Online Supplemental Methods and Data

Activation of SIRT1 Attenuates Klotho Deficiency-induced Arterial Stiffness and Hypertension by Enhancing AMPKα Activity

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Online Supplemental Methods

Optimization of SRT1720 doses

The specificity of SRT1720 has been validated in previous studies.[1-3] It has been reported that at the dose of 100 mg/kg (oral gavage), SRT1720 is a specific and potent activator of SIRT1.[1-3] We also performed a dose-response test for intraperitoneal (IP) injection. At the dose of 30 mg/kg.bw (IP), SRT1720 led to a quick loss of body weight, which was not chosen for this study. We chose 15 mg/kg as an optimal dose for this study which attenuated hypertension but did not affect body weight or cause obvious side effects.

Measurement of brachial-ankle pulse wave velocity (baPWV)

Brachial-ankle pulse wave velocity (baPWV) was obtained in hypertensive patients (HP) and normotensive control (NC) participants in the supine position using an automated device (BP-203RPE III BP Monitor; Omron Healthcare, Inc. Japan). baPWV on both right and left sides was automatically recorded by the device, and the average of the two sides was calculated. Occlusion and monitoring cuffs were placed around both arms and both ankles of the participant following 10 min of rest in the supine position. The arm and ankle cuffs were placed on the skin, and electrocardiography (ECG) electrodes were placed on both wrists. The baPWV was automatically calculated using a height-based formula[4] (the distance between the arterial sites, divided by the time between the feet of their respective waveforms as recorded by the device.

Measurement of blood pressure (BP) by the tail-cuff method

This study was performed according to the guidelines of the National Institute of Health on the care and use of laboratory animals, and was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Oklahoma Health Science Center.

BP was monitored by the volume-pressure recording (VPR) tail-cuff method, with a slight warming (28℃) but not heating of the tail using a CODA 6 BP Monitoring System (Kent Scientific). This method has been validated by using a telemetry system[5, 6]. Animals were gently handled and well trained to minimize the handling stress for the VPR tail-cuff measurement. No signs of stress were observed during BP measurements. The operator was also rigorously trained for the measurement procedure. At least 20 stable cycle data sets were obtained for analysis for each animal at every measurement time point. We demonstrated that the VPR tail-cuff procedure can reliably monitor BP and is a common method for monitoring BP in our laboratory.[7 8 9 10] Nevertheless, we confirmed the BP result by direct intra-arterial cannulation.

Direct arterial blood pressure measurement

Briefly, mice were anaesthetized using isoflurane (4% induction and 1% maintenance). The carotid artery was cannulated using a Scisense 1.2F pressure catheter for blood pressure measurement and connected to an ADV 500 data-acquisition system (Transonic Systems Inc, NY). After 15 min of stabilization, the blood pressure was recorded, and the data were analyzed using Lab Chart v7 software (ADI instruments, CO).

Measurement of aortic pulse wave velocity

Aortic PWV was assessed as previously described.[11-12,13] 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 placed supine on a heating board (37°C) with legs secured to ECG electrodes. Velocities were measured using 6-mm crystal 20-MHz Doppler probes (Indus Instruments, Webster, TX) at the transverse aortic arch and at the abdominal aorta using a Doppler signal-processing workstation (Indus Instruments). Absolute pulse arrival times were indicated by the sharp upstroke, or foot, of each velocity waveform. Aortic PWV was calculated by the thoracic–abdominal distance, divided by the pulse transit time between flow pulses recorded at the thoracic and abdominal aortic sites.

Immunohistochemical analysis and histological staining

Thoracic aortas were quickly excised and placed in cold (4°C) normal saline. The aortic rings (5 mm) with perivascular tissue intact were removed from the thoracic aorta directly distal to the greater curvature of the aortic arch. Aortic tissue was post-fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at a 5-μm thickness. At least 5 sections of each mouse (6 mice per group) were processed for staining, and the sections were then incubated overnight (4℃) with antibodies against SIRT1 (diluted 1:50, Abcam Inc., Cambridge, MA, USA), AMPKα (diluted 1:50, Sigma-Aldrich Corp., MO, USA), phospho-AMPKα (diluted 1:25, Sigma-Aldrich Corp., MO, USA), eNOS (diluted 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and phospho-eNOS (Ser1177, diluted 1:100, EMD Millipore, MA, USA). After incubation with primary antibodies, the sections incubated with AMPKα, p-AMPKα, and p-eNOS were stained by a staining kit protocol (ImmunoCruz™ Rabbit ABC Staining System, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) according to the manufacturer's instructions. For SIRT1 and eNOS, sections were incubated with secondary antibody (diluted 1:2000, goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP, respectively, Santa Cruz Biotechnology, Inc., CA, USA) for 1 hour. Semi-quantitative analysis of positive staining (the intensity of positive staining over the total area in the field) was performed using NIH Image J software. Collagen was quantified by Masson's trichrome stain as described previously[7], and blue staining represents collagen deposition. Elastin was stained by Vehoeff's elastic stain kit (American Master Tech Scientific Inc., CA, USA), and black staining represents elastin in the aorta. Semi-quantitative analysis of relative collagen (the percentage of blue-stained collagen area over the total area in the field) was performed using NIS-Elements BR 3.0 software. For semi-quantitative analysis of the relative amount of elastin, the numbers of elastic fiber breaks were counted, then normalized by the total area of the field.

Western blot analysis

Mouse aortas were homogenized in RIPA buffer containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail (Research Products International Corp., Mount Prospect, IL 60056 USA) and centrifuged (15,000 x g) for 15 min at 4℃. The supernatants were

collected, and the protein concentration was measured using the Pierce bicinchoninic acid assay (Thermo Scientific). An equal amount of protein (20 μg) was loaded in a gradient SDS-PAGE gel (4–20% ExpressPlus PAGE Gel, GenScript USA Inc. NJ, USA), and the protein was transferred onto nitrocellulose filters after separation. Blots were blocked in 5% BSA in Tris-buffered saline and Tween 20 (TBST) for 1 hour, and the membranes were incubated overnight (4℃) with antibodies against SIRT1 (diluted 1:500, Abcam Inc., Cambrige, MA, USA), p53, acylated-p53 (diluted 1:500, Cell Signaling Technology, Inc., MA, USA), AMPKα, phospho-AMPKα (diluted 1:500, Cell Signaling Technology, Inc., MA, USA), eNOS (diluted 1:250, BD Transduction Laboratories Inc., Mississauga, ON, Canada), phospho-eNOS (Ser1177, diluted 1:250, EMD Millipore, MA, USA), collagen I (diluted 1:500, Sigma-Aldrich Corp., MO, USA), elastin (diluted 1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and β-actin (diluted 1:10,000, Abcam Inc., Cambrige, MA, USA).

For determining the circulatory Klotho level, plasma was diluted 20 fold and immediately mixed with an equal volume of electrophoresis loading buffer. Fifteen micrograms protein per well was loaded for western blot analysis, blots were blocked in 5% BSA in TBST for 1 hour, and the membranes were incubated overnight (4℃) with antibodies against Klotho (diluted 1:300, R&D Systems, Inc. Minneapolis, MN, USA). The intensity of total protein stained with Ponceau stain served as loading control.

Measurement of *in situ* superoxide production

In situ superoxide production was measured in aortas using the oxidation-sensitive dye dihydroethidium (DHE, Sigma-Aldrich, Atlanta, GA, USA), as previously described.[7] Briefly, unfixed thoracic aortic rings were embedded in optimal cutting temperature (OCT) buffer, frozen at −80℃, and cut into 5 μm sections using a cryostat. Sections were incubated in PBS (37°C) in a humidified chamber for 30 min followed by incubation with DHE (10^{-5} M in PBS) in the dark for 30 min. 6-diamidin-2-phenylindol dichlorohydrate (DAPI, 3×10^{-7} M, Santa Cruz Biotechnology, Inc., CA, USA) at 37°C for 5 min and mounted on slides. The images were captured using an Olympus IX73 P1F fluorescence microscope, and the average intensity[14] was determined by measuring three continuous sections at 10X magnification and then normalizing by the total area using Image J software.[14,15]

Quantification of NADPH oxidase activity

NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activity in aortas was measured by the lucigenin-enhanced chemiluminescence method.[14, 16-17] Briefly, the aorta was homogenized for quantification of NADPH oxidase activity, and the homogenate was incubated with lucigenin in the dark for 15 min. To prevent auto-oxidation of lucigenin, a low concentration was used (5 μmol/l, Sigma-Aldrich, Atlanta, GA, USA). Background counts were obtained by measuring chemiluminescence in a luminometer for 5 min (with a 2-min dark adjustment). After a 20-min recording of light emission and a stable basal value obtained, the homogenate was treated with 10 μM of the enzyme substrate NADPH (Sigma-Aldrich, Atlanta, GA, USA). The light emission was again recorded for 20 min, and the background counts (with lucigenin) were subtracted from each value. Lucigenin chemiluminescent counts were adjusted on the basis of tissue weight, and the activity was expressed as relative light units (RLU)/mg tissue.

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Online Supplemental Data

Supplemental Table 1. Clinical and laboratory data in normotensive controls (NC) and hypertensive patients (HT).

Parameter	NC	HP
Number(Male/Female)	13(10/3)	14(11/3)
Age(years)	46.77 ± 0.78	49.36 ± 2.41
History of hypertension(years)	0	3.43 ± 1.17
TC(mmol/L)	5.29±0.78	5.47 ±0.86
LDL(mmol/L)	3.30 ± 0.21	3.409 ± 0.17
HDL(mmol/L)	1.31 ± 0.08	1.35 ± 0.07
TG(mmol/L)	1.517 ± 0.16	2.037 ± 0.45
Glucose(mmol/L)	5.95 ± 0.67	5.22 ± 0.18
Creatinine(μmol/L)	73.54 ± 3.64	77.64 ± 3.79
BMI （kg/m²）	25.17 ± 0.80	25.91 ± 0.60

Data=means±SEM. BMI, body mass index; TC, total cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, total triglyceride.

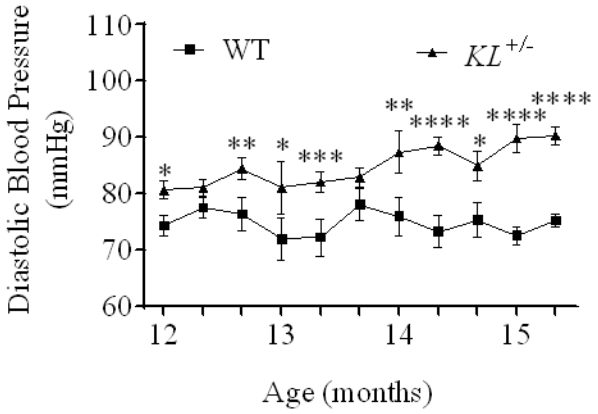

Figure S1. Time course of diastolic blood pressure (DBP) change in WT and *KL*$^{+/-}$ mice. n=10–12 mice/group. Data=means±SEM. *P＜0.05, **P＜0.01, ***P＜0.001, ****P＜0.0001 *vs.* WT.

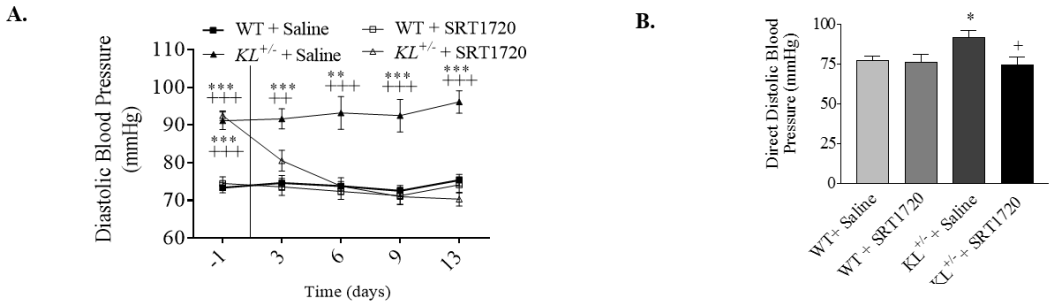

Figure S2. Activation of SIRT1 by SRT1720 attenuated diastolic blood pressure (DBP) in *KL*$^{+/-}$ mice. (A) DBP measured using tail-cuff method. **(B)** DBP measured by intra-arterial cannulation under anesthesia. n=10–12 mice/group. Data=means±SEM. **P＜0.01, ***P＜0.001 *vs* WT + Saline; $^{+}$P＜0.05, $^{++}$P＜0.01, $^{+++}$P＜0.001 *vs* *KL*$^{+/-}$ + Saline.

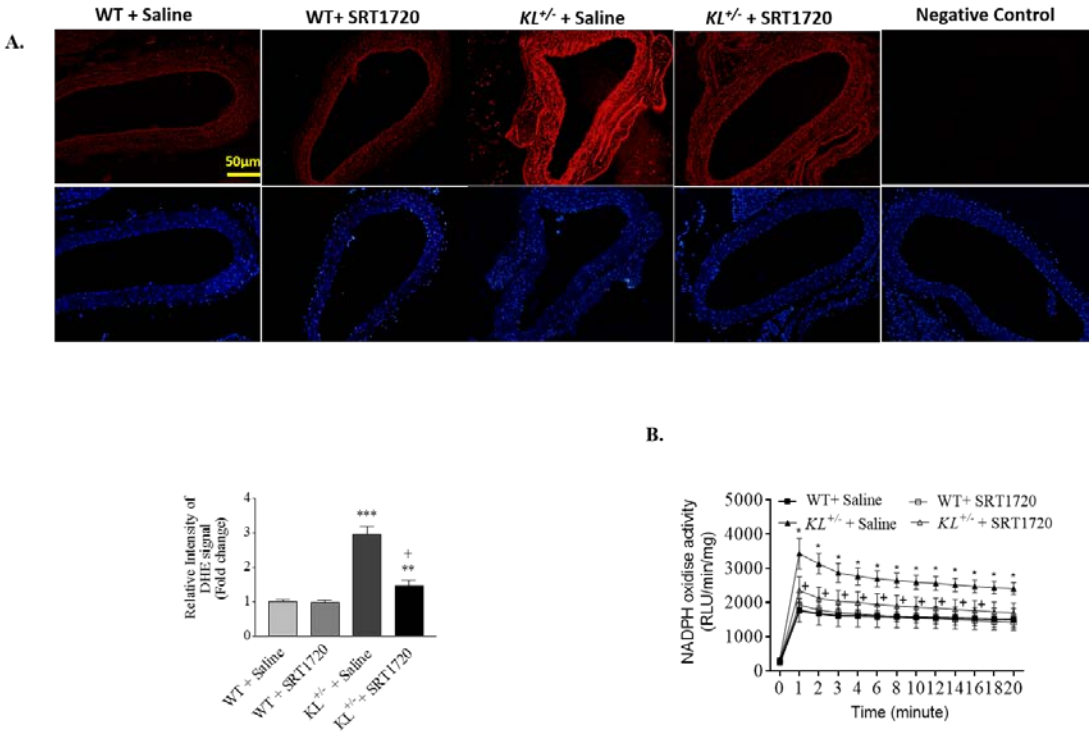

Figure S3. Activation of SIRT1 by SRT1720 attenuated *in situ* superoxide levels in the aortas of *KL*$^{+/-}$ mice. (A) Representative immunofluorescence and semi-quantification of oxidized DHE stain (red fluorescence) in aorta sections. Data were calculated as fold change relative to the control (WT + Saline). **(B)** Time course of NADPH oxidase activity change. n=4-6 mice/group. Data=means ± SEM. *P＜0.05 **P＜0.01 *vs.* WT + Saline; $^{+}$P＜0.05 $^{++}$P＜0.01 *vs.* *KL*$^{+/-}$ + Saline.

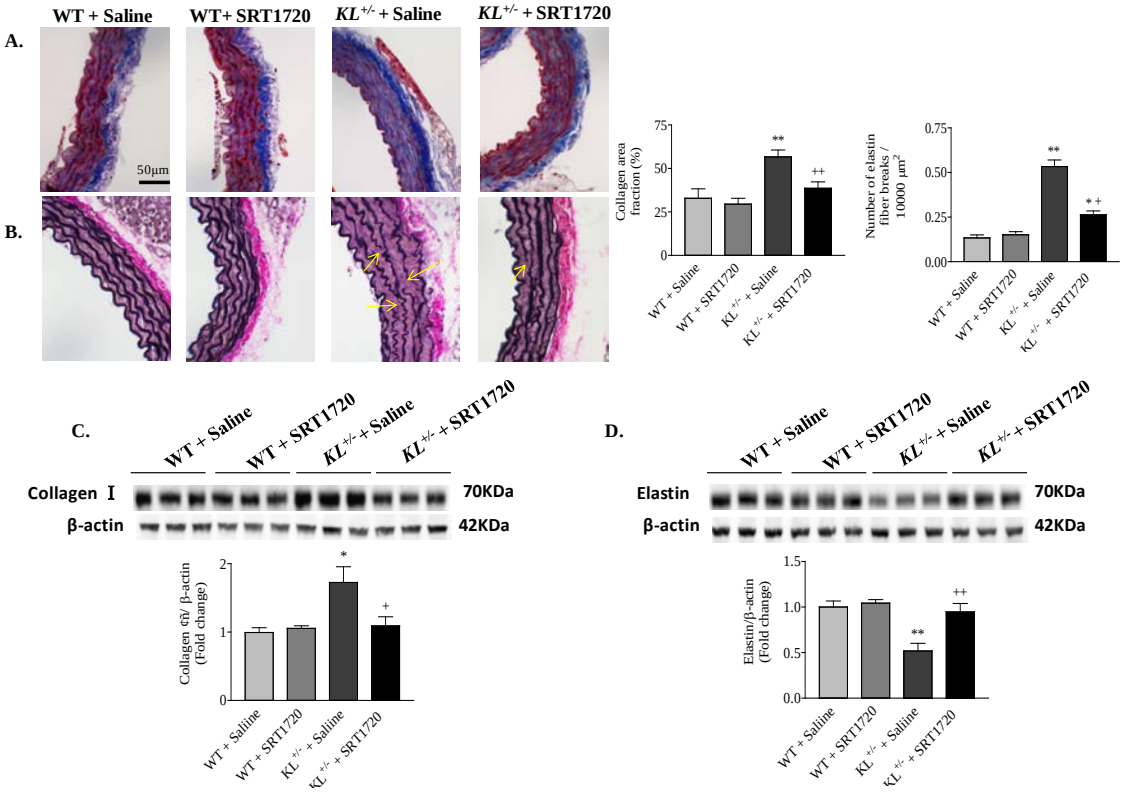

Figure S4. Activation of SIRT1 by SRT1720 attenuated Klotho-deficiency-induced collagen deposition and elastin fragmentation in aortas. (A) Representative photomicrographs of Masson's trichrome staining of collagen (blue staining indicating collagen deposition) and semi-quantification of the area fraction of collagen deposition in aortas. **(B)** Representative photomicrographs of Verhoeff's elastin staining in aortic sections (dark brown) and semi-quantification of the number of elastic fibers (per 1000 μm^2). Arrows indicate breaks in aortic elastic fibers. Representative western blot bands and quantitative analysis of collagen Ⅰ **(C)** and elastin **(D)** in aortas. Protein expression was normalized to β-actin, and the relative expression calculated as fold change relative to the control (WT + Saline). n=6 mice/group. Data=means±SEM. *P＜0.05, **P＜0.01 *vs.* WT + Saline; $^{+}$P＜0.05, $^{++}$P＜0.01 *vs.* *KL*$^{+/-}$ + Saline.

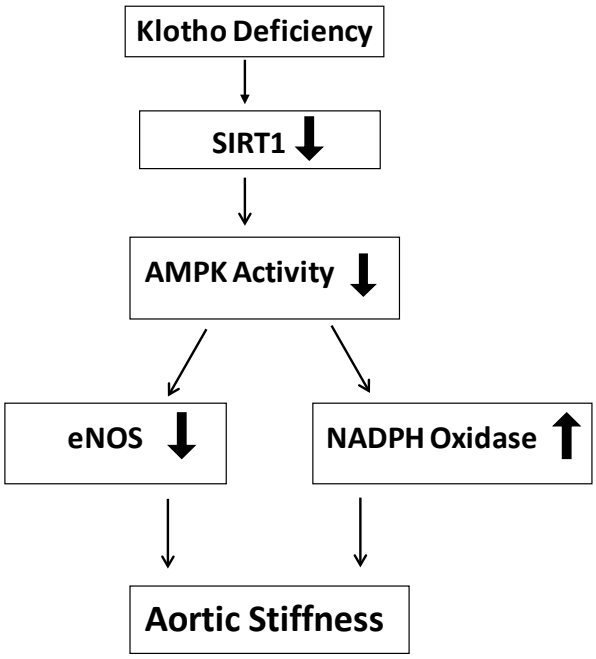

Figure S5. The molecular pathway of Klotho deficiency-induced arterial stiffness. SIRT1, sirtuin 1; AMPKα, AMP-activated protein kinase alpha; eNOS, endothelial nitric oxide synthase.