Online Supplemental Methods and Data

Haplodeficiency of Klotho Gene Causes Arterial Stiffening *via* Upregulation of Scleraxis Expression and Induction of Autophagy

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Supplemental Materials and Methods

Animal Study Protocols

This study was performed according to the guidelines of the National Institute of Health on the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee (IACUC) of University of Oklahoma Health Science Center. klotho mutant heterozygous (klotho$^{+/-}$) mice with more than 9 generations in 129Sv background were kindly provided by Dr. Makoto Kuro-o [1]. All mice were housed in cages at room temperatures (25±1°C) and were provided with Purina laboratory chow (No. 5001) and tap water ad libitum. Following measurement of pulse wave velocity, klotho$^{+/-}$ mice and their aged-matched wild-type (WT) littermates (14 mice/group, 15 mo) were euthanized (ketamine/xylazine, 90/10 mg, IP). Blood was collected for measuring serum levels of aldosterone.

For the interventional study, 14 klotho$^{+/-}$ mice and 14 aged-matched WT littermates were used (15 mo). Each strain of mice was divided into two subgroups. One subgroup received eplerenone (6 mg/kg/day, IP) while the other received an equal volume of DMSO (dimethyl sulfoxide, 5%) and served as a control. Pulse wave velocity (PWV) was measured before and after treatment with eplerenone for 3 weeks. All animals were sacrificed and perfused transcardially with PBS under deep anesthesia (ketamine/xylazine, 90/10 mg, IP). Before perfusion, blood was collected for measuring plasma klotho level. The aortas were then quickly removed, washed, and cut into pieces for subsequent analyses.

Cell Culture and Treatment

MOVAS (ATCC$^{®CRL-2797}$) is a continuous mouse aortic vascular smooth muscle cell line that has been demonstrated to retain a VSMC-like phenotype, including a spindle cell morphology and the expression of VSMC-specific markers such as smooth muscle α-actin and SM22-α. MOVAS were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2mM L-glutamine. The silencer select siRNAs targeting mouse scleraxis (catalog no. sc-153257), Beclin-1 (catalog no. sc-29798), and a silencer negative control siRNA-A (catalog no. sc-37007) were obtained from Santa Cruz (Austin, TX). For transfection, cells were plated in 60-mm tissue culture dishes at 0.7×10^6 cells per dish, grown for 24 h, and then transfected with siRNA at a final concentration of 10 nM using Lipofectamine RNAiMAX and Opti-MEM in serum-free and antibiotic-free medium, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The media were replaced 48 h later with fresh 2% serum containing DMEM, and the cells were then treated with 10 μM aldosterone for another 16 h.

Measurement of Pulse Wave Velocity

Aortic PWV was measured as described previously.[2-3] Briefly, mice were anesthetized under 2% isoflurane in a closed chamber anesthesia machine (SomnoSuite, Kent Scientific, Torrington, CT) for ~1–3 min. Anesthesia was maintained *via* nose cone, and mice were secured in a supine position on a heating board (~37°C) to maintain body temperature. Velocities were measured with 6-mm crystal 20-MHz Doppler probes (Indus Instruments, Webster, TX) at the transverse aortic arch and ~ 3.5 cm distal at the abdominal aorta and collected using Doppler signal processing workstation (Indus Instruments). Absolute pulse arrival times were indicated by the sharp upstroke, or foot, of each velocity waveform. Aortic PWV is then calculated as the quotient of the separation distance, assessed to the nearest half millimeter by engineering caliper and difference in absolute arrival times.

Measurement of Serum Aldosterone Level

Serum aldosterone levels were measured using an aldosterone ELISA kit (10004377, Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instruction.

Histological and immunohistochemical staining

Thoracic aortas were quickly excised and placed in cold (4°C) physiological saline solution. Three millimeter rings with perivascular tissue intact were removed from the thoracic aorta directly distal to the greater curvature of the aortic arch. Aorta rings were post-fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm thickness. Collagen was quantified by masson's trichrome staining as described previously.[4] The blue staining represented collagen deposition. A series of 10-15 sections of each mouse (5 mice per group) were examined and photographed using an Olympus BH-L microscope coupled with a digital color camera. Blue-stained collagen areas were quantified with ImageJ (NIH, Bethesda, MA) from 4-5 regions per section. The same threshold was used for each photo to make sure they are comparable.. Elastin was assessed by immunohistochemical visualization. Briefly, sections are washed and incubated in primary antibody against elastin (1:50, Abcam, Cambridge, MA, USA) or negative control (2.5% horse serum, Vector Labs) overnight and elastin was visualized using the appropriate secondary antibody. Finally, a series of 5-10 sections were examined and photographed using an Olympus BH-L microscope coupled with a digital color camera. Elastin levels were quantified using the Image J software (NIH, Bethesda, MD) from 4-5 regions per section. The threshold for positive staining was determined based on the negative staining (background). The same procedure was used for quantifying staining density for each animal to ensure that the data are comparable. MMP2 and MMP9 expression and myofibroblasts differentiation were also assessed by immunohistochemical staining with their primary antibody against MMP2, MMP9 and α-SMA (1:100, Abcam, Cambridge, MA, USA), respectively.

Western Blot Analysis

Protein samples from the thoracic aorta were prepared in lysis buffer as described previously.[5-6] The proteins (40–50 mg) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was then incubated overnight (4°C) with a primary antibody against collagen-1, elastin, elastase, MMP2, MMP9 (Abcam, 1:1000), TGFβ-1, scleraxis (Santa Cruz, 1:100), Beclin-1, LC3B, or α-Tubulin (Cell Signaling, 1:1000). Goat anti-mouse or goat anti-rabbit horseradish peroxidase (1:2000–1:5,000; Santa Cruz Biotechnology) was used as a secondary antibody and incubated for 1 hour at room temperature. Specific proteins were detected by chemiluminescent methods using Amersham™ ECL™ western blotting detection reagents (GE Healthcare, UK). Protein abundance on western blots was quantified by densitometry using Image lab software (Bio-Rad, Hercules, CA).

Statistical Analysis

Quantitative data were presented as the Means±SE. Differences between experimental groups were examined by one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni post-test using Prism software (GraphPad). For all analysis, $p<0.05$ were considered statistically significant.

References

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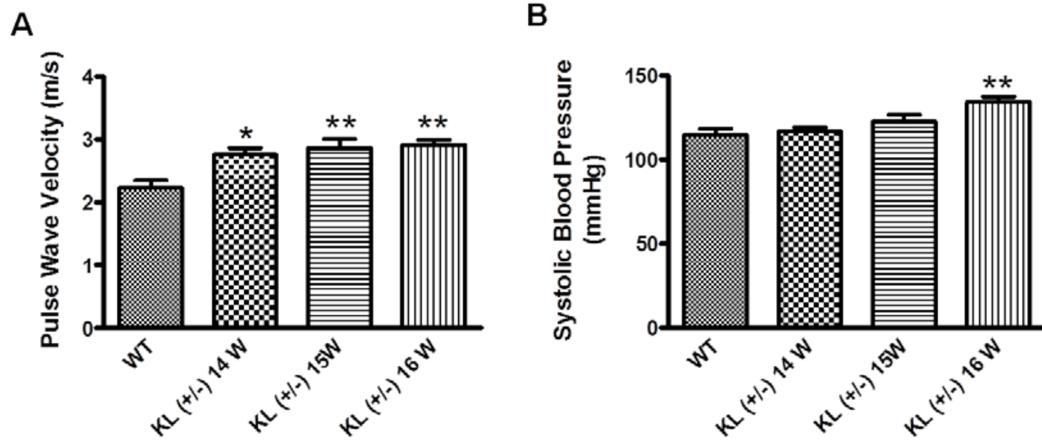
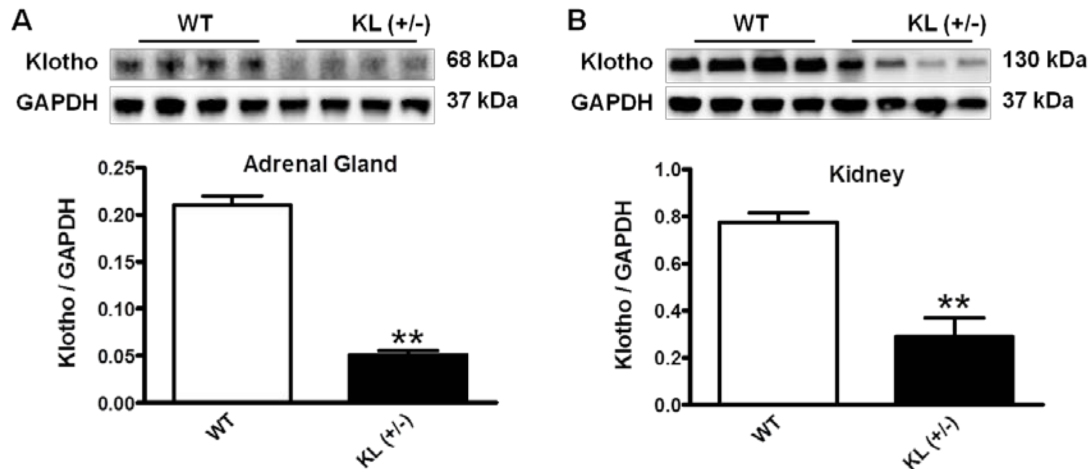
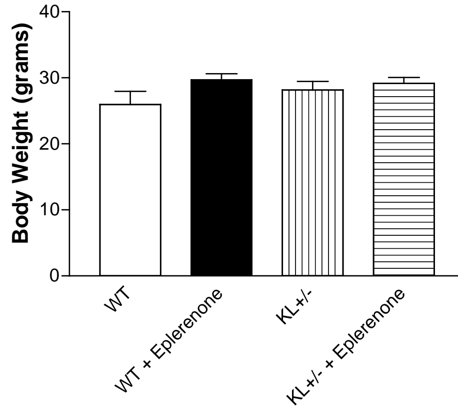
Supplemental Figures



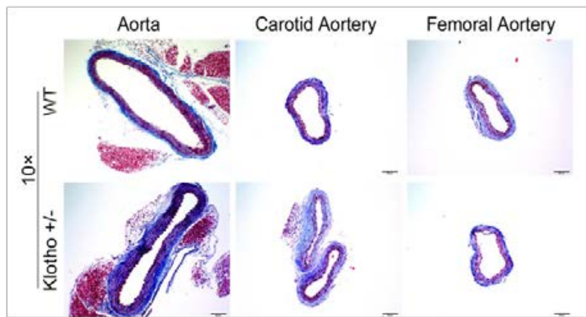
S1. Haplodeficiency of klotho gene increased arterial pulse wave velocity (PWV) prior to the elevation of blood pressure (BP). Pulse wave velocity (PWV) and blood pressure (BP) were monitored in klotho$^{+/-}$ and WT mice from 14-16 weeks of age. **(A)** PWV was measured by 10-MHz Doppler probes. **(B)** BP was measured by the volume-pressure recording (VPR) tail-cuff method using a CODA 6 BP Monitoring System. Data are expressed as mean±SE and analyzed by a one-way ANOVA. n=4. *p<0.05, **p<0.01 *vs.* WT group.

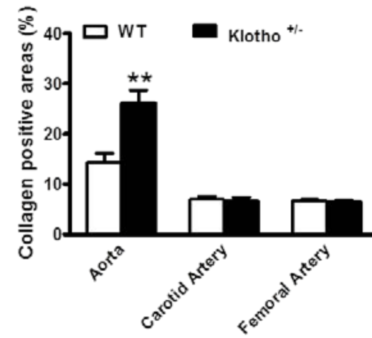


S2. Klotho expressions were reduced in adrenal glands and kidneys in klotho$^{+/-}$ **mice. (A)** Secreated klotho protein expression (68 kDa) in adrenal gland. **(B)** Full-length klotho protein expression (130 kDa) in kidneys. Data are expressed as mean±SE and analyzed by a one-way ANOVA. n=4. **p<0.01 *vs.* WT group.

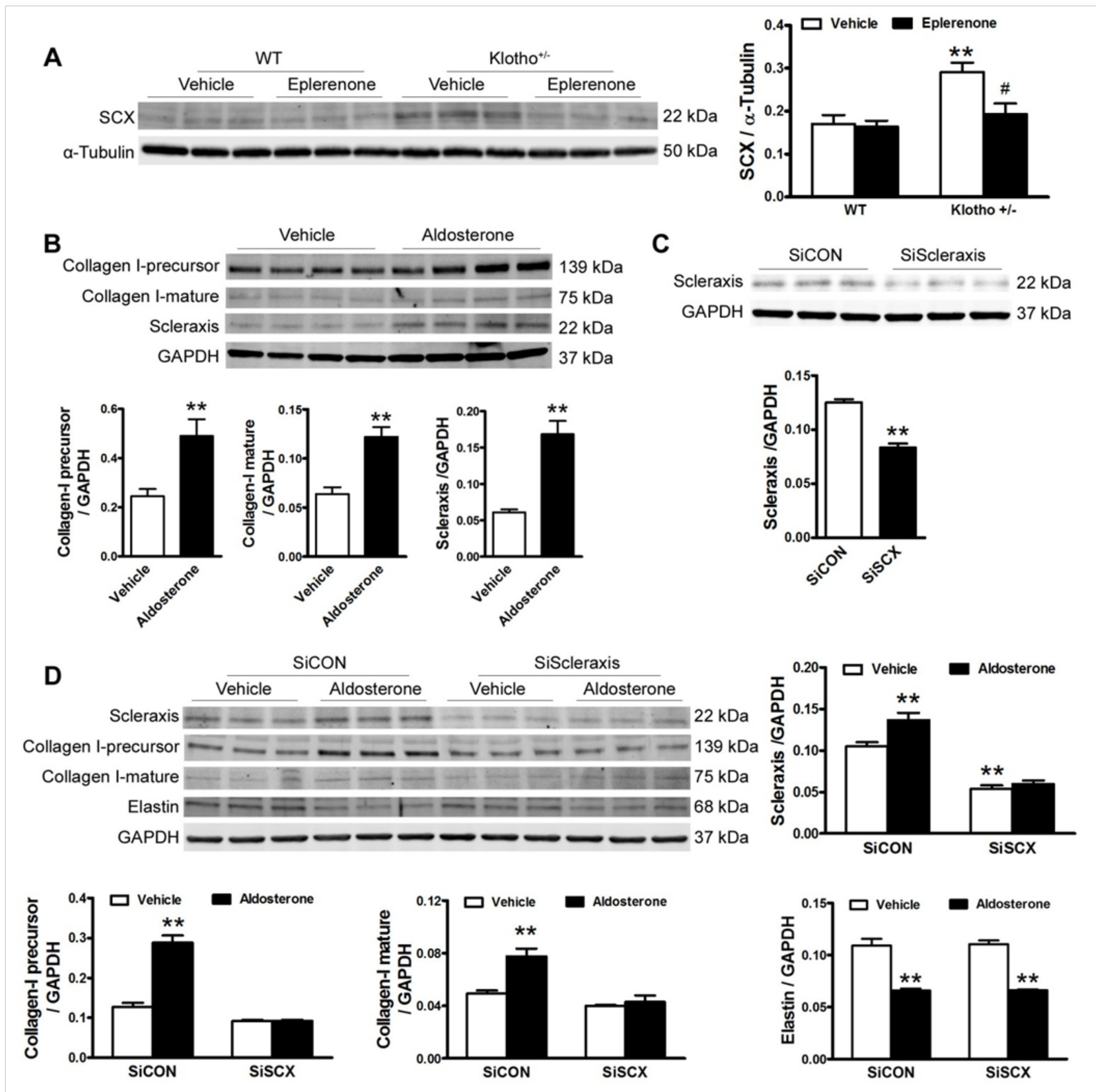


S3. Eplerenone did not alter body weight of WT and klotho +/- mice.
Data=mean±SE. N=7.

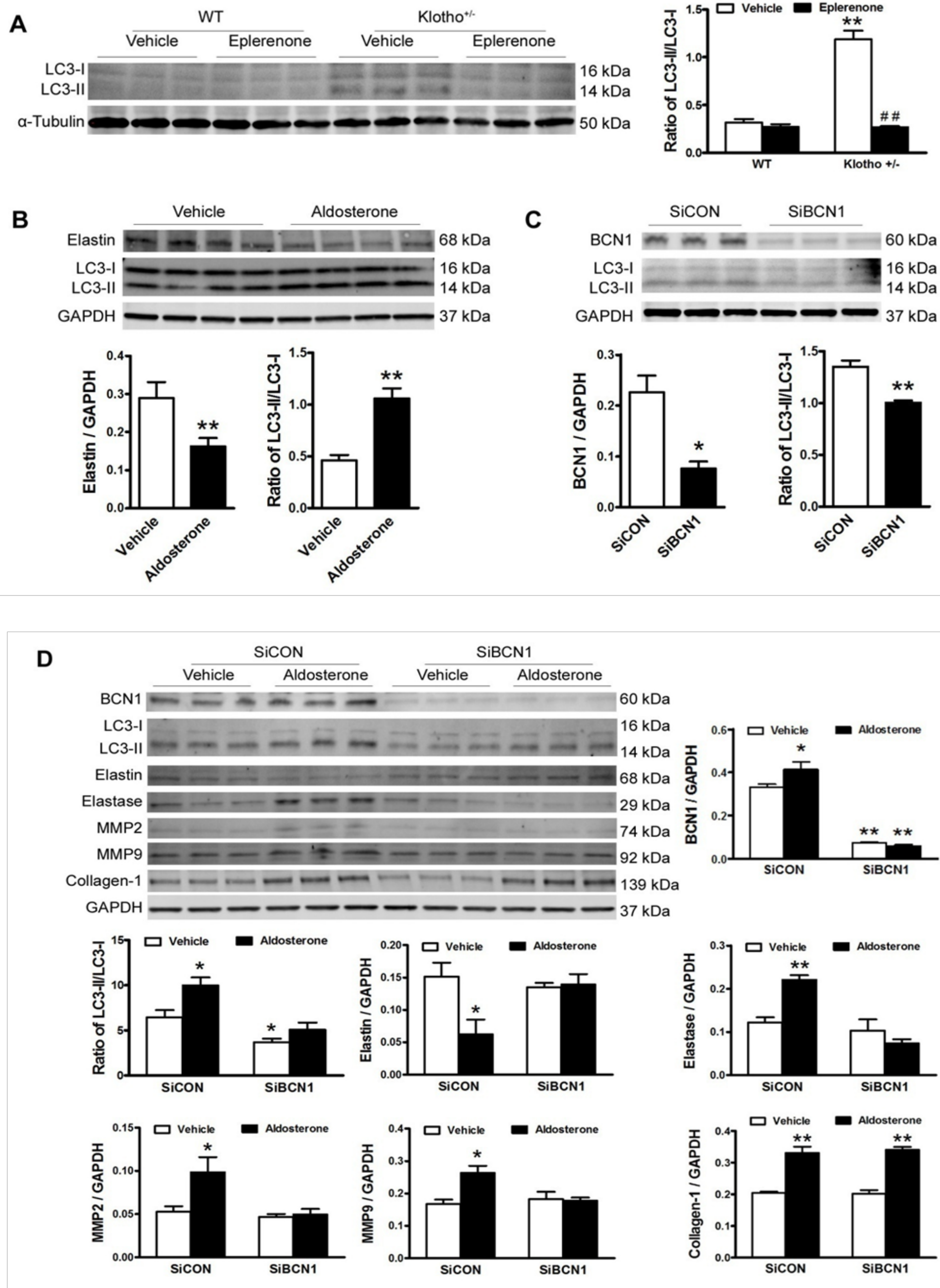




S4. Klotho deficiency increased collagen staining in aortas but not in small arteries (carotid and femoral arteries). Data=mean±SE. N=5. **p<0.01 *vs.* WT group.



S5. Aldosterone increased collagen-1 expression in smooth muscle cells by upregulation of transcriptional factor scleraxis. (**A)** Western blot analysis of scleraxis in the aorta of klotho$^{+/-}$ and WT mice. **(B)** Western blot analysis of scleraxis and collagen-1 in MOVAS treated with aldosterone. **(C)** The effectiveness of 10 nM siRNA in knocking down scleraxis in MOVAS. **(D)** Western blot analysis of scleraxis, collagen-1 and elastin in MOVAS treated with siSCX and aldosterone. Data are expressed as mean±SE and analyzed by two-way ANOVA. n=5, *$p<0.05$, **$p<0.01$ vs WT group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs klotho$^{+/-}$-vehicle group.



S6. Aldosterone decreased elastin levels in smooth muscle cells through induction of autophagy. (A) Western blot analysis of LC3 in the aorta of klotho$^{+/-}$ and WT mice. **(B)** Western blot analysis of elastin and LC3 in MOVAS treated with aldosterone. **(C)** The effectiveness of 10 nM siRNA in knocking down Beclin and inhibition of autophagy in MOVAS. **(D)** Western blot analysis of Beclin, LC3, elastin, elastase, MMP2, MMP9 and collagen-1 in MOVAS treated with Beclin-1 siRNA (siBCN1)

and aldosterone. Data are expressed as mean±SE and analyzed by two-way ANOVA. n=5. *p<0.05, **p<0.01 vs WT group; #p<0.05, ##p<0.01 *vs* $klotho^{+/-}$-vehicle group.