373L mutant lacked 17α-hydroxylase and 17,20-lyase activities in transfected COS-1 cells, and membrane preparations from *E. coli* expressing H373L had a spectral peak at 420 nm rather than at 450 nm. These results suggested that the H373L mutant does not incorporate the heme group properly, suggesting a role for H373 in heme binding. Computational modeling of P450c17 shows that H373 does not interact directly with the heme, but instead forms a hydrogen bond with the carboxylic acid group of E391, which lies in an adjacent β sheet near the membrane-attachment domain (19). Thus mutation of H373, either to an aliphatic residue (H373L) or to a polar one (H373D) appears to create a global alteration in the structure of P450c17 that

secondarily prevents heme binding. The change of histidine to aspartic acid maintains a polar residue but increases its electronegativity, apparently causing a change in protein folding.

The complete loss of both 17α-hydroxylase and 17,20-lyase activities in these two different point mutations in H373 indicates that this locus is essential for enzyme activity. Identification and biochemical characterization of novel *CYP17A1* mutations provides useful information about the structure and enzymatic activity of P450c17. Although rare, 17α-hydroxylase deficiency should be considered in any phenotypic female with delayed puberty and low renin hypertension; the diagnosis is made by an ACTH test with a complete profile of adrenal steroids, especially the precursors and products of the 17α-hydroxylase and 17,20-lyase reactions.

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