SUPPLEMENTAL DATA

METHODS

Hormonal assays

Serum dehydroepiandrosterone sulfate (DHEAS) was determined by a DPC Immulite Assay System; assay sensitivity was 0.052 nmol/L; interassay coefficient of variation ranged from 6.3 to 8.8%. Serum androstenedione (A) was determined by RIA [Diagnostic Systems Laboratories (DSL), Inc., Webster, TX]; assay sensitivity was 0.35 nmol/L; interassay coefficient of variation ranged from 7 to 9.8%. Serum 17-hydroxy-progesterone (17OHP) was determined by RIA (DSL); assay sensitivity was 0.30 nmol/L; interassay coefficient of variation ranged from 8.5 to 11%. ACTH was determined by the DPC Immulite Assay System; assay sensitivity was 5 pg/ml (16.6 pmol/l); interassay coefficient of variation ranged from 6.1 to 10%. Serum renin was determined by IRMA; assay sensitivity was 0.7 pg/ml; interassay coefficient of variation ranged from 1.78 to 2.64%. Serum LH and FSH levels were determined by the Imx systems (Abbott Laboratories, Abbott Park, IL); assay sensitivity was 0.3 IU/L for LH and 0.2 IU/L for FSH; interassay coefficient of variation ranged from 3.1-8.7% for LH and from 3.8-12% for FSH. Serum testosterone (T) was determined by a DPC Immulite Assay System (Diagnostic Products, Los Angeles, CA); assay sensitivity was 0.17nmol/L; interassay coefficient of variation ranged from 7.4 to 13%. Serum cortisol was determined by a DPC Immulite Assay System; assay sensitivity was 0.028 nmol/L; interassay coefficient of variation ranged from 5.2 to 5.9%. Hormonal parameters were determined periodically.

Genetic analysis

Genomic DNA from the patient and 60 healthy controls was isolated from peripheral blood lymphocytes by conventional methods. The coding and flanking intronic regions, including 5'-flanking noncoding regulatory region, of \( \text{HSD3B2} \) were amplified by PCR using specific primers (1). PCR products were purified (QIAquick Gel Extraction Kit, Qiagen, Buenos Aires, Argentina) and sequenced with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Buenos Aires, Argentina) using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Buenos Aires, Argentina). The primers used for sequencing were the same as those used for PCR. The sequences were compared with the NCBI entries of \( \text{HSD3B2} \) (NG_013349.1). Sequence variants were designated according to recommendations by the Human Genome Variation Society (www.hgvs.org/rec.html) using the reference sequences, GenBank number NM_000198.3 (cDNA), and GenBank number NP_000189.1 (protein).
**Site-directed mutagenesis, transient transfection, and enzymatic activity assays**

Full-length cDNA encoding WT human *HSD3B2* (NM_000198.3) was generated by RT-PCR from total RNA isolated NCI-H295R human adrenocortical cells using primers spanning the coding region of *HSD3B2* (forward: 5´-gccacgatgggctggagctgccttgtg-3´; reverse: 5´-tcactgagtcttgactcaggtctcc-3´) carrying EcoRI and XhoI restriction enzyme site, respectively. The purified 1127-bp PCR product was digested with EcoRI/XhoI and cloned into the EcoRI and XhoI sites of the pcDNA3 vector. The accuracy of the construct was confirmed by sequencing. Site-directed mutagenesis was used to generate the pG250V mutant using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla CA, USA) following a standard protocol. Insertion of the mutation and the integrity of the cDNA were checked by direct sequencing.

Nonsteroidogenic COS-7 cells (fibroblast-like cell line derived from monkey kidney tissue, ATCC CRL-1651) were grown in DMEM supplemented with 10% fetal calf serum and antibiotics at 37 C in a humidified 5% CO₂ incubator. Cells were divided into 24-wells plates and transiently transfected using lipofectamine 2000 (Invitrogen, Buenos Aires, Argentina) with WT 3βHSD2 or mutant G250V-3βHSD2 constructs or the empty vector, as negative control, at approximately 80% confluence following the manufacturer’s instructions. To monitor transfection efficiency, cells were also cotransfected with 60ng of firefly luciferase reporter plasmid (pGL3-Promoter Vector) (Promega, Buenos Aires, Argentina) per well.

The 3βHSD2 activity was determined by the conversion of DHEA to Δ⁴-A and Pregnenolone to progesterone in intact transfected COS-7 cells. Briefly, 48hs after transfection, cells were incubated with 0.5µM of pregnenolone or DHEA for 60, 180 and 360 min in serum-free DMEM media. Subsequently, we collected the media and measured the level of steroid products, progesterone and Δ⁴-A, respectively, by competitive chemiluminescent enzyme immunoassay (Immuliite 2000 System, Siemens Healthcare USA). Progesterone assay sensitivity was 0.032 nmol/liter; interassay coefficient of variation was 17.8%. Δ⁴-A assay sensitivity was 0.105 nmol/liter; interassay coefficient of variation was 10.6%. Cells were lysed in passive lysis buffer (Promega). Protein content was determined using the Bradford method. Luciferase assay was performed following the standard protocol of the manufacturer (Luciferase assay System E1500, Promega, Madison, WI). The efficiency and reproducibility of the transfection were verified by determining the ratio of luciferase activity to the total protein content.

To determine apparent kinetic constants of WT 3βHSD2 and mutant G250V-3βHSD2, transfected COS-7 cells were incubated as describe above with 0.125, 0.25, 0.5, 1.0, 2.0 or 4.0 µM of pregnenolone for 30 min. Postincubation treatment and analysis were performed as described above. Equal expression levels of WT and mutant G250V-3βHSD2 proteins in each well were confirmed by western blot. Kinetic parameters were established using the Michaelis-Menten equation to determine
the $K_m$ and $V_{max}$. Catalytic efficiency was defined as the ratio $V_{max}/K_m$ expressed as percentage of wild type.

Data are presented as the mean ± SD for three independent transfection experiments, each performed in triplicate.

**Western blot analysis**

To ensure comparable levels of expression and translation of WT 3βHSD2 and mutant G250V-3βHSD2 proteins, Western blot analysis was performed in a standard protocol. Briefly, cell lysates were obtained from cells transfected with the WT or mutant 3βHSD2 plasmid. Equal amounts of protein (50 μg) were subjected to SDS-PAGE and electroblotted to PVDF membranes (Invitrogen, Buenos Aires, Argentina). A prestained protein MW marker (Novex Sharp Pre-stained Standard, LC5800, Invitrogen) was used for monitoring the progress of SDS-PAGE and for assessing transfer efficiency. Nonspecific sites were blocked with 5% nonfat dry milk in 0·2% Tween 20 in Tris-buffered saline (TBS-T) overnight at 4 °C and then incubated overnight at 4 °C with a rabbit polyclonal antihuman 3βHSD antibody (sc-28206; Santa Cruz Biotechnology, Inc), diluted 1µg/mL in blocking solution. After washing, membranes were incubated 2 h at room temperature with HRP conjugated goat antirabbit antibody (Amersham, Buenos Aires, Argentina) diluted 1 : 5000 in blocking solution. Bound secondary antibody was detected using ECL Plus Western blotting detection system (Amersham, Buenos Aires, Argentina) according to the manufacturers’ instructions. The approximate size of the protein that has been made visible with Western blotting detection system was estimated by overlapping PVDF membrane and the film. Steroidogenic Human adrenocortical H295R cell lysate was used as 3βHSD2 positive control. We used a mouse monoclonal anti-β-actin antibody (Monoclonal anti-β-actin clone AC-15, Sigma-Aldrich, Buenos Aires, Argentina) to ensure equal loading of protein for WT and mutant.

**Immunofluorescence analysis**

COS-7 cells transfected with the WT or mutant 3βHSD2 plasmid were incubated with ER tracker Red reagent (Molecular Probes, Eugene, OR) 1µM for 25 min to identify endoplasmic reticulum (red emission). After washing cells were harvested by trypsinization and fixed using PBS containing 4% formaldehyde for 5min. Fixed cells were then cytocentrifugated in a Cytospin 3 (Shandon, ThermoElectron Corporation, Waltham, MA, USA) centrifuge for 5 min at 750 rpm. After blocking with 1.5% normal goat serum in PBS for 1 h, slides were incubated with rabbit polyclonal antihuman 3βHSD antibody (4µg/mL, sc-28206; Santa Cruz Biotechnology, Inc) for 1 h at room temperature, followed by the secondary Alexa Fluor 488 goat anti-rabbit antibody (5 µg/ml) (Molecular Probes, Eugene, OR) for 1 h. After the last wash, slides were mounted and examined in an Olympus
FV300 Confocal laser scanning microscope. To control for non-specific immunofluorescent staining, cells were also incubated in solutions in which the primary antibody was omitted.

**Molecular modelling of the 3βHSD2 structure**

The molecular 3D model structure of 3βHSD2 (NP_000189.1) was built by homology modelling using PHYRE2 (2). The Gal10 fusion protein galactose mutarotase/UDP-galactose 4-epimerase from Saccharomyces cerevisiae (PDB ID 1Z45) was chosen as template. The modelling was performed with NAD+ inside the protein. Further refinement of the model was performed by minimizing for 2,000 steps in implicit solvent using an harmonic restraint of $d^2 10 \text{kcal/molÅ}^2$ on all protein atoms (3) with the Amber Package (4). Protein parameters were taken from Amber99SB forcefield while NAD+ parameters were taken from Walker et al (5, 6). Protein models structure were visualized using VMD and Tachyon (7, 8). PROCHECK was run in both the template and the model with no significant deviations, in the geometrical and topological parameters.

**REFERENCES**


**SUPPLEMENTAL TABLE 1: Hormonal data of the patient at 7 months of age**

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<thead>
<tr>
<th>Parameter</th>
<th>Dexamethasone&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Reference values</th>
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<tr>
<td>LH (M IU/ml)</td>
<td>0.21</td>
<td>0.78-4.2</td>
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<tr>
<td>FSH (M IU/ml)</td>
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<tr>
<td>Progesterone (nmol/liter)</td>
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<td>0.16-1.11</td>
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<td>Testo (nmol/liter)</td>
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<td>0.17-6.14</td>
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<td>17OHP (nmol/liter)</td>
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<td>3.69-6.30</td>
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<td>Δ4 (nmol/liter)</td>
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<td>&lt;0.35-1.57</td>
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<td>Cortisol (nmol/liter)</td>
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<td>149.0-651.4</td>
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<td>ACTH (pmol/liter)</td>
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<td>Na&lt;sup&gt;+&lt;/sup&gt;/K&lt;sup&gt;+&lt;/sup&gt; (meq/l)</td>
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<td>133-145/3.5-5</td>
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<td>Renin (pmol/liter)</td>
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<td>DHEAS (µmol/liter)</td>
<td>143</td>
<td>&lt;0.03-1.1</td>
</tr>
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* High-dose dexamethasone suppression Test (test dose: 120µg/kg/d) (9).

**LEGENDS FOR SUPPLEMENTAL FIGURES**

**SUPPLEMENTAL FIGURE 1: Patient’s genital features at 7 months of age** showing clitoromegaly.

**SUPPLEMENTAL FIGURE 2: Patient’s growth curve under glucocorticoid and mineralocorticoid replacement therapy since 7 month of age.** Small circles depict body length in cm, corresponding bone ages (small squares) being connected by a straight line.

**SUPPLEMENTAL FIGURE 3: Mutation analysis by direct DNA sequencing.** Patient’s electropherogram showed a homozygous G to A substitution at position c.749G>T in exon 4 of *HSD3B2* gene, resulting in the G250V missense mutation. Location of the codon 250 is indicated on a schematic of the *HSD3B2* gene map.

**SUPPLEMENTAL FIGURE 4: Three-dimensional molecular model of 3βHSD2 protein (template gal10 fusion protein galactose mutarotase/UDP-galactose 4-epimerase from *Saccharomyces cerevisiae* complexed with NAD, PDB accession code 1Z45).** The protein is composed by two domains one that binds to NAD+ and the other that recognizes the substrate. In the closeup view of the site distinctive interactions between NAD+ and the protein are depicted. Putative hydrogen bonds are represented in dashed lines. Protein is represented in New cartoon representation, Alpha helix colored in purple, 3-10 Helix in blue, Beta sheets in yellow, loops in cyan and turns in white. NAD+ is drawn in ball and sticks and protein residues in sticks. Carbon atoms are colored in cyan, Oxygen atoms in red, Nitrogen atoms in blue and phosphorous atoms in brown.
HSD3B2 gene
5’

Codon#:

1 47 48
102 10

3’

Wild type

Patient

c.749G>T, p.G250V

Ala Pro Ser Val Arg Gly Gln Phe Tyr Tyr Ile

GCCCCAAG T GTCCGA GGTCAATTCCTATTA CATC

GCCCCAAG T GTCCGA GGTCAATTCCTATTA CATC