Supporting Information

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SI Materials and Methods

Urinary Steroid Metabolite Analysis. Steroids were enzymatically unconjugated and, after extraction, chemically derivatized before GC/MS–selected ion monitoring analysis. The metabolites quantified were cortisol (F), THF, 5α-THF, α-cortol, β-cortol, cortisone (E), THE, α-cortolone, and β-cortolone. The total metabolites of cortisol is represented as the sum of THF, 5α-THF, F, and cortols, and the total metabolites of cortisone is represented as the sum of THE, E, and cortolones. The total cortisol and cortisone metabolites provide an accurate measure of the 24-h cortisol secretion rate and, by inference, an index of hypothalamic-pituitary-adrenal axis activation (1, 2). The urinary THF+5α-THF/THE and cortol/cortolone ratios were used as measures of 11β-HSD1 activity, as described previously (3). Adrenal androgen secretion was inferred from the summation of urinary androsterone, etiocholanolone, and DHEA levels.

Gene Sequencing. PCR amplification of the coding region of *HSD11B1* and *H6PD* genes, including exon/intron boundaries, was performed using genomic DNA from cases A and B with previously published primers (4). We compared *HSD11B1* sequences with GenBank entries for two overlapping clones covering chromosome 1q32.2–41 (PAC 28010 and PAC 43014) and *H6PD* sequences to clone RP3-510D11 on chromosome 1p36.2–36.3.

Functional Analysis: Mammalian Cell Expression of Homodimers and Heterodimers. The QuikChange Site-Directed Mutagenesis kit (Stratagene) was used to mutate WT HSD11B1 cDNA, contained in pCR3 (Invitrogen), to the respective mutant sequence for expression of homodimers. In addition, WT and mutant HSD11B1 sequences were cloned into the pIRES vector (Clontech) to allow high-level expression of both sequences from the same bicistronic mRNA transcript. All constructs were sequenced. HEK293 cells were transfected with WT or with mutant HSD11B1-pCR3 constructs or pIRES constructs using a 293-cell specific transfection reagent (Mirus). Stably transfected cells were selected using G418 (Sigma), and four cell lines were derived from four separate transfection experiments for WT and mutant enzyme studies. HEK293 cells were mock-transfected with empty vector as a further control.

Total RNA was extracted from transfected cell lines using a single-step extraction method (Trireagent; Sigma) and 1 μg of total RNA reverse-transcribed to cDNA (Applied Biosystems). Quantitative real-time PCR was performed to determine *HSD11B1* expression levels using commercially available primers and probes (Applied Biosystems). Real-time mRNA quantification of 11β-HSD1 mRNA was carried out using the ABI Prism 7700 Sequence Detection System (Applied Biosystems), which uses TaqMan chemistry for highly accurate quantitation of mRNA levels. PCR reactions were carried out in 20-μL volumes on 96-well plates with equal amounts of cDNA (10 ng). All PCR reactions were multiplexed with the housekeeping gene 18S rRNA, provided as a preoptimized control probe (PE Biosystems). This enabled data to be expressed in relation to an internal reference to allow for differences in reverse transcription efficiency. Measurements were taken at least three times for each sample. According to the manufacturer's guidelines, data were expressed as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔCt values (ΔCt = Ct of the target gene minus Ct of the housekeeping gene). Relative gene expression data were calculated and are presented as arbitrary units ($AU = 10^6 \times 2^{-\Delta Ct}$). To exclude potential bias due to averaging data that had been transformed through one of the foregoing equations, all statistical analyses were performed on ΔCt values. Statistical analysis of comparisons among groups was undertaken using paired and unpaired *t* tests where appropriate, provided that the data were normally distributed. For nonparametric data, the Wilcoxon rank-sum and Mann–Whitney *U* tests were used.

For the 11β-HSD1 assays on homodimers, intact cells were incubated with 1 mL of serum-free culture medium with 100 nM cortisone and tritiated tracer for 2 h (5). After incubation, the medium was transferred to a glass tube, and steroids were extracted with 5 mL of dichloromethane, the aqueous phase was removed and the dichloromethane phase was concentrated to 60 μL. Steroids were separated by TLC using a mobile phase of ethanol and chloroform (8:92) and quantified using a Bioscan 2000 image analyzer (Lablogic). Protein levels were assayed using a 96-well plate assay kit (Bio-Rad).

For 11β-HSD1 activity measurements on heterodimers, soluble protein extracts were made using Cytobuster Protein Extraction Reagent (Novagen) and after desalting using PD10 columns (GE Healthcare), dehydrogenase activity assays were carried out as for the bacterially expressed protein (see below) using a saturating concentration (60 μM) of cortisol.

Bacterial Expression and Purification of Recombinant 11β-HSD1 Homodimers. All experiments used an N-terminally truncated version of 11β-HSD1 (i.e., minus the transmembrane anchor), which contains an additional mutation (F278E) designed to promote solubility and monodispersity of the protein without affecting enzyme activity (6). Constructs (WT, R137C, or K187N) were introduced into either the pRSF-1b or the pET-51b(+) vector (Novagen). pRSF-1b encodes an N-terminal His_6-tag, an RSF origin of replication and kanamycin resistance, whereas pET-51b(+) encodes an N-terminal Strep tag, an F1 origin of replication, and ampicillin resistance. After sequence verification, constructs were used individually or in combinations to transform the BL21(DE3) *E. coli* expression strain (Novagen). Cells were cotransformed with the pAJW-3 plasmid (a gift from Dr. P. Lund; ref. 7), which contains the genes for the *E. coli* chaperonins GroEL/ES under chloramphenicol resistance. Cells were grown with shaking (220 rpm, at 37 °C) in LB broth supplemented with either 30 μg mL^{-1} of kanamycin and/or 50 μg mL^{-1} of ampicillin in addition to 50 μg mL^{-1} of chloramphenicol. Protein expression was induced, and His-tagged protein was purified by affinity chromatography and gel filtration as described previously (6). For Strep-tagged protein, the cleared cell lysate was loaded onto a Strep-Tactin Superflow Column (Novagen) and washed with buffer containing 25 mM sodium phosphate, 300 mM NaCl, 5% glycerol, 10 mM imidazole, and 0.005% Anapoe X-100 (pH 8.0) (Anatrace). Protein was then eluted with 6 volumes of buffer containing 2.5 mM desthiobiotin, a reversible binding analog of biotin. Fractions containing protein were separated by size-exclusion chromatography as described previously (6).

Bacterial Expression and Purification of 11β-HSD1 Heterodimers. To enable expression of WT and mutant 11β-HSD1 heterodimers, *E. coli* BL21(DE3) cells were cotransformed with pET-51b(+) containing the WT 11β-HSD1 construct pAJW3 (encoding chaperonins) and with pRSF-1b containing either WT 11β-HSD1 or one of the mutants (R137C or K187N). Cells were grown with shaking (220 rpm at 37 °C) in LB broth supple-

mented with 30 μg mL^{-1} of kanamycin (pRSF-1b), 50 μg mL^{-1} of ampicillin [pET-51b(+)], and 50 μg mL^{-1} of chloramphenicol. Protein expression was induced, and His-tagged proteins were purified by affinity chromatography and gel filtration as described previously (6). Protein-containing fractions were quantified (A_{280}), diluted to a final protein concentration of 40 μg mL^{-1}, and incubated for 15 h at 15 °C in a buffer containing 25 mM sodium phosphate, 300 mM NaCl, 5% glycerol, 10 mM imidazole, and 0.005% Anapoe X-100 (pH 8.0). The sample was then loaded onto a His-Select column, and the His-tagged protein was eluted as described previously (6). Protein-containing fractions were again diluted to a final protein concentration of 40 μg mL^{-1} and incubated for 15 h at 15 °C in the foregoing buffer. The sample was then loaded onto a Strep-Tactin Superflow Column, and Strep-tagged protein was eluted as described in the previous section. Neither tagged construct was seen to bind to the heterologous affinity column as long as 10 mM imidazole was included in the wash buffer.

Measurement of 11β-HSD1 Activity. Dehydrogenase activity of bacterially expressed 11β-HSD1 was assayed at 37 °C in 25 mM sodium phosphate (pH 8.0), with 200 μM $NADP^{+}$ and cortisol concentrations ranging from 0.5 μM to 32 μM, using a Hitachi F-7000 spectrofluorimeter to monitor NADPH production. Excitation and emission wavelengths were 340 nm and 456 nm, respectively. The amount of added enzyme was adjusted to obtain a linear rate of reaction for 5 min after the addition of cortisol. A calibration curve was constructed using 0–1 μM NADPH. Results were analyzed by nonlinear regression using Visual-Enzymics (Softzymics).

Western Blot Analyses. Western blots were prepared by electroblotting 11% SDS-polyacrylamide gels onto Immobilon PVDF membranes (Millipore) at 100 V for 1 h in a buffer containing 25 mM Tris, 200 mM glycine, and 20% (vol/vol) methanol. Membrane was blocked in PBS containing 0.1% (vol/vol) Tween-20 and 20% (wt/vol) skimmed milk powder, washed, and then incubated with either an anti-His, anti-Strep, or anti–11β-HSD1 polyclonal antibody in PBS containing 0.05% (vol/vol) Tween-20. Membrane was then washed and incubated with secondary antibody diluted 1:25,000 in PBS containing 0.05% (vol/vol) Tween-20. Detection was by enhanced chemiluminescence (Amersham Biosciences), and band intensities were quantified using ImageMaster 1D (Amersham Biosciences). Standards with known amounts of 11β-HSD1 protein were included on each blot.

Statistical Analysis. Statistical analyses of comparisons among groups were performed using one-way ANOVA with Tukey's post hoc testing for normal distributions and the Mann–Whitney rank-sum test for nonnormal distributions. All analyses were performed using the SigmaStat 3.1 software package (Systat Software).

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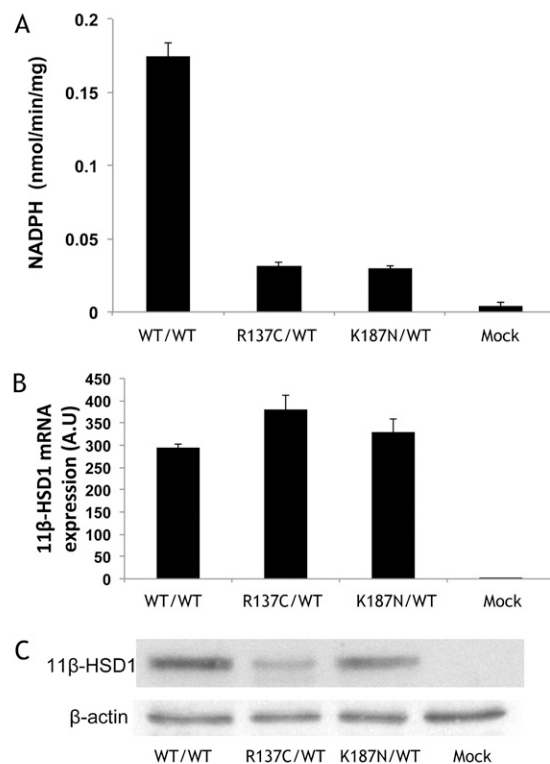

Fig. S1. Effect of coexpression of mutant and WT 11β-HSD1 constructs in stably transfected mammalian cells. (*A*) Specific enzyme activity of cleared lysates of HEK293 cells transformed with the bicistronic pIRES vector containing mutant and WT 11β-HSD1 constructs. 11β-HSD1 enzyme activity was measured in the dehydrogenase direction (i.e., conversion of cortisol to cortisone) at saturating substrate concentrations. Mock treatments used empty vector as a control. The reduction in enzyme activity observed on inclusion of either of the mutant constructs was statistically significant ($P < 0.001$). Bars represent SEM ($n = 3$). (*B*) Real-time PCR assessment of *HSD11B1* gene expression in the stably transfected clones compared with mock-transfected controls, indicating overexpression of *HSD11B1* mRNA in transfected lines with similar expression levels in all construct combinations. Results are expressed in arbitrary units (AU). Bars represent SEM ($n = 3$). (*C*) Western blot analysis of cleared lysates from HEK293-transfected cells, using anti–11β-HSD1 antibody or anti–β-actin as a control. Expression of either mutant caused a reduction in the amount of 11β-HSD1 protein detected in the extracts, but the effect was greater with R137C. All data shown are representative examples from three experiments.

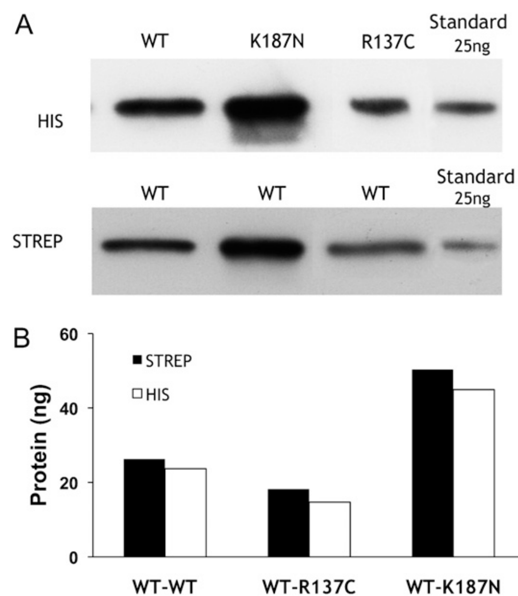

Fig. S2. Western blot analysis of the hybrid dimers of 11β-HSD1 purified from the bacterial expression system. (*A*) Western blot of the samples analyzed in Table 2. Anti-His and anti-Strep antibodies were used to detect the two different monomers in the purified heterodimers. (*B*) Densitometry estimation of concentrations of His and Strep tags in the purified heterodimers. The intensity of bands in *A* was analyzed and used to calculate the amount of protein (in ng) present on the blots. In all samples, there were equal amounts of Strep- and His-tagged protein on the blot, in agreement with the production of hybrid dimers.