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Anti-aging Gene Klotho Regulates Endothelin-1 Levels and ET_B Receptor Expression in Kidneys of Spontaneous Hypertensive Rats

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Running title: Klotho Regulates the ET system

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Animal Study Protocols

The animal use protocol was approved by the institutional animal care and use committee of the University of Oklahoma Health Sciences Center. Male spontaneous hypertensive rats (SHR of 12 wk) of age were used in the experiment. Four groups of spontaneous hypertensive rats (SHR) (5 rats/group) and 1 group of age-matched WKY rats (5 rats/group) were used. Briefly, 4 groups of SHR received intravenous (IV) injection of AAV.mKL, AAV.LacZ, AAV.GFP, and phosphate buffered saline (PBS), respectively, while the WKY group received PBS as a control. AAV.mKL (2×10^8 PFU/rat, 0.5 ml) and PBS (0.5 ml) were administered through tail vein. At the end of week 12 after gene delivery, all animals were euthanized with sodium pentobarbital (100 mg/kg, intraperitoneal). Following blood collection, animals were perfused with saline. One kidney was removed and frozen at -80°C for molecular assays. The other kidney was fixed in paraformaldehyde (PFA) for histological examination as described previously [1].

Western Blot Analysis of Klotho and ET Receptors Expression

Western blot analysis was performed as described previously [2-4]. Briefly, tissues were homogenized in lysis buffer (50 mM Tris, 150 mM NaCl, 1% sodium dodecyl sulfate, 1% sodium deoxycholic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylene diamine tetracetic, and 1% Triton X-100) containing protease inhibitor complex and centrifuged for 5 min at 10,000 *g*. The supernatants were collected and immediately mixed with an equal volume of electrophoresis loading buffer for western blot analysis of klotho, ET_A, ET_B, Mn-SOD protein expression. The equal amount of protein was loaded in a 4-20% gradient SDS–PAGE gel, the protein was transferred onto nitrocellulose membranes after gel separation. Blots were blocked in 2% BSA in TBST for 1 hour; the membranes were incubated with antibodies (diluted in 2% BSA in TBST) at 4ºC overnight against klotho (1:300 dilution, R&D Systems, Inc. Minneapolis, MN, and USA), ET_A (1:200 dilution) or ET_B (1:500 dilution; Alomone Labs, Jerusalem, Israel), SOD (1;1000 dilution, Millipore, Billerica, Massachusetts), β-actin (1:10000 dilution, Abcam Inc., Cambrige, MA, USA) followed by washing 3 times (15 min/each) in TBST. The membranes were incubated with HRP conjugated secondary anti-goat, anti-mouse or anti-rabbit antibodies (1:2000-1:5000) for 1 hour at room temperature. Proteins were visualized by ECL (Amersham), exposed to an X-ray film and developed with X-ray processor (Konica Minolta, SRX-101A). Protein expression was normalized with the expression of β-actin, which was served as an internal control. The films were imaged by using a Bio-Rad transilluminator, protein band intensities were quantified using Image J software (NIH).

Immunohistochemical Analysis of ET Receptors Expression in Kidneys

The immunohistochemical procedure was described in our previous study [1-2,5]. Briefly, the kidneys were fixed with 4% paraformaldehyde (PFA) in PBS at 4°C overnight followed by 70% ethanol at 4°C overnight, and then embedded with paraffin wax. Tissue sections were cut at 5 μm and incubated with peroxidase-blocking solution (Dako) for 5 min and subsequently with protein blocker (Biocare, CA, USA) for 10 min. The sections were then incubated with antibodies of ET-1 (1:200 dilution, Ambion, Austin, TX, USA) ET_A (1:100 dilution) or ET_B (1:200 dilution; Alomone Labs, Jerusalem, Israel), Mn-SOD (1;200 dilution, Millipore, Billerica, Massachusetts, USA) at 4°C overnight and then with a secondary antibody (dilution 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 30 min, then tissue sections were used for counterstaining using hematoxylin staining. Staining was visualized and digital photographs were taken using a Nikon microscope.

Statistical Analysis

The data were analyzed by a one-way analysis of variance (ANOVA). Tukey's test was used to assess the significance of difference between means. The significance was set at 95% confidence limit.

References

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2. **Wang X, Sun Z, Cade R.** Prolonged attenuation of cold-induced hypertension by adenoviral delivery of renin antisense. Kidney Int. 2005 Aug;68(2):680-7.
3. **Sun Z, Wang X, Wood CE, Cade JR.** Genetic AT1A receptor deficiency attenuates cold-induced hypertension. Am J Physiol Regul Integr Comp Physiol. 2005 Feb;288(2):R433-9.
4. **Bello Roufai M, Li H, Sun Z.** Heart-specific inhibition of protooncogene c-myc attenuates cold-induced cardiac hypertrophy. Gene Ther. 2007 Oct;14(19):1406-16.
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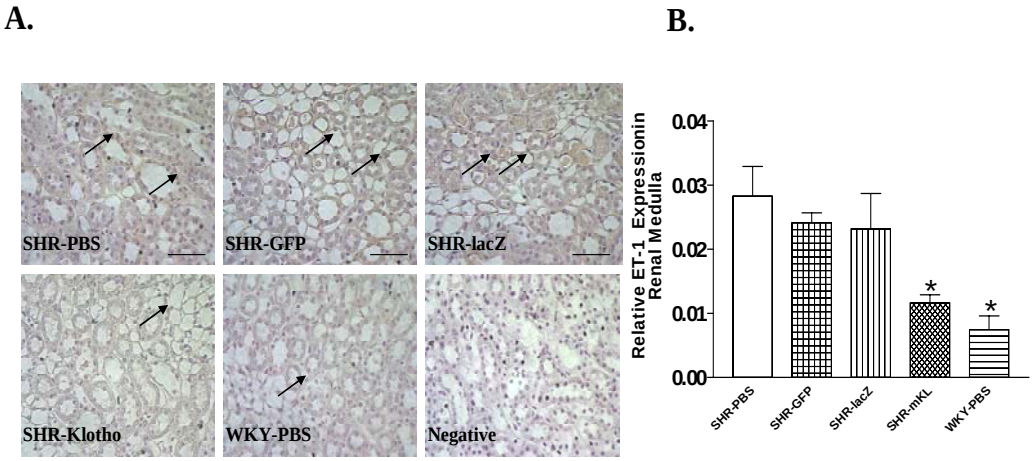
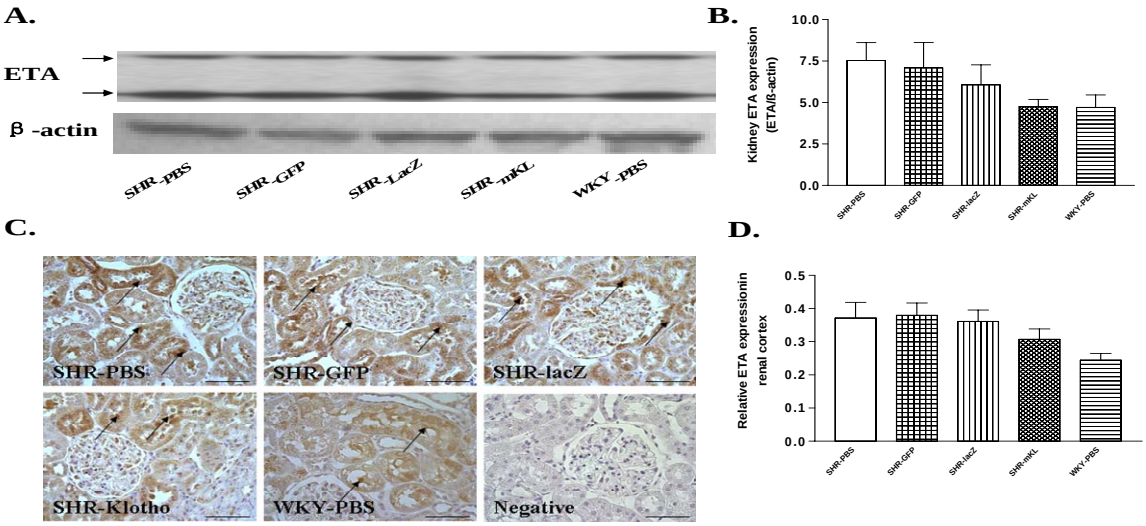
Supplemental Figure 1. Effects of klotho gene delivery on ET-1 protein expression in renal medulla. ET-1 protein expression was increased in renal medulla in SHRs which was attenuated by expression of klotho.



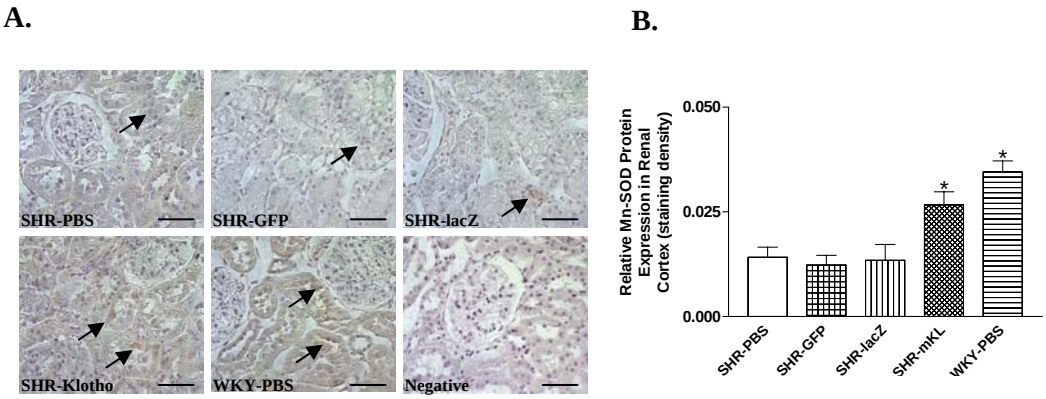
S1A, Immunohistochemical (IHC) analysis of ET-1 expression (brown staining) in the renal medulla. Arrows indicate ET-1 expression. **B,** Semi-quantitative analysis of ET-1 expression in medulla. Negative control, without the first antibody. Data are mean±SE. *p<0.05 vs SHR-PBS group. N=5 animals per group.

Supplemental Figure 2. Effects of klotho gene delivery on ET_A protein expression in kidneys. ET_A protein expression was not altered in kidneys of SHRs or by in vivo expression of klotho.



S2A, Representative western blot bands of ET_A. **B,** Quantitative analysis of ET_A protein expression. **C,** Immunohistochemical analysis of ET_B expression in kidneys. **D,** Quantitative analysis of ET_A protein expression. Arrows indicate ET_A protein expression in renal tubule epithelial cells (brown staining). Negative control, without the first antibody.

Supplemental Figure 3. Effects of klotho gene delivery on Mn-SOD protein expression in renal cortex. Mn-SOD protein expression was downregulated in renal cortex of SHRs, which was rescued by klotho gene delivery.



S3A, Immunohistochemical analysis of Mn-SOD expression in renal cortex. **B,** Quantitative analysis of SOD protein expression. Arrows indicate Mn-SOD protein expression (brown staining). Negative control, without the first antibody. Data are mean±SE. *p<0.05 vs SHR-PBS group. N=5 animals per group.