Online Data Supplement

Klotho Gene Deficiency Causes Salt-sensitive Hypertension *via* Monocyte Chemotactic Protein-1/CC Chemokine Receptor 2--mediated inflammation

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Running title: Klotho and Salt-sensitive Hypertension

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METHODS

Animal Study Protocols

This study was carried out according to the guidelines of the National Institute of Health on the care and use of laboratory animals. The project was approved by the Institutional Animal Care and Use Committee. Klotho mutant heterozygous (KL(+/-)) mice in 129Sv background were kindly provided by Dr Makoto Kuro-o[1]. The wild type (WT) littermate 129Sv mice were used as controls. 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.*

Sixteen KL(+/-) mice and 16 WT mice (9 weeks, n=16) were used. Blood pressure (BP) was measured weekly from the age of 9 to 23 weeks. After BP was stabilized (5 mo), each strain of mice was divided into 2 groups. One group of each strain received 1% saline followed by 2% saline as drinking fluid while the remaining groups received regular tap water. BP and body weight were measured twice a week. After the BP level reached stable again, each group of was further divided into 2 sub-groups at the age of 6 months (n=4). Under each diet condition in each strain, one sub-group received INCB3284 (15 mg/kg/day, IP, Tocris Bioscience, Bristol, UK) while the other subgroup received an equal dose of vehicle (dimethyl sulfoxide, DMSO) and served as a control. INCB3284 is an effective CCR2 antagonist.[2] BP and body weight were measured twice a week. Urine was collected three times for assessing renal function (urea, creatinine and albumin) during the treatment. After 10 days of the treatment, animals were euthanized (halothane). Blood was collected for measuring urea and creatinine concentration. Animals were then perfused transcardially using heparinized saline. One kidney was collected and embedded in paraffin for histological and immunohistochemical analysis. The other kidney was saved in -80°C for molecular assays.

Measurements of Blood Pressure

BP was measured by the volume-pressure recording (VPR) tail-cuff method with slight warming (28°C) 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.[3-4] Animals were gently handled and well trained for the VPR tail-cuff measurement to minimize the handling

stress. No signs of stress were observed during BP measurements. The operator was also strictly trained for the measurement procedure. At least 20 stable cycle data were obtained for the analysis of the result of each animal at every measurement time. The VPR tail-cuff procedure can reliably monitor BP and is a common method for monitoring BP in our laboratory.[5-8] Nevertheless, we realized the potential limitation of this method which is not optimal *vs* the telemetry system.

Morphological and Immunohistochemical Investigations

A series of paraffin-embedded kidney sections (5μm) were processed for the following stainings. At least 3 random fields of each section were observed and analyzed (15 sections).

Immunohistochemical (IHC) staining. Staining was performed as we described previously.[5] Briefly, the sections were incubated overnight (4°C) with primary antibodies against mouse monoclonal (KP1) CD68 (1:100, Abcam Inc., Cambridge, MA, USA), CD4 (GK1.5) (1:100, Santa Cruz Biotechnology, Inc., CA, USA) and CD8-α(H-160) (1:100, Santa Cruz, USA). Subsequently, the sections were incubated with secondary antibody including goat anti-mouse, goat anti-rabbit, and chicken anti-rat IgG-HRP (1:1000-2000, Santa Cruz) for 1h. The sections stained without the primary antibody served as negative controls. The sections were examined and photographed at equal exposure conditions and magnificationusing a Nikon Eclipse Ti-U microscope coupled with a digital color camera. The numbers of CD68, CD4 and CD8 positive cells infiltrated in kidneys were directly counted under microscope at equal magnification (X400).

Hematoxylin and Eosin (HE) staining. HE staining was performed in kidney sections for histological structure examination. Images for each section were randomly collected at equal magnification (X400) under a Nikon Eclipse Ti-U microscope. Tubular cast formation was defined as the red deposition of proteinaceous material in renal tubules. The semi-quantitative analysis of relative tubular cast area fraction in medulla (percentage of cast area over the total area in a given field) was measured using NIS-Elements BR 3.0 software (Nikon, Melville, NY, USA).

Masson's trichrome staining. Trichrome staining was performed in kidney sections for

detecting renal fibrosis. The blue staining indicated collagen deposition. The semi-quantitative analysis of relative collagen area fraction (percentage of blue-stained collagen area over the total area in one field) was measured using NIS-Elements BR 3.0 software.

Measurements of Renal Function

Plasma urea level was detected with a quantichrom™ urea assay kit (DIUR-500, BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instruction. Urine creatinine level was detected with a quantichrom™ creatinine assay kit (DICT-500, BioAssay Systems) according to the manufacturer's instruction. Urine albumin concentration was measured with a mouse-specific microalbuminuria ELISA kit (Albuwell M, Exocell, Philadelphia, PA, USA) according to the manufacturer's instruction. Urine albumin excretion was normalized to urine creatinine.

Western Blotting Analysis

Western blotting analysis was performed as described previously.[5] Briefly, the membranes were blocked in 3% BSA or 5% milk in TBST for 2h, and then incubated overnight (4°C) with primary antibodies against MCP-1 (1:500, Cell Signaling Technology, Inc., Danvers, MA, USA), TNF-α (1μg/ml, Abcam), CCR2 (1μg/ml, Abcam), MR (1:250, Abcam), Sgk1 (1:500, Santa Cruz), NCC (1:1000, EMD Millipore Corporation, Billerica, MA, USA), ATP synthase β (1:20000, BD Transduction Laboratories Inc., Mississauga, ON, Canada) and β-actin (1:7500; Abcam). Goat anti-mouse or goat anti-rabbit with HRP (1:2000–1:15000, Santa Cruz) was used as a secondary antibody and incubated for 1 h at room temperature. Proteins were visualized by ECL, exposed to an X-ray film and developed with a X-ray processor (Canon, SRA-101A). The relative protein expression of each sample was first normalized to β-actin and then calculated as fold change of the controls (WT).

Supplemental Experiment. One group of WT mice and three groups of KL(+/-) mice were used (7-8 months) to test the effects of blockade of CCR2 by RS102895. RS102895 is an effective CCR2 antagonist.[9-10] The groups and treatments are as follows: WT; KL(+/-); KL(+/-) + HS (2% NaCl in drinking water); and KL(+/-) + HS + RS102895 (CCR2-selective

antagonist).

Blood pressure was measured using the VPR tail-cuff method. Following a 2-week treatment with RS102895 (Tocris, 30 mg/kg.day, IP injection twice a day), animals were euthanized for testing vascular relaxing responses to acetylcholine and sodium nitroprusside (SNP) using the myography (Danish Myo Technology, Ann Arbor, MI). Blood was collected for measuring serum creatinine and plasma urea as we described previously.[11-12]

Statistical Analysis

BP was analyzed using one-way ANOVA repeated in time. All other data were analyzed by one-way ANOVA. The student unpaired *t* test was used for comparisons between 2 groups. Tukey's multiple comparison tests were used to assess the significance of differences between means. Significance was set at a 95% confidence limit.

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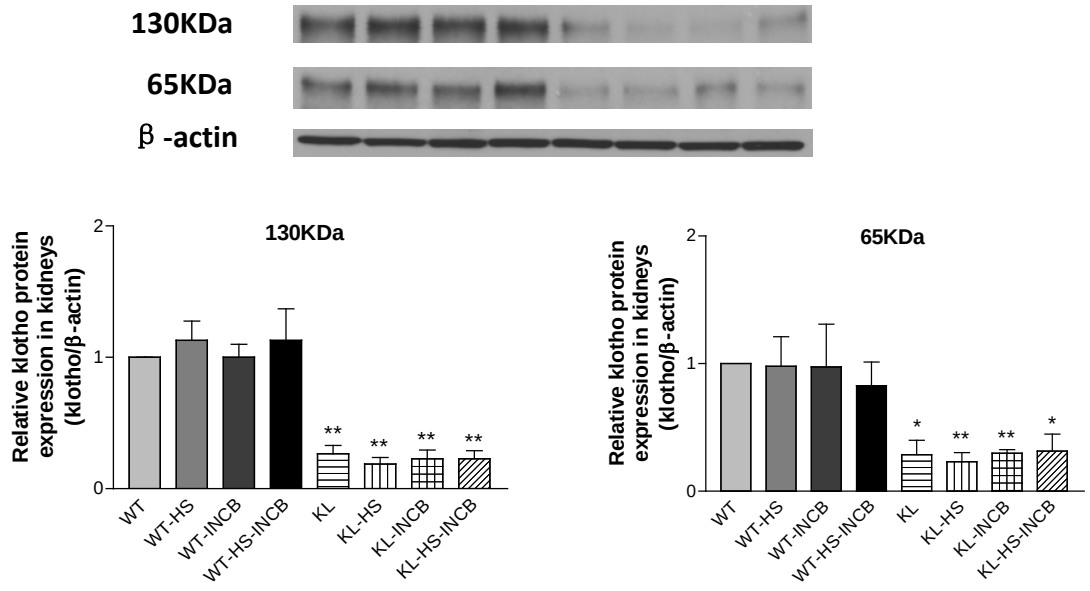
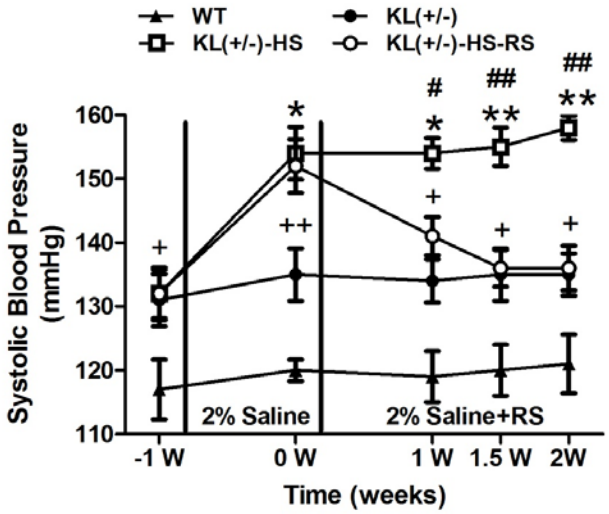
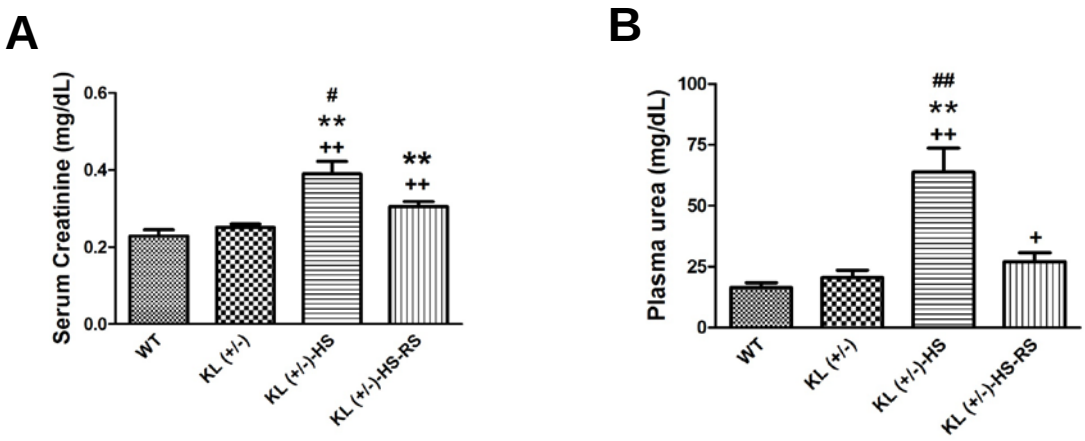
Online Supplemental Data



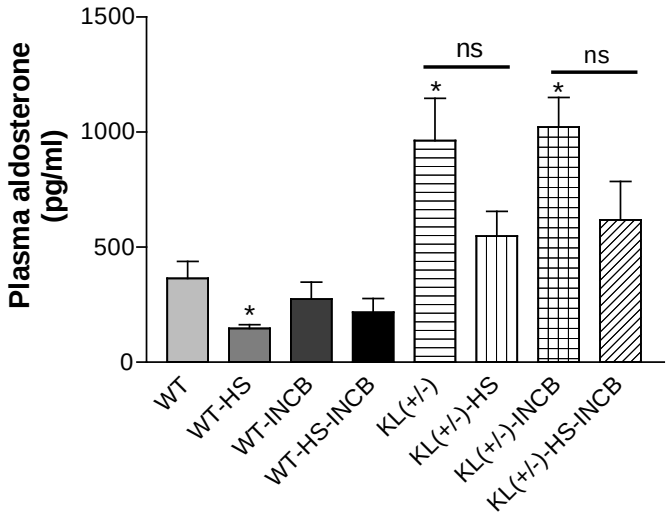
Supplemental Figure 1. Klotho protein expression in kidneys. Klotho has two transcripts: full-length klotho (130 KDa) and short-form klotho (65KDa). Relative klotho protein expression was first normalized to β-actin, and then calculated as fold changes of the controls. *p<0.05 , **p<0.01 *vs* the WT group.



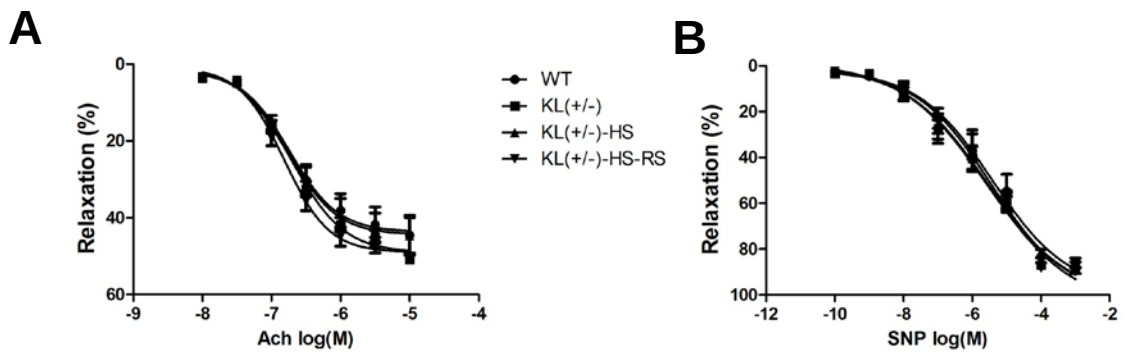
Supplemental Figure 2. Blockade of CCR2 by a CCR2-selective antagonist, RS102895, abolished the HS-induced increases in blood pressure in KL(+/-) mice. Data=means±SEM. N=5. +*p*<0.05 *vs* WT; **p*<0.05, **p<0.01 *vs* KL(+/-); #*p*<0.05, #p<0.01 *vs* KL(+/-)-HS-RS.



Supplemental Figure 3. Blockade of CCR2 by RS102895 significantly attenuated the HS-induced increases in serum creatinine **(A)** and plasma urea **(B)**. Serum creatinine and plasma urea were increased in KL(+/-) mice by HS, indicating that HS impaired kidney function. RS102895 improved kidney function in KL(+/-)+HS mice. Data=means±SEM. N=5. +p<0.05, ++p<0.01 *vs* WT; **p<0.01 *vs* KL(+/-); #p<0.05, ##p<0.01 *vs* KL(+/-)-HS-RS.



Supplemental Figure 4. Half klotho deficiency (+/-) increased plasma aldosterone levels. HS tended to decrease plasma aldosterone levels but did not reach significant difference. *p<0.05 *vs* the WT group. ns, no significant difference (p>0.05). N=4-5.



Supplemental Figure 5. Blockade of CCR2 did not affect vascular relaxant responses to acetylcholine (Ach) **(A)** or sodium nitroprusside (SNP) **(B)** in mesenteric arteries. Vessels were preconstricted with norepinephrine (NE, $1x10^{-5}$ M). RS102895 did not alter endothelium-independent vasorelaxant response to NO donor (SNP, 10^{-10} to 10^{-3} M) or endothelium-dependent vasorelaxing response to Ach (10^{-8} to 10^{-5} M). Data=means±SEM. N=5.