Partial Defect in the Cholesterol Side-Chain Cleavage Enzyme P450scc (*CYP11A1*) Resembling Nonclassic Congenital Lipoid Adrenal Hyperplasia

Taninee Sahakitrungruang,* Meng Kian Tee,* Piers R. Blackett, and Walter L. Miller

Department of Pediatrics (T.S., M.K.T., W.L.M.), University of California, San Francisco, San Francisco, California 94143; and Department of Pediatrics (P.R.B.), University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

Context: The cholesterol side-chain cleavage enzyme (P450scc), encoded by the *CYP11A1* gene, converts cholesterol to pregnenolone to initiate steroidogenesis. Genetic defects in P450scc cause a rare autosomal recessive disorder that is clinically indistinguishable from congenital lipoid adrenal hyperplasia (lipoid CAH). Nonclassic lipoid CAH is a recently recognized disorder caused by mutations in the steroidogenic acute regulatory protein (StAR) that retain partial function.

Objective: We describe two siblings with hormonal findings suggesting nonclassic lipoid CAH, who had a P450scc mutation that retains partial function.

Patients and Methods: A 46,XY male presented with underdeveloped genitalia and partial adrenal insufficiency; his 46,XX sister presented with adrenal insufficiency. Hormonal studies suggested nonclassic lipoid CAH. Sequencing of the *StAR* gene was normal, but compound heterozygous mutations were found in the *CYP11A1* gene. Mutations were recreated in the F2 plasmid expressing a fusion protein of the cholesterol side-chain cleavage system. P450scc activity was measured as Vmax/Km for pregnenolone production in transfected COS-1 cells.

Results: The patients were compound heterozygous for the previously described frameshift mutation 835delA and the novel missense mutation A269V. When expressed in the P450scc moiety of F2, the A269V mutant retained 11% activity of the wild-type F2 protein.

Conclusions: There is a broad clinical spectrum of P450scc deficiency. Partial loss-of-function *CYP11A1* mutation can present with a hormonal phenotype indistinguishable from nonclassic lipoid CAH. **(*J Clin Endocrinol Metab* 96: 792–798, 2011)**

Steroidogenesis is initiated by converting cholesterol to pregnenolone by the cholesterol side-chain cleavage enzyme, P450scc, encoded by the single *CYP11A1* gene on chromosome 15q23-24 (1–3). Mitochondrial cytochrome P450scc catalyzes three consecutive reactions: 20α-hydroxylation, 22-hydroxylation, and scission of the C20,22 carbon bond. Each of these reactions requires a pair of electrons donated by reduced nicotinamide adenine dinucleotide via an electron transfer chain consisting of a flavoprotein termed ferredoxin [adrenodoxin (Adx)] reductase (AdRed) and an iron-sulfur protein termed ferredoxin (Adx) (reviewed in Ref. 4). Congenital lipoid adrenal hyperplasia (lipoid CAH) is a severe autosomal recessive disorder that disrupts the synthesis of all adrenal and gonadal steroids, including glucocorticoids, mineralocorticoids, and sex steroids (reviewed in Ref. 5) and typically features massively enlarged adrenals filled with lipids (6, 7). Because these patients appeared to have a

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Abbreviations: Adx, Adrenodoxin; AdRed, Adx reductase; lipoid CAH, congenital lipoid adrenal hyperplasia; Km, Michaelis constant; StAR, steroidogenic acute regulatory protein; Vmax, maximum velocity.

lesion in the conversion of cholesterol to pregnenolone, it was initially thought to be caused by a lesion in the enzyme(s) converting cholesterol to pregnenolone, and hence was termed "20,22 desmolase deficiency" (7–9). Studies of the biochemistry of this reaction showed that it was catalyzed by P450scc (reviewed in Ref. 10), but analysis of the *CYP11A1* gene encoding P450scc found no mutations (11–13). Instead, patients with lipoid CAH have mutations in the gene encoding the steroidogenic acute regulatory protein (StAR) (14–17), which facilitates the transport of cholesterol into mitochondria (reviewed in Ref. 18), where cholesterol is converted to pregnenolone by P450scc. A milder form of this disease, termed nonclassic lipoid CAH, is a recently recognized disorder caused by StAR mutations that retain partial activity (19–21). StAR is expressed in the adrenals and gonads but not in the placenta (22). Because placental production of progesterone is essential for the maintenance of human pregnancy, mutations in P450scc were thought to be incompatible with term gestation. Nevertheless, beginning in 2001 several patients with defects in *CYP11A1* gene causing P450scc deficiency have been reported (23–28). Most of these cases were caused by severe loss-of-function mutations and presented with severe, early onset adrenal failure and complete phenotypic 46,XY sex reversal in genetic males (27), although one patient with late-onset nonclassic disease has been reported (28). We now describe two siblings who appeared to have nonclassic lipoid CAH, but instead had a mutation in P450scc that retained partial activity.

Patients and Methods

Case reports

The patients were siblings from a nonconsanguineous Hispanic family. Patient 1 was a 9-yr-old 46,XY male with micropenis, severe hypospadias, bifid scrotum, and cryptorchidism. He was previously evaluated for his abnormal genital development and had six surgical operations at ages 1–5 in Mexico for repair of the genitalia, but no medical records from Mexico were available. Attempted orchidopexy had been unsuccessful on the left, but the right testis was brought down to the inguinal region. The scrotum had always been small. At about 8 yr of age, he had a severe viral infection treated with hydrocortisone. There was no family history of hypospadias or ambiguous genitalia. His family emigrated to the United States, and he was first evaluated at the University of Oklahoma Children's Hospital at age 9 yr and 9 months for hypogonadism and hyperpigmentation. At that time, he had been untreated; his height was 141.1 cm (+0.58 SD), his weight was 34.0 kg, (+0.65 SD), blood pressure was 107/66 (75/75 percentile), and pulse rate was 84. The physical examination was normal except that the penis was 2.3 cm long (< −2.5 SD) and 1.3 cm wide; the scrotum was bifid; the left testis had been removed, but the right testis was palpable in the inguinal region and was approximately 0.5 ml in volume. The areola and palmar creases were pigmented bilaterally. An ACTH stimulation test at age 9 yr showed normal basal cortisol [9 μg/dl (250 nmol/liter)] with grossly elevated basal ACTH [1507 pg/ml (335 pmol/liter)]. After receipt of 250 μg of cosyntropin, the cortisol was 10 μg/dl (276 nmol/liter), pregnenolone was below 20 ng/dl (<630 pmol/liter), progesterone was below 10 ng/dl (<318 pmol/liter), 17 OH-pregnenolone was below 10 ng/dl (<300 nmol/liter), 17 OH-progesterone was below 5 ng/dl (<150 pmol/liter), dehydroepiandrosterone sulfate was 19 μg/dl (517 nmol/liter), and androstenedione was 23 ng/dl (803 pmol/liter). The serum sodium was 134 mmol/liter, and plasma renin activity shortly after starting treatment was 6400 ng/dl · /h (normal range, 70–330). He was treated with hydrocortisone, 5 mg three times daily (10 mg/m^2 · d), and 9α-fluorocortisol, 0.1 mg daily, and referred for urological assessment. When seen at age 14 yr, the genitalia were prepubertal. Testosterone was less than 3.0 ng/dl (<104 pmol/liter), LH was 40.9 mIU/ml, and FSH was 87.2 mIU/ml. The right testis, still located near the inguinal canal, was 2 ml in volume, and the penis measured 3 cm in length and was 0.5 cm in width. He was treated with testosterone cypionate, 100 mg every 4 wk for 3 months, which was then increased to 200 mg every 4 wk. By age 16 yr, the penile length had increased to 6 cm but was retracted into pubic fat tissue. Abdominal computed tomographic imaging showed slightly small adrenals with stippled calcifications.

Patient 2 is the 5-yr-old sister of patient 1. She was being treated with hydrocortisone, 5 mg twice daily (14.5 mg/m^2 · d), and 9α-fluorocortisol, 0.1 mg/d for Addison's disease, which had been previously diagnosed in Mexico at the age of 12 months, but medical records were not available. When first seen at age 5 yr and 4 months, her height was 116.2 cm (+1.29 SD), and weight was 21.1 kg (+1.05 SD). Her physical examination was normal with normal prepubertal female genitalia. Mild pigmentation appeared consistent with her ethnicity. Her pulse rate was 99 and blood pressure was 107/68 (75/75 percentile). The ACTH was 275 pg/ml (61.1 pmol/liter), indicating incomplete suppression by hydrocortisone. The ACTH at a subsequent visit was 156 pg/ml (34.7 pmol/liter) but increased again to 446 pg/ml (99.1 pmol/liter) with plasma renin activity below 20 ng/dl · h. When seen at the age of 11.5 yr, she remained prepubertal and her growth was tracking just below the 25th percentile for height and weight while continuing treatment with adjusted doses of hydrocortisone at 15 mg/m^2 · d and 9α-fluorocortisol at 0.1 mg/d. Her karyotype was 46,XX, and abdominal computed tomographic imaging showed small adrenals with diffuse multiple punctate calcifications. Adrenal hypoplasia congenita due to an X-linked DAX-1 mutation was excluded by the presence of an affected female sibling and by the genital abnormality in the male, and steroidogenic factor 1 mutations typically cause 46,XY disordered sexual development without adrenal insufficiency (29); therefore, a lesion in StAR or P450scc was considered.

DNA sequencing

With Institutional Review Board approval and informed consent, leukocyte genomic DNA was extracted and all protein-coding exons and at least 100 bp of flanking intronic DNA of the *StAR* and *CYP11A1* genes were amplified by PCR using previously described primers and conditions (23, 25, 30). 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 us-

ing Sequencher version 4.2 (Gene Codes, Ann Arbor, MI), compared with reference cDNA (1) and gene (2) sequences.

To determine whether the identified mutations lie on different DNA strands, a 1005-bp fragment encompassing exons 4 and 5, which spanned both mutations, was amplified from genomic DNA using primer pair sense 5′-ATAGGATCCCCCGAGGCCCAGCGATTCA and antisense 5′-ATACTCGAGGTGGGCACAGGGGGCAACAAG. The PCR products were digested with *Bam*HI and *Xho*I, ligated to pcDNA3.1, and propagated in *Escherichia coli*. Plasmid DNAs isolated from four individual colonies were sequenced on both strands.

Construction of P450scc expression vectors

The A269V mutant of P450scc was recreated in the F2 plasmid expressing a fusion protein of the cholesterol side-chain cleavage system (H_2N-P450scc-adrenodoxin reductase-adrenodoxin-COOH) (31) by PCR-based, site-directed mutagenesis using the primers 5′-GACCATGTGGCTGTATGGGACGTGATT and 5′-AATCACGTCCCATACAGCCACATGGTC. 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 *E. coli* DH5α cells. The mutagenized cDNAs were verified by sequencing.

Functional studies of P450scc activity

Nonsteroidogenic monkey kidney COS-1 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 divided into 12-well plates (Falcon; BD Biosciences, Lincoln Park, NJ) and transfected at approximately 50% confluence with either the wild-type or mutant F2 plasmid using Effectene (QIAGEN, Valencia, CA). To monitor transfection efficiency, cells were also cotransfected with 5 ng of *Renilla* luciferase reporter plasmid (pRL-CMV) (Promega, Madison, WI) per well. After 24 h, the culture medium was replaced with fresh serum-free medium containing 0.3, 1, 3, or 5 μM 22R-hydroxycholesterol, which bypasses the action of StAR and is converted to pregnenolone by P450scc (14, 32). Culture media were collected 24 h later and analyzed for pregnenolone production by enzyme immunoassay (ALPCO Diagnostics, Salem, NH). Assays were done in the linear range of the standard curve. Cross-reactivity with progesterone, dehydroepiandrosterone, and 5α-androstenediol were less than 6%, and the sensitivity of this assay was 5.4 ng/dl. The data were normalized to *Renilla* luciferase measurements, thus normalizing for the efficiency of transfection. Lineweaver-Burke analyses were done using the GraphPad Prism 3 (GraphPad Software, San Diego, CA), and data are presented as the mean $\pm$ SEM for three independent experiments. Statistical analyses were done with two-tailed, unpaired *t* tests.

Results

Mutational analysis

DNA sequencing of coding regions and splice sites of the *StAR* gene showed no mutations in both siblings. Both patients were compound heterozygous for the novel *CYP11A1* missense mutation 806C>T in exon 4 (as numbered from the first base in the ATG start codon) (1), changing alanine 269 to valine (A269V) and the frameshift mutation 835delA in exon 5, which was reported previously (25, 27). According to NCBI reference sequence NC_000015.9, 806C>T is at position 23928 and 835delA is at 24608. Parental DNA was not available for mutation analysis. Therefore, to determine whether the patients were compound heterozygotes, we amplified adjacent exons 4 and 5 that contained the mutations as a single fragment, cloned the DNA, and then sequenced four clones. All clones carried only one of the two identified mutations, proving that they lie on separate alleles.

Studies of P450scc enzyme activity

To catalyze the conversion of cholesterol to pregnenolone, P450scc requires the presence of two electron transfer proteins, Adx and AdRed (4). Because the relative abundances of Adx and AdRed influence P450scc activity in transfected cells (31, 33), we used a single plasmid, termed F2, expressing a fusion protein of the cholesterol side-chain cleavage system (H_2N-P450scc-AdRed-Adx-COOH) (31). This fixes the molar ratio of the three components at 1:1:1, thus eliminating variations in the level of cofactor expression as a source of potential variation in P450scc activity. The A269V mutation was recreated in the P450scc moiety of the F2 vector by site-directed mutagenesis; then wild-type and mutant F2 plasmids were separately transfected into COS-1 cells, incubated with 0.3, 1, 3, and 5 μM 22R-hydroxycholesterol for 24 h, and assayed for the production of pregnenolone by enzyme immunoassay. Because the assays involve intact cells rather than purified enzyme of known mass, the data yield only "apparent" kinetic parameters. Lineweaver-Burke analyses (Fig. 1) showed that the differences in the apparent Michaelis constant (Km), maximal velocity (Vmax), and Vmax/Km of F2 compared with A269V-F2 were significantly different (Table 1). After 24-h incubation, only 3.2% of the 22R-hydroxycholesterol was converted to pregnenolone in cells transfected with the wild-type F2 construct; hence, substrate was not limiting, and thus the mutant activities were not overestimated. As assessed by Vmax/Km, the A269V mutant had 11% activity of wild-type activity. One might consider that the presence of a normal StAR protein will result in conditions of substrate excess, so that only the reduced Vmax indicates loss of P450scc activity; by this assessment, the mutant retained 29% of wild-type activity.

Discussion

P450scc deficiency is a rare disorder, with only seven patients described to date (23–28). Most cases of P450scc deficiency have presented with the hormonal and pheno-

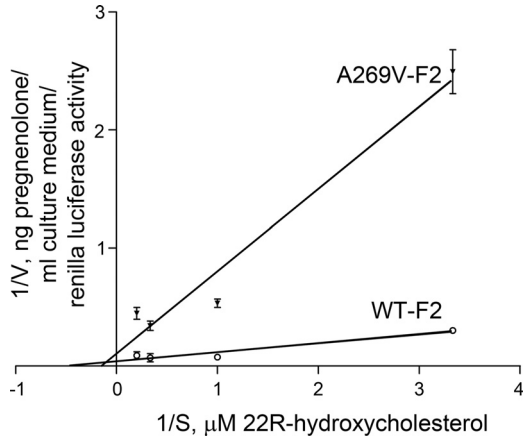

FIG. 1. Lineweaver-Burke analyses of the side-chain cleavage activities of F2 and A269V-F2. COS-1 cells were cotransfected with either the wild-type F2 construct and *Renilla* luciferase or the A269-F2 construct and *Renilla* luciferase, and incubated with 0.3, 1, 3, and 5 μM 22R-hydroxycholesterol for 24 h. Pregnenolone in the medium was measured by enzyme immunoassay and normalized to the *Renilla* luciferase activity, and the data are displayed as Lineweaver-Burke plots. The data are expressed as means ± SEM from three independent experiments.

typic features of classic lipoid CAH, although none have been described with the massive adrenal enlargement typical of lipoid CAH (27). However, the most recent report of P450scc deficiency described a nonclassic presentation in a 46,XY male with hypospadias, cryptorchidism, and adrenal failure at 9 yr associated with a homozygous L222P mutation (28). We describe two patients with nonclassic P450scc deficiency, who carried the novel missense mutation A269V on one allele and a frameshift mutation devoid of activity on the other allele.

Lipoid CAH, which closely resembles P450scc deficiency clinically, is caused by StAR mutations. Both are very severe forms of CAH (14, 15, 27), leading to the severe impairment of all adrenal and gonadal steroid hormone synthesis. Patients with the "classic" forms of both P450scc deficiency and lipoid CAH typically present with adrenal failure and salt wasting in infancy and are phenotypically female irrespective of genetic sex. Nonclassic lipoid CAH is a recently recognized disorder caused by partial loss-of function mutations in StAR (19–21). Affected individuals can present with late onset adrenal insufficiency resembling Addison disease with only mild or minimally disordered sexual development. Similarly, nonclassic P450scc deficiency presents after infancy with varying degrees of retained adrenal and gonadal function.

Functional assays using the F2 fusion protein of the P450scc system in transfected COS-1 cells showed that P450scc A269V retained 11% of wild-type activity as assessed by Vmax/Km, or 29% of wild-type activity as assessed by Vmax alone; the 835delA mutation is devoid of activity, as expected (21). A previously reported 46,XY phenotypically female patient (patient 5) was a compound heterozygote for the P450scc mutations V415E and L141W; as assessed by Vmax alone in the F2 system, V415E had no measureable activity and L141W retained 39% of wild-type activity (27). The retained activity in that phenotypically female patient was slightly greater than that in our partially virilized patient. It is not uncommon to find apparent discrepancies between genotype and phenotype when examining individual patients. Such comparisons in CAH due to 21-hydroxylase deficiency plagued that field for several years, until comparisons of large numbers of patients showed excellent genotype/phenotype correlations. However, this is not yet possible with P450scc, with less than a dozen reported cases.

There has been a relatively poor correlation between the clinical findings and the enzymology of the mutations in the previously reported cases of P450scc deficiency (27). This may be attributable to technical dif-

TABLE 1. Catalytic activities of wild-type F2 and F2 carrying the A269V P450scc mutant in the conversion of 22R-hydroxycholesterol to pregnenolone in transfected COS-1 cells

	Wild-type F2	A269V-F2	*P* value
Km ± SEM (μM)	2.57 ± 0.622	6.43 ± 0.273	0.0031
Vmax ± SEM (ng · ml^{-1} · *Renilla* luciferase activity-1)	32.6 ± 6.23	9.30 ± 1.02	0.0006
Vmax/Km ± SEM	12.8 ± 0.751	1.44 ± 0.104	<0.0001
% Wild type	100	11	

human CYP27A1	--FWKRYLDGWNAIFSFGKKL
human CYP24A1	TKVWQGHTL**A**WDTIFKSVKAC
human CYP27B1	G-PWGRLCRDWDQMFAFAQRH
human CYP11B1/B2	PKVWKEHFE**A**WDCIFQYGDNC
human CYP11A1	TKTWKDHVA**A**WDVIFSKADIY
bovine Cyp11a1	TKTWRDHVA**A**WDTIFNKAEKY
sheep Cyp11a1	TKTWRDHVA**A**WDTIFNKAEKY
mouse Cyp11a1	TKTWKDHAA**A**WDVIFNKADEY
chicken Cyp11a1	TKTWRDHVH**A**WDAIFTQADKC
zebra finch Cyp11a1	SRTWREHVH**A**WDAIFCQADKC
lizard Cyp11a1	SKTWQDHIT**A**WDIIFMQADKC
crocodile Cyp11a1	SKTWQDHVK**A**WDVIFMQADKC
zebrafish Cyp11a1	SNIWKNHVE**A**WDGIFNQADRY

FIG. 2. Sequence alignment of human mitochondrial P450 enzymes with human P450scc and with the P450scc proteins from other species. The alignment of the human mitochondrial P450 enzymes is taken directly from Fu *et al.* (39). CYP27A1 is sterol 27-hydroxylase (40); CYP24 is 1,25 dihydroxyvitamin D 24-hydroxylase (41); CYP27B1 is 25-hydroxyvitamin D 1α-hydroxylase (39); CYP11B1 and CYP11B2, whose sequences are identical in the region shown, are steroid 11β-hydroxylase and aldosterone synthase, respectively (42). Residue 269 in the human protein and corresponding residues in the other species are *bold and underlined*. The alignment of P450scc from the indicated species was performed by a BLAST search of the NCBI database (http://www.ncbi.nlm.nih.gov) using *Homo sapiens* P450scc, accession NP_000772.2, as the reference sequence (1).

ferences in the assays and the small number of patients studied to date. Among the reports of P450scc deficiency, three (24, 26, 28) used a triple transfection with equimolar amounts of three plasmids expressing P450scc, Adx, and AdRed, whereas our studies used the F2 system (Refs. 23, 27 and the present report). We find that using the single F2 plasmid expressing a fusion protein of the cholesterol side-chain cleavage system facilitates studying the functional consequences of P450scc mutations because the F2 system eliminates potential variations in the levels of cofactor expression, ensuring that any variations in mutant P450scc activity are due to the amino acid change rather than to variations in cofactor expression. Cells transfected with the F2 construct produce substantially more pregnenolone than do cells transfected with either P450scc alone or triply transfected with three vectors separately expressing P450scc, AdRed, and Adx, because the F2 fusion protein increases enzymatic activity by increasing the maximum velocity of the reaction (31). This provides a better ratio of signal to background, permitting more reliable measurement of the low activities of P450scc mutants. Nevertheless, assays in transfected cells may not fully reflect the complexity of events *in vivo*.

Ala269 is a highly conserved residue, directly preceding a tryptophan residue that is preserved among P450scc sequences from fish, birds, reptiles, and mammals and that is also conserved in all human mitochondrial P450 enzymes (Fig. 2). A crystal structure of P450scc with bound 22R-hydroxycholesterol has recently been posted on the Protein Data Bank (http://www.pdb.org/pdb/explore/explore.do?structureId=3NA0) (34). By assessing this structure with the program DSSP (Define Secondary Structure of Proteins) (35), Ala 269 is buried in an α-helix and is not accessible to the protein's surface, and hence probably does not affect redox partner interactions. Thus, whereas substitution of Val for Ala is a conservative mutation that typically elicits minor effects on protein folding, we would hypothesize that the bulkier valine residue would interfere with a function of the conserved adjacent tryptophan, but the reason for this mutation's loss of activity is not clear. Similarly, the L, R, and V residues in the previously reported mutants L141W, L222P, R353W, and V415E (24, 27, 28) are also inaccessible to the protein's surface, whereas the A residues mutated in A189V and A359V (24, 26) are partially surface-exposed and hence might alter redox-partner interactions.

Human pregnancy depends on progesterone from the mother's corpus luteum during the first trimester, following which the placenta becomes the major source of progesterone (36). P450scc is needed for placental progesterone production. Residual mutant P450scc activity seems to explain the term gestation in several cases of P450scc deficiency, although one patient with frameshift mutations that ablated all activity was born at term (27). That case may be explained by an unusually long survival of the maternal corpus luteum. The exact mechanism of "turning on" placental progesterone synthesis is unclear, but it may relate to a shift of oxygen concentration (37) and requires the expression of *grainyhead* (LBP) transcription factors rather than steroidogenic factor-1 to induce *CYP11A1* gene transcription (38).

Adrenal enlargement has been reported in virtually all patients with classic lipoid CAH caused by StAR mutations, although one patient with normal-sized adrenals has been reported (17). By contrast, such adrenal enlargement has not been reported in patients with P450scc deficiency, which is clinically indistinguishable from StAR mutations (27). Similarly, no patients with nonclassic lipoid CAH have had adrenal enlargement (19–21). By contrast, small adrenals with calcifications, as seen in our patients, suggesting cirrhotic, end-stage fat deposition, have been reported in nonclassic lipoid CAH (20). Therefore, in the setting of adrenal failure, enlarged adrenals indicate lipoid CAH due to StAR mutations; normal-sized adrenals cannot exclude it (17), but instead suggest nonclassic lipoid CAH or P450scc deficiency. Our cases illustrate that P450scc mutations can present with a broad spectrum of phenotypes, extending from classic lipoid CAH through a nonclassic phenotype presenting in males with hypogonadism and partial adrenal insufficiency.

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Address all correspondence and requests for reprints to: Walter L. Miller, M.D., Professor of Pediatrics and Chief of Endocrinology, HSE 1427, University of California San Francisco, San Francisco, California 94143-0978. E-mail: wlmlab@ucsf.edu.

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