Role of GRK4 in the Regulation of Arterial AT_1 Receptor in Hypertension

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Supplementary Methods

Generation of transgenic mice

Two constructs were used to generate the transgenic mice. The full-length WT *hGRK4γ* cDNA was obtained by PCR by using the *GRK4γ* cDNA in pTRE plasmid as the template. The 142V polymorphism was generated using site-directed mutagenesis. The two cDNAs were subcloned into pcDNA3.1. Expression of the cDNA insert was under the control of the cytomegalovirus promoter and bovine growth hormone (BGH) poly (A) signal. Full-length cDNA was verified by sequencing. The GRK4γ transgenic mice were generated by microinjecting the cDNA constructs into fertilized eggs obtained from the mating of a (C57BL/6J) F1 female mouse and a (SJL/J) F1 male mouse at the Institute of Animal Laboratory Science, Chinese Academy of Medical Sciences, Comparative Medicine Center, Peking Union Medical College. The presence of the transgene in the transgenic mice was verified by PCR and sequencing studies. The DNA samples were obtained from toes or tails of the hGRK4γ WT or hGRK4γ 142V transgenic mice. The forward primer of *hGRK4γ* was 5'-GATGAGGACCGAAGTGATTGT-3' and the reverse primer was 5'-TTGCCCAGGTTGTAAATGTG-3' (GenBank accession no. NM_001004057, 560bp). The amplification was performed with the following conditions: 35 cycles of denaturation at 95°C for 2 min, annealing for 30 sec at 58°C, and extension for 40 sec at 72°C. The PCR products were sequenced using the Applied Biosystem 377 DNA sequencer (Perkin Elmer, Wellesley, MA) and compared with the NCBI sequence (NM_001004057).

Arterial sample of AT_1R knock-out mice

AT_1R knock-out ($AT_1R^{-/-}$) mice in this study were obtained from laboratory of Dr Yanfang Chen (Wright State University, Dayton, OH, US). The genotype was determined by PCR analysis of genomic DNA which isolated from mice tail tissue according to previously reported methods[1]. The artery tissues were isolated and washed with ice-cold PBS for several times, and lysed by homogenizer in ice-cold lysis buffer. After centrifugation, the supernatant was mixed in 6×sample buffer and boiled for 5 min, stored at -70^o until use for immunoblotting.

Cell culture

Immortalized RPT cells from WKY [2, 3] were cultured at 37^o C in 95% air and 5% CO_2 atmosphere in FBS% DMEM/F-12 culture media, as previously described.[4]

3T3-L1 preadipocytes, obtained from the American Type Culture Collection (CL-173, ATCC, Manassas, VA), are confirmed as a cell line of mouse preadipocytes[5]. The cells were cultured in high-glucose Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum in 5% CO_2 humidified atmosphere at 37^o C. The differentiation of adipocytes was induced according to

previously reported methods.[6] Rat2 cells were obtained from the American Type Culture Collection (CL-1764, ATCC, Manassas, VA), which are confirmed as a cell line of rat fibroblast[7].

The cells (80% confluence) were extracted in ice-cold lysis buffer, sonicated, kept on ice for 1hr, and centrifuged. The supernatants were used for immunoblotting.

Confocal microscopy of double-stained transfected A10 cells and artery

For the aorta, GRK4 was visualized using the goat anti-GRK4 antibody (1:400) followed by a fluorescein isothiocyanate (FITC); α-smooth muscle actin was visualized using rabbit anti-smooth muscle actin antibody (1:50-1:100), followed by a rhodamine (TRITC)-conjugated affinity-purified goat anti-rabbit secondary antibody (red; Molecular Probes).

For the transduced A10 cells, GRK4 (1:100) was visualized using a monoclonal mouse anti-GRK4 receptor antibody (Abcam, Cambridge, UK), followed by AMCA-goat anti-mouse IgG antibody (blue; Jackson ImmunoResearch Laboratory, West Grove, PA). AT_1R was visualized using a polyclonal rabbit anti-AT_1R antibody (1:100), followed by rhodamine–conjugated goat anti-rabbit IgG antibody (red; Jackson ImmunoResearch Laboratory, West Grove, PA). Cells that were treated with only AMCA-goat anti-mouse IgG antibody or rhodamine-conjugated goat anti-rabbit IgG antibody revealed no immunofluorescence, and omission of the anti-AT_1R antibody showed no red or purple color after merging the images (data not shown).

RT-PCR of GRK4 and AT_1R

For *β*-actin, the forward primer was 5'- CCACTGCCGCATCCTCTT -3' and the reverse primer was 5'-GTCAGCAATGCCTGGGTA-3' (GenBank accession no. NM_031144, 251bp). For the AT_1R, the forward primer was 5'-AAATTGAGTGGCTGTATG-3' and the reverse primer was 5'-CTTGACCTCCCATCTCCT -3' (GenBank accession no. NM_031009, 160bp). The amplifications were both performed with the following conditions: 35 cycles of denaturation at 94°C for 2 min, annealing for 30 sec at 59°C, and extension for 45 sec at 72°C. For GRK4, the forward primer was 5'- TGTCCTGATCCTGAGGC -3' and the reverse primer was 5'- ACACACCCTGTCGCAAAT -3' (GenBank accession no. NM_022928.1, 125bp). The amplification was performed with the following conditions: 35 cycles of denaturation at 95°C for 2 min, annealing for 30 sec at 59°C, and extension for 45 sec at 72°C.

Intracellular calcium measurement

Transduced cells (1×10^6/ml) were seeded in 60 mm glass dish and incubated with 5μM calcium-dependent fluorescent indicator Fura-2 in DMEM medium at 37°C. The cells were then washed twice with Krebs buffer (140 mM NaCl, 5.4mM KCl, 0.5mM $CaCl_2$, 1.2mM $MgSO_4$, 0.3Mm NaH_2PO_4, 10 mM HEPES, 5mM glucose, pH 7.4 with Tris base). The cells were dispersed by trypsin-EDTA solution (0.25% trypsin and 0.02% EDTA). The cell suspensions (200 μl, 1×10^5/ml) were transferred into a

96-well cell culture plates (Thermo, Rochester, NY), which were placed in the Thermo Varioskan flash instrument (Thermo Fisher Scientific, Waltham, MA). Fluorescence was detected every 5 sec alternating between 340 and 380 nm excitation (2 nm slit size) at 510 nm emission (5 nm slit size).

Aorta ring study

Aorta rings were mounted between a post- and a force-transducer (AD Instruments, Sydney, Australia) that was attached to a micrometer, then immersed into a 10 ml isolated organ chamber (Scientific Instruments, Barcelona, Spain) containing Krebs-Henseleit solution (K-H solution; in mM): 118 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.03 KH2PO4, 0.45 MgSO4•7H2O, 2.5 CaCl2, and 11.1 glucose at pH 7.35 to 7.45, which was continuously bubbled with 95% O2/5% CO2 gas mixture, and the temperature was maintained at 37℃. Preload tension was 0.5g, and the K-H solution was replaced every 20 min. After a 2-hr equilibration, Ang II (final concentrations, 10-8-10-5M) was added into the organ chamber with or without a 15 min-candesartan (10-6M) pre-incubation. The tension of the aorta rings was recorded and expressed by change in amplitude from baseline.

1. Ito M, Oliverio MI, Mannon PJ, Best CF, Maeda N, Smithies O, Coffman TM. Regulation of blood pressure by the type 1A angiotensin II receptor gene. *Proc Natl Acad Sci USA*. 1995;92:3521-3525.
2. Zheng S, Yu P, Zeng C, Wang Z, Yang Z, Andrews PM, Felder RA, Jose PA. $G\alpha_{12}$- and $G\alpha_{13}$-protein subunit linkage of D_5 dopamine receptors in the nephron. *Hypertension*. 2003;41:604-610.
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4. Zeng C, Liu Y, Wang Z, He D, Huang L, Yu P, Zheng S, Jones JE, Asico LD, Hopfer U, Eisner GM, Felder RA, Jose PA. Activation of D_3 dopamine receptor decreases angiotensin II type 1 receptor expression in rat renal proximal tubule cells. *Circ Res*. 2006;99:494-500.
5. Green H, Meuth M. An established pre-adipose cell line and its differentiation in culture. *Cell*. 1974;3:127-133.
6. Chawla A, Lazar MA. Induction of Rev-ErbAα, an orphan receptor encoded on the opposite strand of the α-thyroid hormone receptor gene, during adipocyte differentiation. *J Biol Chem*. 1993;268:16265-16269.
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S1

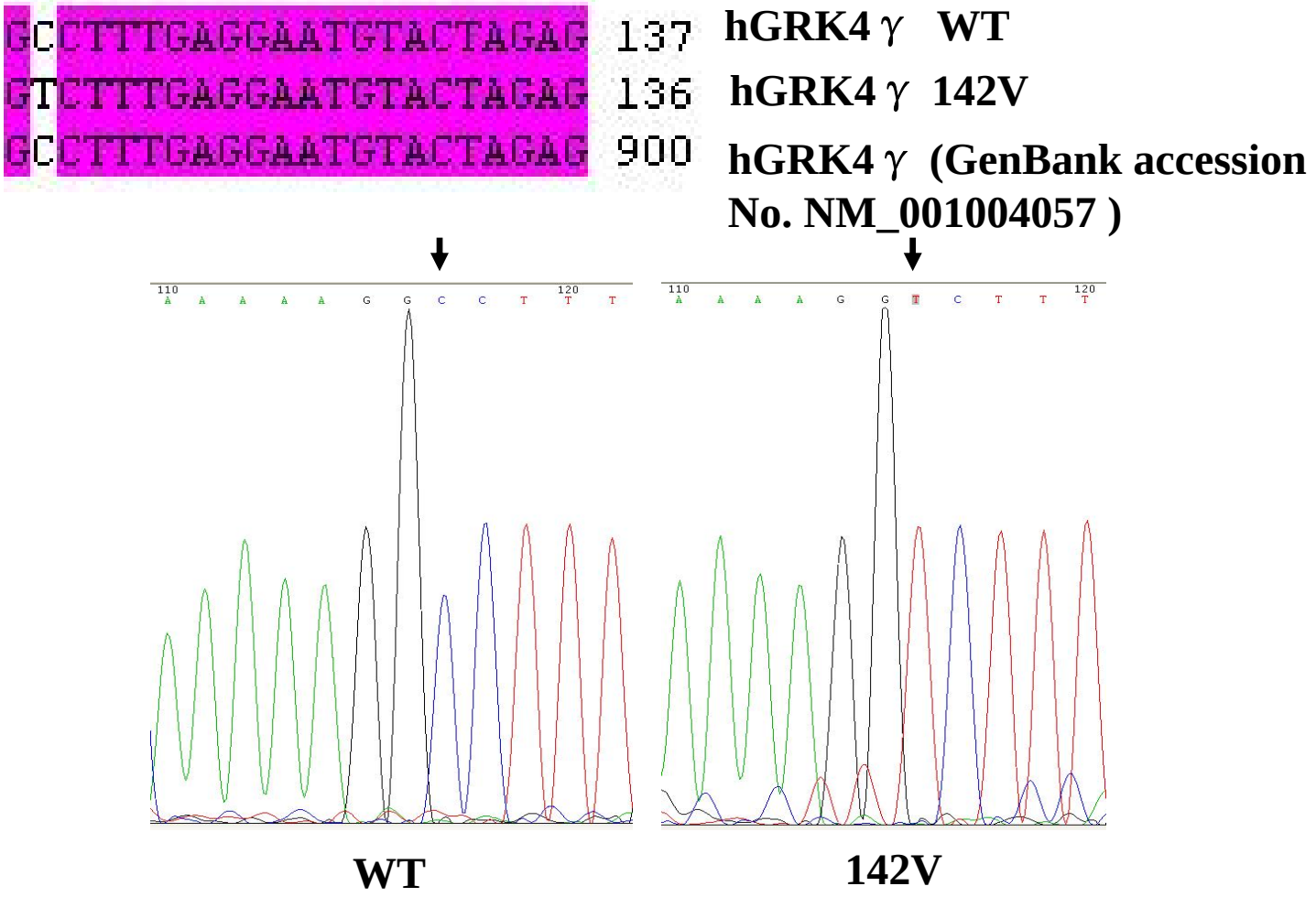
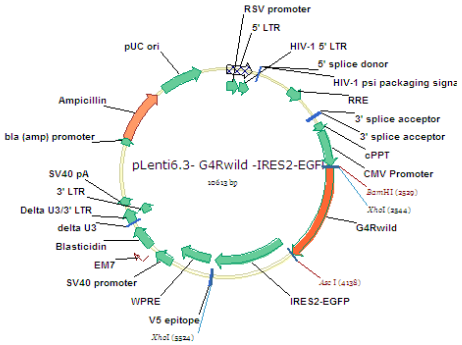


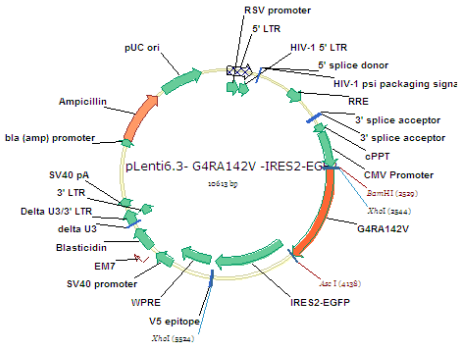
Figure S1. Expression of hGRK4 γ WT and hGRK4 γ 142V in mice, verified by gene sequencing. The variant nucleotide is indicated in the figure.

S2

A

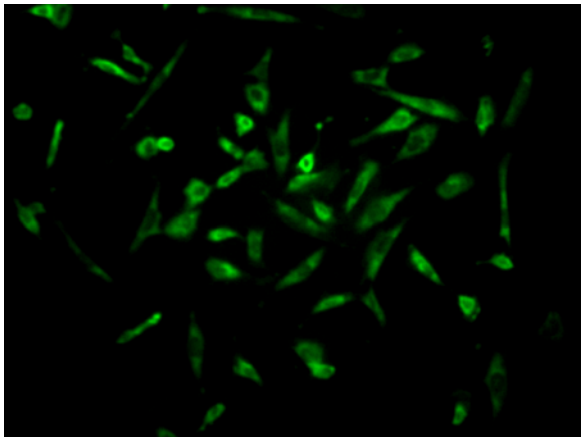


Lv-GRK4 WT-EGFP vector

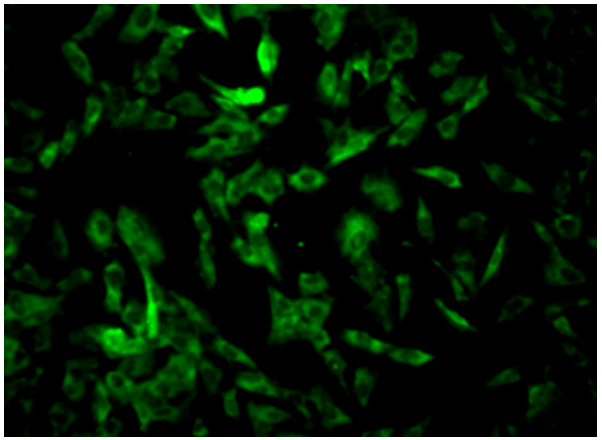


Lv-GRK4 142V-EGFP vector

B



WT



142V

Figure S2. Transduction of hGRK4 γ WT and hGRK4 γ 142V in A10 cells **A:** The constructs of pLenti6.3-hGRK4 γ -IRES2-EGFP plasmids. **B:** GFP expression in hGRK4 γ -transfected cells: GFP expression (green) was evident in A10 cells transduced with hGRK4 γ WT and hGRK4 γ 142V A10 cells.

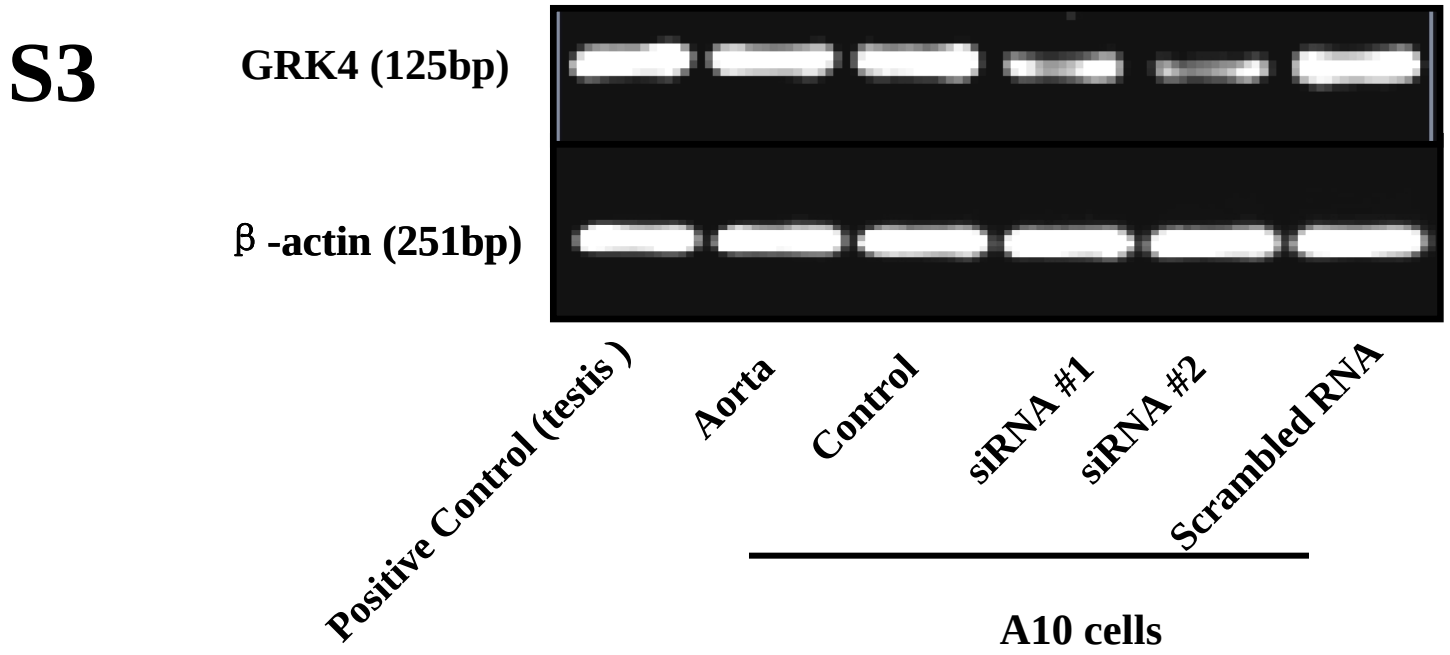


Figure S3. Effect of GRK4 siRNA on GRK4 mRNA expression. GRK4 RT-PCR products from testis (positive control), aorta, and A10 cells (control, siRNA#1, siRNA#2, and scrambled RNA) were analyzed in 10% polyacrylamide gel stained with ethidium bromide. An amplification product of the predicted size (125 bp) is seen in RT-PCR reaction using RNA (1 μ g). The band was attenuated after transfection with the specific GRK4 siRNA into A10 cells (GRK4 siRNA sequence: #1: 5'-AUCUAAAGAGGUGCAUUGAAUUCUUdTdT-3'; #2: 5'-AAGGACCUCAAUGAAUAUGAAGAUAdTdT-3') compared with the band of A10 cells without siRNA transfection (scrambled RNA sequence: 5′ -TGACGATAAGAACAATAACdTdT-3′).

S4

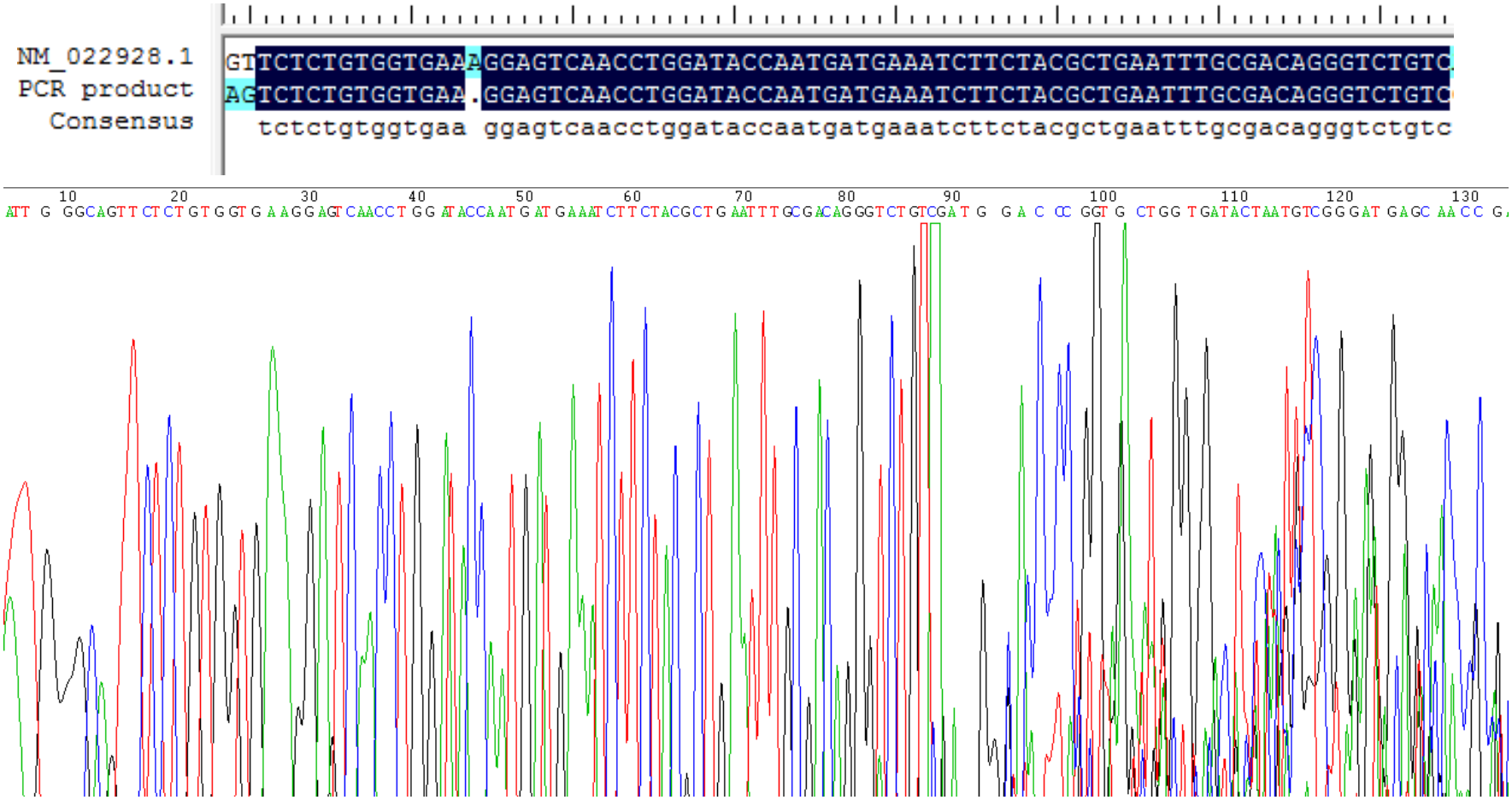


Figure S4. Sequencing of 125 bp GRK4 RT-PCR product. The RT-PCR products were identified by PAGE; the 125bp GRK4 bands were cut, extracted (by DNA gel extraction kit, Omega, US), and sequenced. The sequence of the PCR product almost completely aligned with the sequence of GRK4 (NM_022928.1).

S5

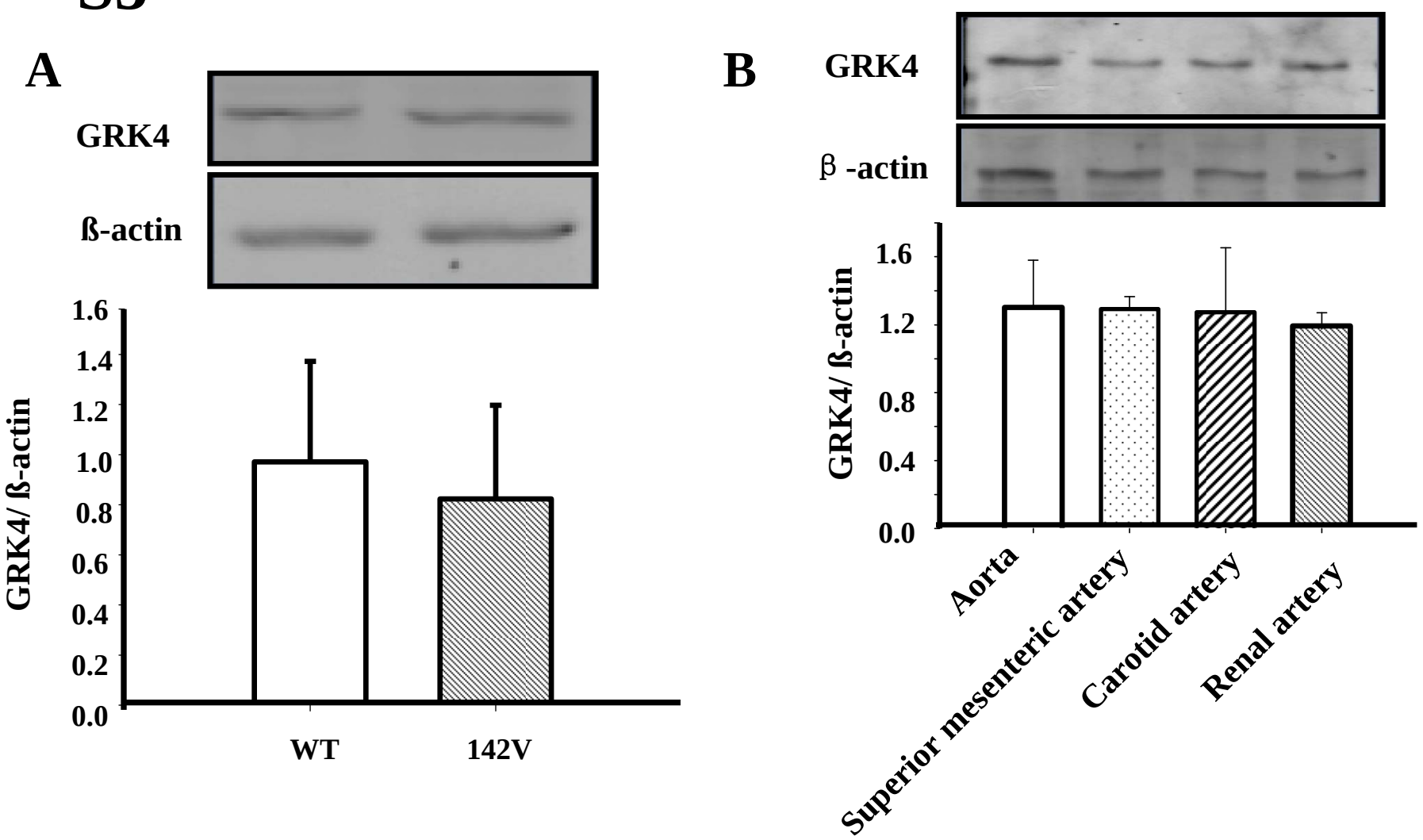


Figure S5. A: Expression of GRK4 in hGRK4 γ WT and 142V transgenic mice. Results are expressed as the ratio of GRK4 and β -actin (n=4, P=NS); **B:** Expression of GRK4 in large and small arterial vessels. Results are expressed as the ratio of GRK4 and β -actin (n=4, P=NS).

S6

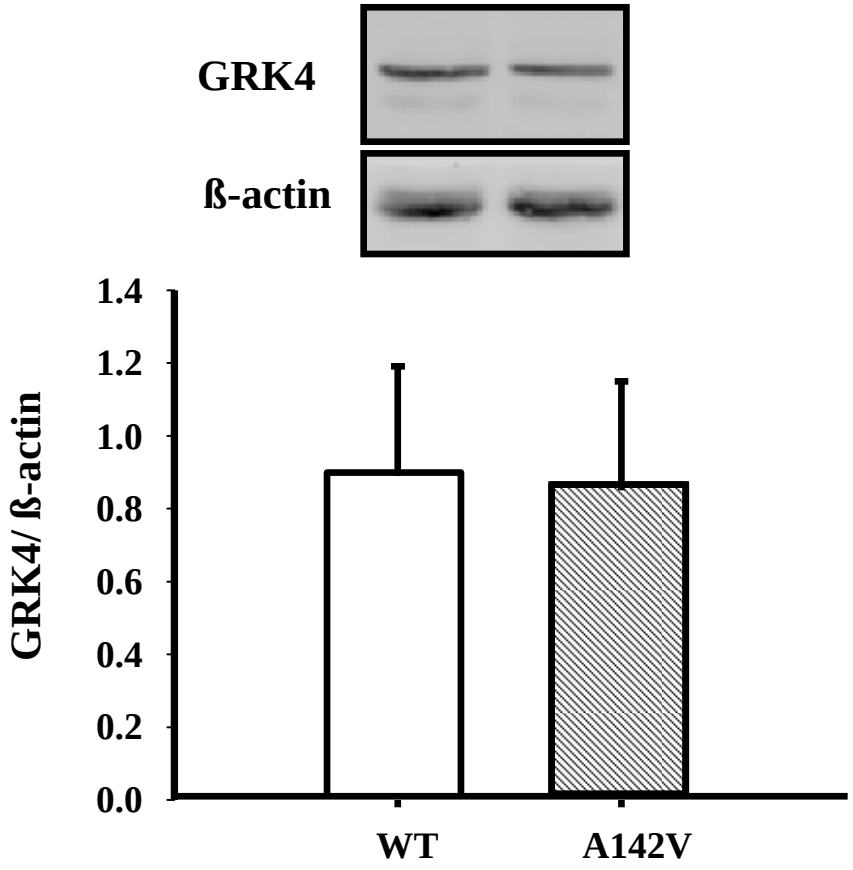


Figure S6. Expression of GRK4 in hGRK4 γ WT- and hGRK4 γ 142V-transduced A10 cells. Results are expressed as the ratio of GRK4 and β -actin (n=4, P=NS)

S7

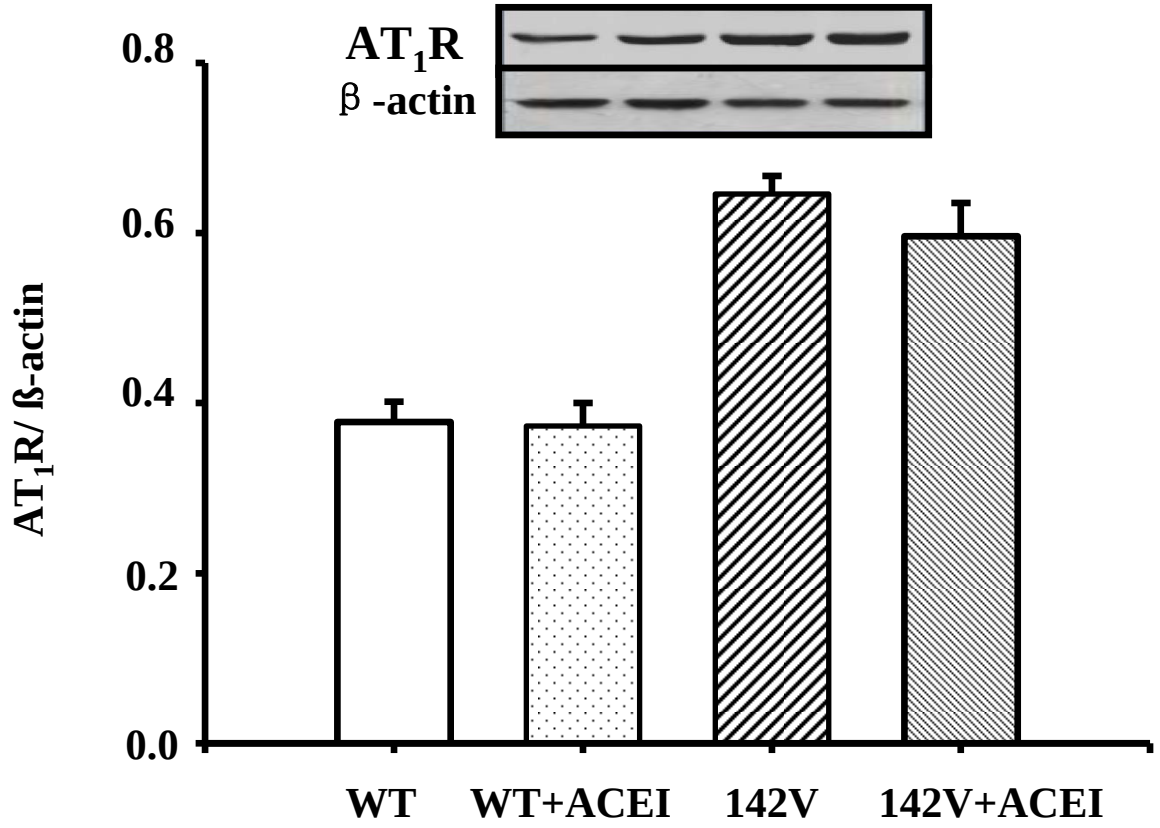


Figure S7. Effect of the angiotensin converting enzyme inhibitor (ACEI), captopril, on AT_1R expression in hGRK4 γ WT- and hGRK4 γ 142V-transduced A10 cells. Results are expressed as the ratio of AT_1R and β -actin (n=4, P=NS).

S8

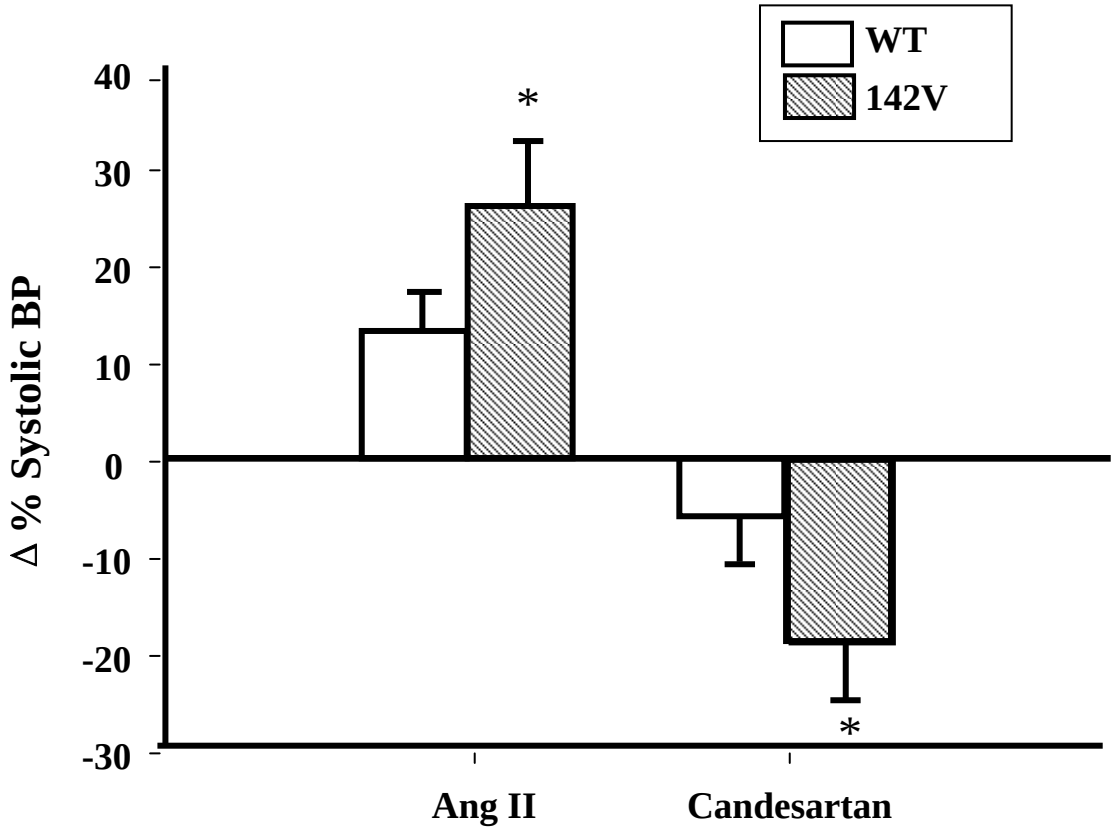


Figure S8. Effect of Ang II and the AT_1R antagonist, candesartan, on systolic blood pressure (BP) in hGRK4 γ WT and hGRK4 γ 142V transgenic mice. Ang II (1 mg/kg/min at the rate of 10 μl/hr) increased systolic BP to a greater extent in hGRK4 γ A142V than hGRK4 γ WT transgenic mice. Conversely, an intravenous administration of candesartan (0.139 mg/kg/min at the rate of 10 μl/hr) by mini-pump decreased systolic BP to a greater extent in hGRK4 γ 142V than hGRK4 γ WT transgenic mice. The mice were anesthetized with pentobarbital and BPs measured from the femoral artery. BPs were obtained after a 1-hr stabilization period (n =11, *P<0.001 vs. WT mice).