Supporting Information

Schmid et al. 10.1073/pnas.1110965108

SI Text

Quantitative Real-Time PCR. For quantification of rat 11β-hydroxysteroid dehydrogenase (11β-HSD)-1 and 11β-HSD-2 mRNA, cDNA was synthesized as described and amplified by PCR to generate standards of each sample. PCR products were cloned into plasmid vectors (pCRIITOPO) and transformed into competent *Escherichia coli* using the TOPO TA Cloning kit (Invitrogen). Plasmids were purified using a Maxi Plasmid kit (Qiagen) and concentrations of plasmids were measured by spectroscopy. After DNA sequencing, a linear-regression standard curve was created by serial dilutions of plasmid. PCR contained 2 μL of cDNA sample, 5 μL SYBR Green PCR master mix (Qiagen), 5 pmol of sense primer, and 5 pmol of antisense primer (Table S1) and proceeded on a Light Cycler 1.5 (Roche). Each cycle consisted of 40 s at 94 °C, 40 s at specific annealing temperature, and 40 s at 72 °C for a maximum of 40 cycles. Each sample was analyzed along with standards and no template controls and no by-products were present in the reaction, as indicated by the dissociation pattern provided at the end of the reaction. The expression level of rat 11β-HSD-1 and 11β-HSD-2 genes were calculated by relative quantification to the expression of the housekeeping gene β-actin.

Western Blot Analysis. Western blot analysis was performed as previously described (1). Briefly, insulinoma (INS)-1 cells and rat and human islets were washed twice in ice-cold PBS and lysed in ice-cold CelLyticTM M Cell Lysis Reagent (Sigma-Aldrich) containing 1% protease inhibitor mixture (Sigma-Aldrich). Cell lysates were matched for protein content; maximal volume of 30 μL of protein were loaded on each lane, separated by a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. After blocking, membranes were immunostained with goat anticorticotropin-releasing hormone receptor-type 1 (anti-CRHR1) antibody at 1:250 (polyclonal; Imgenex) or rabbit anti-HSD11B1 antibody at 1:800 (polyclonal; Abcam). Bound primary antibody was detected with horseradish peroxidase-coupled anti-goat or anti-rabbit secondary IgG (Santa Cruz) using a Western Breeze Detection Kit (Invitrogen). Chemoluminescence signals were read with the GeneGnome Chemoluminescence detector (Syngene).

Immunohistochemistry and Immunofluorescence. Samples of human and rat pancreas were fixed in 4% paraformaldehyde for 10 h and processed in paraffin. Serial sections of 4 μm were stained using an automated immunostainer (BenchMark; Ventana) according to the manufacturer's protocols. Primary antibodies were goat anti-CRHR1 at 1:250, rabbit anti-HSD11B1 antibody at 1:400, or guinea pig anti-insulin at 1:100 (polyclonal, code A0564; Invitrogen). The signal was amplified using the Ventana amplification kit and visualized using avidin-biotin labeling and 3,3′-diaminobenzidine. Slides were counterstained with hematoxylin. Staining with isotype control antibodies was performed to confirm specificity of staining. INS-1 cells were fixed in 4% paraformaldehyde and nonspecific staining was blocked with PBS containing 0.1% BSA, 0.3% Triton-X-100 and 5% horse serum. Following primary antibodies, goat anti-CRHR1 antibody and rabbit anti-HSD11B1 antibody were applied overnight at 4 °C. Secondary antibodies, donkey anti-guinea pig at 1:200 (DyLight488, code 706–485-148; Jackson Laboratories) and Cy3 donkey anti-goat at 1:500 (Jackson ImmunoResearch), were applied for 3 h. Serial sections of pancreata from *CRH*$^{-/-}$ and wild-type mice (5 μm) were collected on polylysine slides (Fisher Scientific) and stained with mouse antiglucagon (monoclonal; Sigma) and anti–C-peptide (polyclonal; Millipore). Secondary antibodies conjugated with AlexaFluor 488 (green) or 546 (red) were from Molecular Probes. DAPI (Molecular Probes) was applied for cell nucleus specific staining. Slides were imaged using Zeiss Axiovert200M with AxioCamMRc5.

Electron Microscopy. Stimulated INS-1 cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Coverslips were postfixed for 90 min in 1% osmium tetroxide solution, as described previously (2). After dehydration in ethanol, specimens were embedded in epoxy resin. Ultrathin sections (60 nm) were stained with uranyl acetate and lead citrate to obtain suitable contrast and analyzed using an electron microscope (Zeiss 906).

Cell Apoptosis Assay. Cell apoptosis was assessed using Caspase-Glo 3/7 Assay (Promega) following the manufacturer's instructions. This assay provides a homogeneous luminescent signal proportional to caspase-3/7 activity.

Cell Proliferation Assay. BrdU was detected immunochemically allowing the quantification of newly synthesized DNA of actively proliferating cells using BrdU Cell Proliferation Kit (Millipore) following the manufacturer's instructions.

11β-HSD Activity Assay. The supernatants of INS-1 cells were collected and centrifuged, and activity of 11β-HSD was assayed by measuring the conversion of added 500 nM cortisone (Sigma-Aldrich) to cortisol with an immunoassay EIA kit (Enzo Life Science). Absorbance was measured spectrophotometrically using a microplate reader at 405 nm. Because these cells do not produce cortisol, cortisol reflects the activity of 11β-HSD (3).

Statistical Analysis. The data were presented as mean values ± SEM. Significant differences between groups were tested by ANOVA with Bonferroni's or Student *t* test. A value of $P < 0.05$ was considered statistically significant.

RNA Isolation and RT-PCR. Total RNA was extracted from INS-1 cells, rat islets, and human islets using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was eluted in RNase-free water and concentrations were measured photometrically at 260 nm. One microgram purified RNA was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase using oligo-dT primers (Promega) in a final volume of 25 μL at 37 °C for 60 min, as recommended by the manufacturer. One microliter of cDNA was amplified in a 10-μL reaction mixture containing 1.5 mM $MgCl_2$, 1× PCR buffer (Invitrogen), 0.25 mM of each deoxynucleotide (Promega), 1 unit of Platinum Taq DNA Polymerase (Invitrogen), and 0.5 μM of each of the different primers. The PCR reactions were carried out with initial denaturation at 94 °C for 4 min; 35 cycles amplification at 94 °C for 20 s, annealing for 20 s, and elongation at 72 °C for 1 min, followed by final extension at 72 °C for 4 min for qualitative analysis. Primer sequences and annealing temperatures are summarized in Table S1. The housekeeping gene β-actin was used as loading control. After agarose gel electrophoresis and ethidium bromide staining, products were visualized under UV light using the Gene Genius documentation system (Syngene). Rat pituitary, a known site for the expression of CRH mRNA was included as a positive control.

1. Ziegler CG, et al. (2008) Dehydroepiandrosterone induces a neuroendocrine phenotype in nerve growth factor-stimulated chromaffin pheochromocytoma PC12 cells. *Endocrinology* 149:320–328.
2. Ziegler CG, et al. (2009) Expression of neuropeptide hormone receptors in human adrenal tumors and cell lines: Antiproliferative effects of peptide analogues. *Proc Natl Acad Sci USA* 106:15879–15884.
3. Friedberg M, et al. (2003) Modulation of 11 beta-hydroxysteroid dehydrogenase type 1 in mature human subcutaneous adipocytes by hypothalamic messengers. *J Clin Endocrinol Metab* 88:385–393.

Table S1. Oligonucleotide primers used for expression analysis

Gene	Species	Sense	Antisense	Annealing temperature	Size
CRH	Rat	tgatccgcatgggtgaagaatacttcct	cccgataatctccatcagtttcctgttgctg	70 °C	394 bp
CRH-R1	Rat	cttctccttctggggctga	aggtgccaatgaggtccac	60 °C	123 bp
11β-HSD1	Rat	gaagaagcatggaggtcaac	gcaatcagaggttgggtcat	60 °C	133 bp
11β-HSD2	Rat	gctgctgcagatggacct	gcagccaggctggatgatg	60 °C	393 bp
β-actin	Rat	gtgtgatggtgggtatgggtcagaa	accagaggcatacagggacaacaca	65 °C	314 bp
CRH	Human	atctccctggatctcaccttccac	cgcaccgttttatttcccaataatc	60 °C	126 bp
CRH-R1	Human	caaacaatggctaccgggag	acaccccagccaatgcaga	60 °C	475 bp
11β-HSD1	Human	ctcgagtcggatggctttttatg	acttgcttgcagaatagg	60 °C	566 bp
11β-HSD2	Human	accgtattggagttgaacagc	tcactgactctgtcttgaagc	60 °C	477 bp
β-actin	Human	gccgtcttcccctccatcgtg	ggagccacacgcagctcattgtaga	65 °C	209 bp