

Additional experimental approaches to be considered for the online version:

Renin Immunohistochemistry

Formalin fixed kidneys were dehydrated in graduated concentrations of alcohol, embedded in paraffin blocks, sectioned (2-3 μm) and mounted onto slides with Vectabond (Vector Laboratories, Inc., Burlingame, CA). Serial kidney sections were immunostained by the immunoperoxidase technique with modifications as described previously.¹ Renin immunolocalization was assessed using a polyclonal anti-rat specific renin antibody raised in rabbit (generously provided by Dr. Tadashi Inagami, Vanderbilt University) and an automatic robot system (DAKO Corp., Carpinteria, CA) which allowed us to apply identical conditions to all slides. To assist in identification of principal cells, anti-mouse/rat aquaporin-2 polyclonal antibody (1:500 concentration; Calbiochem-Novabiochem, Corp., San Diego, CA) was also assessed in each group of rat kidneys. After dewaxing in xylene, the kidney sections were rehydrated in 100, 95 to 70% alcohols and treated with methanol and 0.3% H_2O_2 for 30 minutes to block peroxidase activity. Then, kidney sections were washed and sequentially incubated with 1) normal blocking rabbit serum for 20 min; 2) primary antibody, anti-peptide renin polyclonal antibody prepared in antibody diluents (DAKO Corp., Carpinteria, CA) containing 1% bovine serum albumin at 1:4,000 or 1:8,000 concentrations (200 μl) for 90 minutes; 3) secondary antibody, biotin-conjugated rabbit anti-mouse IgG for 30 minutes; and 4) avidin DH, biotinylated horseradish peroxidase H complex using the ABC Elite Vectastain kit (Vector Laboratories Inc., Burlingame, CA) for 45 minutes. Peroxidase activity was visualized with 0.1% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO) and/or 3-amino-9-ethylcarbazole (AEC, Sigma, St. Louis, MO) as

chromogens and 0.1% H₂O₂. The slides were counterstained with hematoxylin, mounted using aqueous mounting media (Biomedex, Fisher Scientific), and coverslipped.

The renin antibody specificity was determined by 1) omission of the primary antibody and substitution with normal rabbit serum, and 2) preadsorption of the primary antibody (1:4,000) for 72 hours using 5X excess (1.0×10^{-4} mol/L concentration) of pure porcine renin (Sigma, St. Louis, MO). Renin immunoreactivity was determined in 10 different microscopic fields/tissue section/animal using an Olympus BX51-TRF microscope, 40X objective, and an integrated Magnafire SP Digital "Firewire" Camera System for image processing. The intensity of JGA and distal tubule renin immunoreactivity in AngII-infused and sham rat kidney sections was analyzed using Image-Pro[®] Plus Software (version 4.5.1 for Windows 2000 XP; Media Cybernetics, Inc) which allowed a computerized determination of the area of positive staining (μm) and the intensity of immunoreactivity (sum of density of positive tubules in an analyzed area). For this analysis, immunoreactivity of JGA renin was analyzed separately from the distal tubular renin. The results are expressed in arbitrary units of the relative intensity normalized to the renin immunostaining average of the Sham group.

Western Blot Analysis

To avoid the contribution of JGA renin to distal tubular renin protein expression, renal medullas were dissected from cortex. Dissected renal medullas were immediately homogenized in cold lysis buffer (1% Triton X-100, 20mM Tris-HCl, pH 7.6, 150mM NaCl) containing protease inhibitors (PIC, Sigma, St. Louis, MO) for 10 minutes. Insoluble material was removed by centrifugation at 4 °C. Protein concentrations from

lysates were determined using the Protein Assay Reagent (Pierce, Rockford, IL) and pre-diluted BSA (bovine serum albumin) assay standards (Pierce, Rockford, IL). Proteins (10 µg) were resolved on 10-20% glycine stacking gels at 125 V for 90 minutes, and then transferred to nitrocellulose membranes. Adequacy of protein transfer was assessed by Ponceau-staining of the membranes. Molecular mass markers such as 10- to 250-kDa Rainbow markers (Amersham-Pharmacia-Biotech, Piscataway, NJ) and 20- to 120-kDa Magic markers (Invitrogen Life Technologies, Carlsbad, CA) were used. Nonspecific binding sites were blocked with 5% blocking reagent (Amersham-Pharmacia-Biotech, Piscataway, NJ) in Tris-buffered saline Tween (1X TTBS) overnight at 4 °C. Membranes were then incubated with the rat anti-renin (1:4,000) or β-actin (diluted 1:20,000, Sigma, St. Louis, MO) antibodies at room temperature for 2 and 1 hour, respectively. After three washes in 1X TTBS, the nitrocellulose membrane (Amersham-Pharmacia-Biotech, Piscataway, NJ) was exposed for 1 hour at room temperature to the corresponding secondary antibody. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (ECL, Amersham-Pharmacia-Biotech, Piscataway, NJ) according to manufacturer's recommendations. The immunoblots were then exposed to Hyperfilm-ECL (Amersham-Pharmacia-Biotech, Piscataway, NJ) films and analyzed using Alpha Innotech imaging system (Alpha Innotech, San Leandro, CA) to obtain densitometric values.

PCR

RT-PCR

Total RNA from rat kidney cortex and medulla were extracted using Trizol reagent (Gibco BRL Life Technologies, Carlsbad, CA). After total RNA quantitation, first strand cDNA synthesis was performed using 5 µg of sample and SuperScript II (Invitrogen Life Technologies Co, Carlsbad, CA) RNase H-reverse transcriptase system. Synthetic specific primers (Invitrogen Life Technologies, Carlsbad, CA) located in exons 1 and 5 of *renin 1c* gene (sense 5'-ATGCCTCTCTGGGCACTCTT-3', and antisense 5'-GTCAAACCTTGGCCAGCATGA-3') were used as previously described.^{2,3} Rat β-actin specific primers were used as an internal standard (BD Biosciences Clontech Laboratories, Inc, Palo Alto, CA). An expected 560-bp fragment of rat *renin 1c* gene was obtained using a thermal cycling (denaturing at 94 °C for 45 seconds and extension at 72 °C for 45 seconds) for 34 cycles with a primer annealing temperature of 60 °C for 45 seconds in a DNA Engine Peltier Thermal Cycler Model PTC-200 (MJ Research, Inc., Watertown, MS). Negative controls for the reactions included omission of reverse transcriptase (-RT). Positive controls included rat kidney cortex RNA which is expected to contain the renin mRNA. RT-PCR products for *renin 1c* and β-actin from each animal were electrophoresed on 1.2% agarose gel pre-stained with ethidium bromide and scanned with ultraviolet illumination using a digital imaging analyzer (Alpha Innotech, San Leandro, CA). No PCR products were detected in the absence of the RT enzyme which excluded the possibility of contamination of total RNA samples with genomic DNA. PCR products from two kidney cortex and two kidney medulla samples were sequenced.

References

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