Supplemental

Methods

Gene cloning study to prepare positive control of CYP11B2 in H295R cells

The human CYP11B2 cDNA was prepared from the total RNA of H295R cells by reverse transcription polymerase chain reaction (RT-PCR), amplification of which was carried out by PCR using the primers: for CYP11B2, 5′-TTGAATTCGAGCCACCATGGCACTCAGGGCAAAGGCAGAG -3′ and 5′-GGCTCGAGTGGGTGCAGATGCAAGACTA-3′ (forward with the underlined EcoRI site and reverse with the underlined XhoI site, respectively). The conditions used for the PCR amplification were 94 °C for 2 min; 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 2 min for 35 cycles using KOD plus™ DNA polymerase (Toyobo). The PCR products were then purified by gel extraction kit (Qiagen) and subcloned into TOPO (PCR™ II Blunt-TOPO) vector and transformed into One Shot® Competent *E. coli* DH5ὰ cells (Takara) followed by spreading on a Luria broth (LB) agar plate with kanamycin (50 µg/mL) and incubated overnight at 37 °C. Some colonies were selected and amplified in LB with kanamycin. Amplified plasmids were extracted, purified and sequenced to confirm that the PCR-amplified cDNA was identical to the original sequence. This was then cloned into the EcoRI-XhoI site of the mammalian expression vectors encoding pcDNA3.1(+) and pCMV-Tag2C (FLAG) obtained from Invitrogen. 5 µg of the cloned DNA-vector were transiently transfected in H295R cells (seeded in a 6-well plate and grown up to about 80% confluent) using Lipofectamine3000 (Invitrogen) according to manufacturer's recommendations. After 24 hours, cells were lysed using RIPA buffer, and proteins were assayed by Western blotting.

Trypan blue cell count assay

H295R cells were seeded in 12-well plates until about 80% confluence. The medium was then replaced with fresh reduced serum medium (0.2% Nu-serum), and incubated for an additional 24 hours. Then the culture supernatants were replaced with the reduced serum medium containing vehicle (0.1% dimethyl sulfoxide) or Tolv with and without AngII. 24 hours after incubation, the cells were trypsinized and suspended in the medium. The cell suspension was then mixed with equal volume of 0.4% trypan blue and the percentage of stained cells was determined. The number of cells was counted using TC20 automated cell counter (Bio-Rad Laboratories, Hercules, CA).

MTT Assay

H295R cells were seeded on 96-well plates and treated with the appropriate agents as indicated; cell proliferation was evaluated by MTT (Cell 96 Non-Radioactive Cell Proliferation Assay) (Promega Corporation, Madison, WI, USA), based on the transformation and colorimetric quantification of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide after 24 hours of treatment. Cells were incubated with dye solution for 4 h at 37 °C, and then the conversion reaction of the tetrazolium salt in formazan was arrested by incubating the cells with stop solution for 1 hour at 37 °C. The absorbance of the solution was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA) at a wavelength of 570 nm.

Supplemental Figure legends

Figure S1. Study design for the in vivo experiment. Tolvaptan (Tolv) treatment was started 24 hours after subcutaneous angiotensin II (AngII) infusion because pumping of AngII from mini-pump starts approximately 24 hours after implantation. All the rats were sacrificed after 6 days of concomitant-treatment with AngII and Tolv.

Figure S2. Effects of Tolvaptan on AngII-induced CYP11B2 mRNA expression at different time points up to 24 hours in H295R cells. Cells were treated with 1 μmol/L Tolvaptan for 30 mins and then stimulated by 100 nmol/L AngII for the indicated time periods. CYP11B2 mRNA expression was measured by qRT-PCR. Results are shown as mean±SD (n = 5 separate experiments performed in duplicate). †$P < 0.05$ vs Cont of respective time-point; not significant for AngII vs AngII and Tolv treatment group among all the time-points. C, Cont; A, AngII; A+T, AngII+Tolv.

Figure S3. Effect of Tolvaptan on proliferation or viability of H295R cells. Cells were treated with Tolvaptan at the indicated doses for 30 mins and then stimulated by 100 nmol/L AngII for 24 hours. The proliferation or viability of cells was analyzed by trypan blue assay (A) and MTT test (B). Results are shown as mean±SD (n = 5 separate experiments performed in duplicate).

Figure S4. AVPR2 or arginine vasopressin receptor 2 (V2 receptor) expression in H295R cell lysates analysed by Western blotting using kidney lysates as positive control. V2 receptor protein specific bands were extremely low or even not identified in H295R cells.

Figure S5. Positive controls for CYP11B2. Western blot analysis using the antibodies as indicated to detect pcDNA3.1(+)-CYP11B2 and pCMV-Tag2C-CYP11B2 fusion proteins and their molecular size from homogenates of H295R transiently transfected cells.