Nonclassic Congenital Lipoid Adrenal Hyperplasia: A New Disorder of the Steroidogenic Acute Regulatory Protein with Very Late Presentation and Normal Male Genitalia

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Context: Lipoid congenital adrenal hyperplasia is a severe disorder of adrenal and gonadal steroidogenesis caused by mutations in the steroidogenic acute regulatory protein (StAR). Affected children typically present with life-threatening adrenal insufficiency in early infancy due to a failure of glucocorticoid (cortisol) and mineralocorticoid (aldosterone) biosynthesis, and 46,XY genetic males have complete lack of androgenization and appear phenotypically female due to impaired testicular androgen secretion in utero.

Objective: The objective of this study was to investigate whether nonclassic forms of this condition exist.

Patients and Methods: Sequence analysis of the gene encoding StAR was undertaken in three children from two families who presented with primary adrenal insufficiency at 2–4 yr of age; the males had normal genital development. Identified mutants were tested in a series of biochemical assays.

Results: DNA sequencing identified homozygous StAR mutations Val187Met and Arg188Cys in these two families. Functional studies of StAR activity in cells and in vitro and cholesterol-binding assays showed these mutants retained ~20% of wild-type activity.

Conclusions: These patients define a new disorder, nonclassic lipoid congenital adrenal hyperplasia, and represent a new cause of nonautoimmune Addison disease (primary adrenal failure).

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IPOID CONGENITAL ADRENAL hyperplasia (CAH) (OMIM no. 201710), the most severe form of adrenal hyperplasia, significantly impairs adrenal and gonadal steroidogenesis by a defect in the conversion of cholesterol to pregnenolone (1). Affected infants experience salt loss from impaired mineralocorticoid and glucocorticoid synthesis, but hormonal replacement therapy permits long-term survival (2). Because the fetal testis is affected, 46,XY genetic males fail to produce testosterone and are born with female external genitalia. The defect in lipoid CAH is primarily in the steroidogenic acute regulatory protein (StAR) (3, 4), which facilitates the entry of cholesterol into mitochondria, where it becomes the substrate for the cholesterol side-chain cleavage enzyme, P450scc (1, 5). Unlike abnormalities in StAR, which is not expressed in placenta, defects in P450scc will also disrupt placental progesterone synthesis, presumably interrupting pregnancy, although rare mutations in P450scc have been reported in children with adrenal failure (6–9).

In most steroidogenic disorders, such as steroid 21-hydroxylase deficiency, a spectrum of clinical findings results from different missense mutations. However, in lipoid CAH, the clinical findings are remarkably similar; all individuals reported to date have had female external genitalia irrespective of chromosomal sex and have had evidence of salt loss in the first year of life, usually within the first 2 months (3, 4, 10–13).

We report three children from two families who have a novel, mild form of lipoid CAH with their initial clinical manifestations at 2–4 yr and normal development of the male genitalia in 46,XY individuals. This new disorder, termed nonclassic lipoid CAH, illustrates that the clinical presentations of steroidogenic disorders can vary substantially from their classic descriptions and identifies a new form of nonautoimmune Addison disease (primary adrenal failure).

Patients and Methods

Case histories

Patient 1, a Pakistani (Gujakhan) 46,XX phenotypic female, had an uneventful infancy, experienced fever and vomiting at 2 yr of age, and was hypoglycemic during a viral illness at 4 yr. Progressive hyperpig-
Structural modeling of missense mutants

Our computational model of human wild-type N-62 StAR (17, 18) was modified by introducing missense mutations using the Swiss-Model program (http://expasy.ch/spdbv/). Energy minimization was performed with Amber 7 (http://amber.scripps.edu) (University of California, San Francisco, Computer Graphics Laboratory). The resulting models were checked with WHAT IF (http://swift.cmbi.kun.nl/WIWWWI/), and images were generated with Chimera (http://www.cgl.ucsf.edu/chimera).

Results

The clinical findings of compensated glucocorticoid and mineralocorticoid deficiency in the presence of apparently normal fetal testicular steroidogenesis suggested an abnormality in a gene affecting adrenal function, but sequencing of the ACTH receptor (MC2R), DAX1 (AHC, NR0B1), and SF1 (steroidogenic factor 1, NR5A1) was normal. However, the gene for StAR contained the homozygous mutation Val187Met in patient 1 and the homozygous mutation Arg188Cys in patients 2 and 3 (Fig. 1A). These residues are highly conserved among vertebrate species (Fig. 1B). The patients were heterozygous, and these mutations were not detected in 200 control Pakistani alleles, indicating these were functional mutations and not polymorphisms.

Structural modeling of missense mutations

To visualize the effects of these mutations, we used a validated computational model of human StAR, showing that Val187 and Arg188 lie in sheet β6, which contributes to the sterol-binding pocket (17, 18) (Fig. 1C). Arg188 forms a salt bridge with Glu169, coordinating with the 3-OH group of cholesterol to facilitate its binding (Fig. 1D). Mutation of Glu169 to Gly or Lys causes lipid CAH (4), confirming the essential role of this residue, but the folding of the Glu169Gly mutant is not as greatly disturbed as other severe mutants (19). The adjacent Val187 residue does not have an obvious function, but the modeling shows that its mutation to Met should inhibit cholesterol binding sterically.

Biochemical assays

The modifications of the pTWIN1 intein vector (New England Biolabs, Beverly, MA) for bacterial expression of wild-type and mutant N-62 StAR, the preparation and quantitation of proteins, the steroidogenic assays with isolated MA-10 cell mitochondria, and the assays of cholesterol binding were performed as described previously (17), except that nickel-nitrilotriacetic acid chromatography was added after the chitin-binding column for the Val187Met mutant.

Sequence analysis, mutagenesis, transfection, and steroid measurement

DNA was extracted from blood leukocytes with institutional review board approval. The seven exons of the human StAR gene were amplified by PCR and sequenced as described (11, 14). PCR-based site-directed mutagenesis was used to recreate the mutations found in the patients’ DNA in a full-length StAR cDNA expression vector (15), and the resulting plasmids were sequenced to ensure accuracy. Using Lipofectamine (Invitrogen, Carlsbad, CA), nonsteroidogenic monkey kidney COS-1 cells were cotransfected at 50–80% confluence with a StAR expression vector and the F2 plasmid expressing a fusion protein of the StAR, the preparation and quantitation of proteins, the steroidogenic cholesterol side-chain cleavage system (H2N-P450scc-adrenodoxin reductase-adrenodoxin- COOH) (16). Culture media were immunoas-

TABLE 1. Clinical and biochemical characteristics of patients with nonclassic lipid CAH

<table>
<thead>
<tr>
<th></th>
<th>Val187Met</th>
<th>Arg188Cys</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at investigation (yr)</td>
<td>4.5</td>
<td>2.2</td>
<td>10–50</td>
</tr>
<tr>
<td>Presentation</td>
<td>Hypoglycemia, pigmentation</td>
<td>Pigmentation</td>
<td>Pigmentation</td>
</tr>
<tr>
<td>Adrenal imaging</td>
<td>Normal US</td>
<td>Normal CT</td>
<td>Normal CT</td>
</tr>
<tr>
<td>ACTH (pg/ml)</td>
<td>&gt;1250</td>
<td>&gt;1250</td>
<td>&gt;1250</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;1.0 (μg/dl)</td>
<td>6.2 (μg/dl)</td>
<td>5–15</td>
</tr>
<tr>
<td>Peak (μg/dl)</td>
<td>&lt;1.0</td>
<td>5.6 (μg/dl)</td>
<td>&gt;20</td>
</tr>
<tr>
<td>PRA (ng/ml/h)</td>
<td>5.3 (ng/ml/h)</td>
<td>5.4 (ng/ml/h)</td>
<td>0.3–3.9</td>
</tr>
<tr>
<td>Aldosterone (ng/dl)</td>
<td>15.5</td>
<td>6.7 (ng/dl)</td>
<td>3.1–41</td>
</tr>
<tr>
<td>Treatment</td>
<td>Hydrocortisone</td>
<td>Hydrocortisone, fludrocortisone</td>
<td>Hydrocortisone, fludrocortisone</td>
</tr>
</tbody>
</table>

Conversion to Systeme International units: ACTH, picograms per milliliter × 0.22 for picomoles per liter; cortisol, micrograms per deciliter × 27.6 for nanomoles per liter; PRA, nanograms per milliliter per hour × 0.77 for picomoles per milliliter per hour; aldosterone, nanograms per deciliter × 27.7 for picomoles per liter. US, Ultrasound scan.
Functional analysis

StAR increases the flow of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM), where it is converted to pregnenolone to initiate steroidogenesis. The activity of StAR mutants is typically assayed by transfecting nonsteroidogenic cells with vectors expressing the cholesterol side-chain cleavage enzyme and StAR and measuring the amount of pregnenolone produced. Use of a null StAR vector provides a negative control, whereas addition of 22R-hydroxycholesterol (22R-OH), which bypasses the action of StAR, provides a positive control (3, 4, 13, 15). We cotransfected COS-1 cells with a vector expressing the F2 fusion of the cholesterol side-chain cleavage enzyme system and with a series of StAR expression vectors and assayed pregnenolone (Fig. 2A). The lipofectamine transfection protocol used here is more efficient than the calcium phosphate procedure used in a previous report (13), resulting in greater production of pregnenolone. In the absence of StAR, the F2 enzyme exhibits a low level of StAR-independent steroidogenesis (4, 16) at 10.7 ± 1.7 ng pregnenolone/ml culture medium. In the presence of 22R-OH, cells expressing F2 made 321.7 ± 57.4 ng/ml, indicating the maximal level of steroidogenesis achievable under these conditions. Using their endogenous cellular cholesterol as substrate, cells cotransfected with F2, and wild-type StAR generated 105.2 ± 11.7 ng/ml. In contrast, the Val187Met mutant generated 31.1 ± 6.9 ng/ml, and the Arg188Cys mutant generated 23.5 ± 5.0 ng/ml. Thus, when the background of StAR-independent steroidogenesis is subtracted, the Val187Met and Arg188Cys mutants have 21.6 and 13.6% of wild-type activity, respectively; the activities of the two mutants were significantly different (P = 0.03) by a paired two-tailed Student’s t test.

Biochemical analyses

To explore the mechanism by which these mutants retain partial activity, we expressed each StAR protein in bacteria using a self-splicing bacterial intein system. We purified these proteins to homogeneity and tested their activities in two cell-free systems. First, as an independent assay of the capacity to induce steroidogenesis, we added each StAR protein to mitochondria isolated from steroidogenic mouse Leydig MA-10 cells and measured pregnenolone produced from endogenous mitochondrial cholesterol (Fig. 2B). Wild-type N-62 StAR elicited five times more steroidogenesis than did buffer or the wholly inactive StAR mutant Met144Arg (13, 17). After subtracting the buffer control, the Val187Met and Arg188Cys mutants elicited 28.6 and 17.7% of wild-type activity, respectively; this difference between the two mutants was statistically significant (P = 0.005) by a paired two-tailed Student’s t test. Second, we found that the reduced activity of these mutants was associated with reduced cholesterol-binding capacity (Fig. 2C). Wild-type N-62 StAR could bind either [14C]cholesterol (not shown) or fluorescent nitrobenzoxadiazol (NBD) cholesterol, indicating that the NBD group did not affect binding (17). The cholesterol-binding capacity of the Val187Met mutant was 25.5% of control, and the capacity of the Arg188Cys mutant was 21.0% of the capacity of the wild-type N-62 StAR. Thus, both mutants retained partial cholesterol-binding capacity and partial activity to induce steroidogenesis.

Discussion

StAR mediates the rapid actions of ACTH and angiotensin II on the adrenal and of LH on the gonad, permitting very rapid rises in the circulating concentrations of steroids in response to acute physiological stimuli. StAR elicits this action by facilitating rapid movement of cholesterol from the OMM to the IMM, where it is converted to pregnenolone. The mechanism by which StAR moves cholesterol from OMM to IMM is only partially understood. StAR acts exclusively on the OMM (15, 20), where it undergoes acid-induced conformational changes apparently induced by protonated OMM phospholipids (17, 21, 22). StAR has a sterol-binding pocket that accommodates one cholesterol molecule (17, 18), but each molecule of StAR can mobilize approximately 400 mol-
ecules of cholesterol (23). Although StAR is required for acute steroidogenic responses, low levels of StAR-independent steroidogenesis (e.g., in the placenta) proceed in its absence (3, 4). The presence of StAR-independent steroidogenesis explains the pathophysiology of lipoid CAH by a two-hit model (4). Mutations in StAR ablate the acute response (the first hit), resulting in low circulating steroid levels and compensatory increases in ACTH, angiotensin II, and LH. These increased tropic hormones stimulate cellular uptake of LDL cholesterol and increased de novo cholesterol biosynthesis, leading to accumulation of cholesterol and cholesterol esters in lipid droplets in steroidogenic cells, eventually destroying all steroidogenic capacity (the second hit). The two-hit model explains the unusual findings in lipoid CAH. Salt loss appears relatively late because fetal angiotensin II drive is minimal, so that the adrenal zona glomerulosa remains relatively undamaged until after birth; 46,XX patients spontaneously feminize in adolescence because the fetal ovary is steroidogenically quiescent and first experiences tropic stimulation at puberty. This model has been confirmed by clinical observations (14, 24) and by observations in StAR knockout mice (25).

Most disease-causing StAR mutations are devoid of measurable activity. Only three mutations causing lipoid CAH have had measurable activity in a transfected COS-1 cell assay equivalent to the one we employed. Ala218Val (6% activity) and Leu275Pro (10% activity) were both found in a 46,XY phenotypic female infant with salt loss at 2–4 months (4); hence, this very low level of StAR activity is insufficient to influence phenotype. Met225Thr (29% residual activity) was found in a minimally virilized (clitoromegaly, rugated labia, mild posterior labial fusion) 46,XY female who first experienced salt loss at 10 months of age (10). The coexistence of the severe Glu258Stop null mutant on this patient’s other allele presumably accelerated the course of the second hit, so that the net effect was more severe than would be predicted for a Met225Thr homozygote (10). In contrast, the Val187Met and Arg188Cys mutations described in the present study, which retain approximately 20–25% of activity, were seen in homozygously affected individuals, permitting clear assessment of the phenotypic consequences of retaining this degree of StAR activity. The activities of the Val187Met mutant were slightly greater than those of the Arg188Cys mutant in all three assays, but it is not clear whether these differences were clinically significant. Although the partial activity in vitro correlates well with milder, later onset disease, the presentation of lipoid CAH can vary substantially among homozygotes within a single ethnic group; for example, among seven Saudi patients homozygous for Arg182His, the age of onset of symptoms ranged from 1–14 months, despite this mutant lacking detectable activity in vitro (13). The consequences of these nonclassical mutations on gonadal function cannot be assessed during childhood; long-term follow-up through puberty into adult life will be needed to assess the eventual impact of these mutations on ovarian and testicular function.

Lipoid CAH has been described in most ethnic groups and is common in Japan, Korea, and some isolated populations (1, 3, 4, 10, 11, 13, 24, 26–28). In contrast to previous reports of lipoid CAH, our patients presented late: patient 1, a 46,XX female homozygous for Val187Met, presented at 4 yr of age,
whereas patients 2 and 3, 46,XY phenotypic male siblings homozygous for Arg188Cys, presented at 2–3 yr. These unique clinical courses, which are consistent with the demonstrated partial activity of each mutation, establish a new entity, nonclassic lipid CAH, showing that the clinical spectrum of STAR mutations is substantially broader than had been appreciated previously. Nonclassic lipid CAH is thus a novel form of nonautoimmune Addison disease, which can present with or without salt loss. Other genetic bases of Addison disease probably remain to be identified.

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