ONLINE SUPPLEMENT

Lack of specificity of commercial antibodies leads to misidentification of angiotensin type 1 receptor (AT_1R) protein

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Materials and Methods

Animals- Mice with targeted deletion of the *Agtr1a* and *Agtr1b* genes were generated as previously described.[1, 2] Wild-type 129 SvEv (WT) mice were used as controls. For these experiments, 2- to 3-month old animals were used. All mice were maintained at the animal facility of the Durham Veterans Affairs Medical Center (VAMC), studies approved by the Duke University and Durham VAMC Institutional Animal Care and Use Committees and experiments carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Western blots- Western blotting was performed as previously described.[3, 4] Briefly, mice were anesthetized with 4% isoflurane (Webster veterinary, Devens, MA) and perfused *via* the left ventricle with 20 mL ice-cold physiological saline. Kidneys were removed and dissected under a microscope. Tissue (or HEK cells) were homogenized in a buffer containing 20 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 0.3 mol/L sucrose, 1.0% NP-40, 0.1% sodium dodecyl sulfate and 1:100 dilution of a proteases inhibitor cocktail (Sigma, St. Louis, MO). Equal amount of proteins were denatured for 10 min in sample buffer containing 1.6 % sodium dodecyl sulfate and 100 mM dithiothreitol (Sigma) at room temperature. For kidney tissue, 40 µg homogenates were loaded onto the same 4-12% polyacrylamide gel whereas for HEK cells only 5 µg were used. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Invitrogen, Grand Island, NY). After blocking for 30 min, membranes were incubated over-night at 4°C with primary antibodies as indicated. For detection of the AT_1R the following commercial rabbit polyclonal antibodies were used: antibody #1: Alomone Labs Cat # AAR-011 lot AN-03 (1:1,000); antibody #2: Santa Cruz Cat # sc-1173 N-10 lot L0209 (1:500); antibody #3: Abcam Cat # ab18801 lot GR13003-3 (1:1,000). Anti Penta-His Qiagen Cat # 34660 (1:1,000) was used to detect His-tagged $AT_{1A}R$ and anti GAPDH conjugated Abcam Cat # ab9385 (1:3,000). After incubation for 1 hr at room temperature using a 1:3,000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody, reaction products were detected with the Super Signal West Pico chemiluminescence kit (Thermo Scientific, Rockford, IL). The signal was detected by exposure to Kodak RX film and band intensities quantified by densitometry. Detection of GAPDH as a protein loading control was performed in the same membranes without re-stripping, except the blot from Figure 5B first panel, for which samples for GAPDH were loaded in separate lanes. This was due the similar molecular weight of the AT_1R-HIS protein with GAPDH. The ~51kDa non-specific band revealed by the anti HIS antibody served as second evidence for equal protein loading in these particular experiments

Imunohistochemical staining: Kidneys, livers and adrenal glands were harvested from pentobarbital sodium (50 mg/kg, ip) anesthetized adult $AT_{1AB}KO$ (7) and WT (n=6) mice. Kidneys were prepared by immersion fixation in 10% buffered formalin, processed for paraffin embedding, sectioned at a thickness of 3 µm, and stained by the immunoperoxidase technique as we have previously described.[5, 6] Kidney sections were incubated with the AT_1R rabbit polyclonal antibody (1:300; sc-1173, N-10, antibody #2). Representative color micrographs were obtained from tissue sections

using X100 objectives from AT_{1AB}KO and WT kidneys. Control experiments were performed by omitting the primary or secondary antibodies. Liver tissues were prepared in a similar manner as described above. Slides were imaged using an Olympus DP72 Digital Camera System mounted to an Olympus BX51 TRF Microscope.

Quantitative RT-PCR- Relative levels of mRNA for the AT_{1A}R were measured from the kidney and for the AT_{1B}R from the adrenal gland, where these receptors are most abundant. RNA was isolated and reverse transcription performed using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). SYBR Green-based quantitative PCR was carried out using the SYBR Green PCR Master Mix (AppliedBiosystems, Carlsbad, CA). At the end of PCR cycling, melting curve analyses were performed, and representative PCR products were run on agarose gel and visualized by SYBR® safe staining (Invitrogen). For AT_{1A}R expression, the amount of target gene relative to endogenous control was determined by the ΔCT method and for the AT_{1B} receptor expression was visualized by agarose gel electrophoresis. The following primer sequences were used:
AT_{1a} Forward- 5'-ACTCACAGCAACCCTCCAAG-3',
AT_{1a} Reverse-5'-ATCACCACCAAGCTGTTTCC-3' (Amplicon size: 236bp);
AT_{1b} Forward-5'- ATGGGGAGCAGCCAAGAGGC-3',
AT_{1b} Reverse: 5'-CAGGGCAAGATTCAGAAGGA-3' (Amplicon size: 306bp);
GAPDH Forward-5'-TCACCACCATGGAGAAGGC-3',
GAPDH Reverse-5'-GCTAAGCAGTTGGTGGTGCA-3' (Amplicon size: 168).
Controls tubes lacking cDNA and containing RNA were included in each run.

Pressor effect of angiotensin II- Mice were anesthetized with 2% isoflurane and instrumented as previously described [7]. After a 30-min equilibration period, during which 0.9% NaCl was administered continuously (280 µl/min/Kg), 10 µg/kg of angiotensin II or vehicle (0.9% NaCl) was given as a bolus (1µl/gr). Baseline pressure was taken just before the administration of angiotensin II and peak response at the maximal pressure attained after angiotensin II administration.

Molecular constructs- The Plasmid pAA356 encoding the sequence of the mouse AT_{1A}R fused with the mCherry (AT_{1A}-mCherry) fluorescent protein, was generated by PCR amplification from the plasmid pCMV-SPORT6-AT1 (clon ID #4989471, Open Biosystems, Lafayette, CO) using Platinum Polymerase (Invitrogen) and the direct primers forward GCTAGCATGGCCCTTAACTCTTCTACTGAAG and reverse: GGATCCTCCACCTCAGAACAAGACGCAGGC containing NheI and BamHI sites for directional cloning. A resulting 1.1 kb PCR product was purified from an agarose gel and ligated into the cloning vector pCR-Blunt II-TOPO (Invitrogen). After digestion with NheI and BamHI (Promega, Madison, WI) the released fragment was subcloned into the mammalian expression vector mCherry-N1 (Clontech). To generate the plasmid pAA357 encoding the epitope tagged AT_{1A}-His protein (AT_{1A}-His), the same insert from pAA356 was sub-cloned into pcDNA 3.1/myc-His (Invitrogen) within the same restriction sites. All constructions were confirmed by sequencing at the Hartwell center for Biotechnology at St. Jude Children's Research Hospital.

Expression of AT_{1A} receptors in Human Embryonic Kidney (HEK) cells- The HEK 293 human cell line was obtained from the American Type Culture Collection (ATCC,

Manassas, VA). Cells were cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 U/ml streptomycin (Invitrogen). For imaging experiments, cells were seeded in 4-well chamber slides (Lab-Tek II-CC2 treated, Nunc, Rochester, NY) at low density. Transfections where performed with 0.25–0.5 µg total DNA/chamber using Lipofectamine 2000 (Invitrogen. Cells were used after 24 hours at approximately 70%-80% confluence. For Western blot experiments, approximately 2 x 10^6 cells were seeded in 60 mm plates and the following day were transfected with 0, 2, 4 or 8 µg of pAA357 plasmid DNA as indicated. Mock DNA (empty vector) was utilized to deliver equal amount of plasmid per transfection.

Live cell confocal imaging- HEK cells were co-transfected with pAA356 plasmid (AT_{1A}-mCherry) and the endoplasmic reticulum-specific marker Calreticulin fused with enhanced yellow fluorescence protein (Calreticulim-EYFP) or, the trans-Golgi network-specific marker galactosaminyltransferase fused with EYFP (GalNAcT-EYFP), for approximately 24 hours. Cells were transferred to an environmentally controlled chamber and maintained at ~37°C in a 5% v/v CO_2 and humidified atmosphere. Images were acquired using an Nikon C1si inverted laser scanning confocal microscope (60X apochromatic, 1,45 NA objective) using the EZ-C1 3.20 Viewer (Nikon Corporation). Pictures were exported as TIFF, deconvoluted using ImageJ and pseudo-colored and assembled using Adobe Photoshop CS4.

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