Klotho Gene Delivery Prevents the Progression of Spontaneous Hypertension and Renal Damage

Yuhong Wang, Zhongjie Sun

Abstract—Klotho is a recently discovered antiaging gene. The objective of this study was to test the hypothesis that klotho gene delivery attenuates the progression of spontaneous hypertension and renal damage in spontaneous hypertensive rats (SHRs). An adeno-associated virus (AAV) carrying mouse klotho full-length cDNA (AAV.mKL) was constructed for in vivo expression of klotho. Four groups of male SHRs and 1 group of sex- and age-matched Wistar-Kyoto rats (5 rats per group) were used. Blood pressure was measured twice in all of the animals before gene delivery. Four groups of SHRs received an IV injection of AAV.mKL, AAV.LacZ, AAV.GFP, and PBS, respectively. The Wistar-Kyoto group received PBS and served as a control. AAV.mKL stopped the further increase in blood pressure in SHRs, whereas blood pressures continued to increase in other SHR groups. One single dose of AAV.mKL prevented the progression of spontaneous hypertension for at least 12 weeks (length of the study). Klotho expression and production were suppressed in SHRs, which were reverted by AAV.mKL. AAV.mKL increased plasma interleukin 10 levels but decreased Nox2 expression, NADPH oxidase activity, and superoxide production in kidneys and aortas in SHRs. AAV.mKL abolished renal tubular atrophy and dilation, tubular deposition of proteinaceous material, glomerular collapse, and collagen deposition seen in SHRs, indicating that klotho gene delivery attenuated renal damage. Therefore, the suppressed klotho expression may play a role in the progression of spontaneous hypertension and renal damage in SHRs. AAV delivery of klotho may offer a new approach for the long-term control of hypertension and for renoprotection. (Hypertension. 2009;54:810-817.)

Key Words: klotho □ Nox2 □ blood pressure □ adeno-associated virus □ renoprotection

Klotho is a newly identified antiaging gene.1 It encodes a single-pass transmembrane protein with a long extracellular domain and a short cytoplasmic tail. Klotho protein is predominantly expressed in distal convoluted tubules in the kidneys and in the choroid plexus in the brain.2–4 There are 2 forms of klotho, transmembrane (130-kDa) and secreted (65-kDa) forms.1 Genetic deficiency of klotho causes extensive premature aging phenotypes, including a drastically shortened life span.5 Overexpression of the klotho gene extends the life span.3 Klotho can exert its effects in tissues or cells that do not express klotho, suggesting that it may function as an endocrine hormone.1 It was reported that the klotho gene suppresses the insulin/insulin-like growth factor 1 signaling, increases NO availability, and regulates ion channel activities.2,6,7 Klotho may protect against endothelial dysfunction, although it does not express in blood vessels.8–10 Saito et al10 reported that in vivo klotho gene delivery decreased blood pressure (BP) in Ohtsuka Long-Evans Tokushima Fatty rats but did not affect angiotensin II–induced hypertension.11 However, it is critical to determine whether klotho plays a role in the progression of spontaneous hypertension and renal damage.

The prevalence of hypertension and related cardiovascular diseases increases with age.12,13 Indeed, the mortality from cardiovascular disease is higher in aged versus young people.12,13 Thus, hypertension and related cardiovascular diseases are aging disorders. Cardiovascular aging is an important aging process that determines the lifespan. It was reported recently that the plasma level of klotho decreases with age after 40 years.14 Therefore, it is important to determine whether klotho is involved in the pathogenesis of hypertension. The spontaneously hypertensive rat (SHR) is a well-characterized genetic model of hypertension.15,16 It has many features of human essential hypertension.16 The characteristic of this model is that hypertension progresses spontaneously with age. In addition, SHRs develop end-organ damages, including renal injury, cardiac hypertrophy, and heart failure.16 The purpose of this study was to test the hypothesis that klotho gene delivery attenuates the progression of spontaneous hypertension and renal damage in SHRs.

Methods
A full description of the Materials and Methods section can be found in the online Data Supplement at http://hyper.ahajournals.org.
Construction of Recombinant Adeno-Associated Virus With the Mouse Klotho Gene

The procedure for constructing the recombinant adeno-associated virus (AAV)-2 carrying the mouse full-length cDNA (AAV.mKL) was described in details in the online Data Supplement (Figures S1 to S3). AAV carrying green fluorescent protein (GFP; AAV.GFP) and β-galactosidase (LacZ; AAV.LacZ) were constructed as reporter gene constructs.

Animal Study Protocols

Four groups of SHRs and 1 group of age-matched Wistar-Kyoto (WKY) rats were used (5 rats per group, all males, 12 weeks old). Systolic BP and body weight were measured twice before gene delivery. BP was measured using the tail-cuff method, as described in the online Data Supplement. Briefly, the 4 groups of SHRs received AAV.mKL, AAV.GFP, AAV.LacZ, and PBS, respectively. The viral particles were delivered intravenously via the tail vein at 2×10^8 particles per rat (0.5 mL). The WKY group received PBS and served as a control. BP and body weight were measured weekly after gene delivery. All of the animals were euthanized at the end of week 12. Serum creatinine and kidney collagen deposition were measured. The detailed protocols can be found in the online Data Supplement. The protocol was approved by the institutional animal care and use committee of the University of Oklahoma Health Sciences Center.

Immunohistochemical Analysis of Klotho Expression in Kidneys and Aortas and Histological Examination of Kidneys

The immunohistochemical procedure was described in our previous studies.17–20 For details, refer to the online Data Supplement.

Measurement of In Situ Superoxide Production

The in situ superoxide production was measured in aortas and kidneys using the oxidation-sensitive dye dihydroethidium (DHE), as described in the online Data Supplement.

Quantification of NADPH Oxidase Activity

NADPH oxidase activity in aortas was assessed by the lucigenin chemiluminescence method.21–23 The detailed procedure can be found in the online Data Supplement.

Western Blot Analysis of Klotho and NADPH Oxidase Expression

Klotho and NADPH oxidase protein expressions were measured by Western blot, as described in the online Data Supplement.

RT-PCR Analysis of Mouse Klotho mRNA Expression

Mouse klotho mRNA was analyzed by RT-PCR using mouse-specific primers as described in the online Data Supplement.

Statistical Analysis

The data for BP and BW were analyzed by a 2-way ANOVA (temperature and treatment) followed by a 1-way ANOVA (repeated in time). The remaining data were analyzed by a 2-way ANOVA. The Newman-Keuls procedure was used to assess the significance of
the difference between means. The significance was set at the 95% confidence limit.

Results

Effects of Klotho Gene Delivery on Spontaneous Hypertension

The baseline BP of 12-week–old SHRs was significantly higher than that of the WKY rats (Figure 1). BP of the SHR-GFP, SHR-LacZ, and SHR-PBS groups continued to increase and reached 177/110 mm Hg at 12 weeks after gene delivery. In contrast, BP of the AAV.mKL group did not increase compared with its pretreatment level (Figure 1). One single dose of AAV.mKL prevented progression of hypertension for 12 weeks (length of the study). AAV.mKL did not affect food and water intakes or body weight gain (Figure S4).

Effects of Klotho Gene Delivery on In Situ Vascular Superoxide Production in SHRs

Aortic segments of SHRs showed an increase in superoxide production (red fluorescence or DHE staining) compared with those of WKY rats (Figure 2A). In contrast, vascular superoxide production was markedly decreased in SHRs treated with AAV.mKL. A quantitative analysis confirmed that klotho gene delivery significantly attenuated the in situ vascular superoxide production (Figure 2B).

Effects of Klotho Gene Delivery on Klotho Production and NADPH Oxidase Activity

Nox2 protein expression was increased in aortas of SHRs compared with that of the WKY rats (Figure 3A and 3B). Klotho gene delivery decreased aortic Nox2 expression in SHRs to that of the WKY rats. Nox1, Nox4, and endothelial NO synthase were not affected by klotho gene delivery (data not shown). NADPH oxidase activity was increased significantly in aortas of SHRs, which could be abolished by klotho gene delivery (Figure 3C).

Plasma levels of membrane and secreted forms of klotho were decreased significantly in SHRs compared with WKY rats (Figure 3D and 3E). AAV.mKL increased plasma levels of klotho protein in SHRs to those of WKY rats, indicating that klotho gene delivery increased klotho production. The urine levels of klotho were also decreased in SHRs over a time course (weeks 5 and 8) and were reverted by klotho gene delivery (Figure S5).
The plasma level of interleukin (IL) 10 was decreased significantly in SHRs, which was reverted by klotho gene delivery (Figure 3D and 3F). The IL-10 level was not different between SHR-GFP/SHR-LacZ and SHR-PBS, indicating that the AAV vector may not alter IL-10 expression.

Effects of Klotho Gene Delivery on Superoxide Production in Kidneys in SHRs

Tissue sections of kidneys of SHRs showed increased superoxide production compared with those of WKY rats (Figure 4A). In contrast, superoxide production was markedly attenuated in SHRs treated with AAV.mKL. A quantitative analysis confirmed that klotho gene delivery significantly attenuated superoxide production in kidneys (Figure 4B).

Effects of Klotho Gene Delivery on Klotho and Nox2 Expression in Kidneys

Renal klotho protein expression was decreased significantly in SHRs compared with WKY rats (Figure 5A and 5B). Klotho gene delivery increased renal klotho expression in SHRs to the control (WKY) level. Renal Nox2 expression was upregulated in SHRs, which was reverted by klotho gene delivery (Figure 5A and 5C). Renal expression of endothelial NO synthase, Nox1, and Nox4 was not altered in SHRs or affected by klotho gene delivery (Figure S6).

Immunohistochemical analysis indicated that klotho protein expression (brown staining) was localized in the renal tubule epithelial cells (Figure 5D). Klotho staining was not found in the aorta (photos not shown).

Effects of Klotho Gene Delivery on Kidney Damage in SHRs

A partial loss of kidney medulla was found in some SHRs (SHR-PBS, SHR-GFP, and SHR-LacZ; Figure 6A). Histological examination (staining) indicated that some cortical tubules were atrophic, dilated, and filled with proteinaceous material in SHRs (Figure 6B). Renal damage also included glomerular collapse in SHRs (Figure 6C). These pathological changes disappeared in the AAV.mKL-treated SHRs (SHR-mKL), indicating that klotho gene delivery abolished renal injury in SHRs (Figure 6). AAV.mKL also significantly decreased urinary output of protein (Figure S7), suggesting that klotho gene delivery improved renal function. Thus, klotho gene delivery attenuated kidney damage in SHRs.

Effects of Klotho Gene Delivery on Kidney Collagen Staining and Serum Creatinine

There was a significantly increase collagen deposition (blue staining) in kidneys of SHR-PBS, SHR-GFP, and SHR-LacZ groups (Figure 7A and 7B). Renal interstitial fibrosis was found in these groups. Serum level of creatinine was increased in SHRs (Figure 7C), indicating a decrease in renal clearance function. Klotho gene delivery significantly decreased serum creatinine in SHRs, suggesting improvement of renal function.

Expression of GFP, LacZ, and mKL mRNA in Kidneys and Livers

GFP expression was found in livers and kidneys (Figure 8A and 8B), but not in aortas (photos not shown), in the
The overlap (merge) of GFP and 4',6-diamidino-2-phenylindole (DAPI) further disclosed that GFP expression was localized in the nuclei. The result indicated that the GFP reporter gene was still expressed in rats at 12 weeks after delivery of AAV.GFP. No GFP was found in livers or kidneys in the SHR-mKL groups (negative staining).

In addition, strong X-galactosidase staining was found in kidneys of AAV.LacZ-treated rats (Figure S8), indicating that the LacZ reporter gene expression was active at the time of animal sacrifice. These results suggest that AAV achieved a long-term expression of transgenes.

Mouse klotho mRNA was strongly expressed in the kidney and liver in AAV.mKL-treated rats at 12 weeks after gene delivery (Figure 8C and 8D), indicating successful delivery of the mouse klotho gene. Mouse klotho mRNA was not found in any other groups. Mouse klotho mRNA expression was not detectable in the aorta or mesenteric arteries of the SHR-mKL group (data not shown).

**Discussion**

The present study revealed that klotho expression and production were markedly suppressed in SHRs and were reversed by AAV.mKL. Notably, klotho gene delivery stopped further increases in BP in SHRs. To our knowledge, this is the first study showing that AAV delivery of the klotho gene prevented the progression of spontaneous hypertension. Thus, the suppression of the klotho gene in SHRs may play a role in the pathogenesis of the progression of spontaneous hypertension. It was reported that the level of the circulating klotho decreases with age, whereas the prevalence of hypertension increases with age in humans. Therefore, it will be interesting to evaluate whether klotho is involved in the progression of human essential hypertension. It is noted, however, that klotho gene delivery did not decrease the BP of SHRs to the control level. A separate study is required to determine whether klotho is involved in the initiation of spontaneous hypertension by klotho gene delivery before the elevation of BP.

It is notable that a single dose of AAV.mKL controlled hypertension for ≥12 weeks (length of the study). The prolonged antihypertensive effect of AAV.mKL was probably attributed to the long-lasting AAV vector. Indeed, AAV.mKL was still expressed at 12 weeks after gene delivery, as evidenced by the strong expression of mouse klotho mRNA in the kidneys of SHRs. Consequently, klotho gene delivery resulted in prolonged upregulation of klotho protein expression. In addition, AAV.GFP expression was found in the nuclei, suggesting that the transgene was expressed in the cells. It was reported that AAV-based transgene can integrate into the host genome. AAV has an advantage over other viral vectors, because it produces no or minimal inflammatory and immune responses in vivo. Indeed, no signs of inflammation were seen during autopsy, and no viral effect was observed in the data analysis. AAV also exhibited low vector toxicity both in animal experiments and clinical trials. Thus, AAV delivery of klotho may offer a new approach for long-term control of hypertension.

NADPH oxidases play a critical role in the hemodynamic responses to angiotensin II and in the pathogenesis of hypertension. Vascular and renal superoxide production was increased in SHRs. It is well established that an increase in the reactive oxygen species level is involved in the
pathogenesis of hypertension.\textsuperscript{21,29–35} Klotho gene delivery decreased vascular and renal superoxide production in SHRs, which may contribute to the mechanism of the antihypertensive effect of klotho. A decrease in superoxide production may be attributed to the suppression of Nox2 by klotho gene delivery, which did not affect other isoforms of NADPH oxidases or endothelial NO synthase. The NADPH oxidase is the primary source of reactive oxygen species in the vasculature,\textsuperscript{36} and it can act as a mediator of vascular injury and inflammation in many cardiovascular diseases.\textsuperscript{30,32,33} The suppressing effect of AAV.mKL on Nox2 and superoxide production was not attributed to a drop in BP, because AAV.mKL did not decrease BP compared with the pretreatment level. It is interesting that mouse klotho did not express in vasculatures, but klotho gene delivery suppressed vascular Nox2 and NADPH oxidase activity, supporting the notion that klotho functions as a hormone.\textsuperscript{1} Indeed, klotho gene delivery increased the level of the circulating klotho, providing a basis for the direct action of klotho in the vascular system that does not express klotho.\textsuperscript{1} Although the klotho receptor is unknown,\textsuperscript{1} our most recent study in the cell culture indicated that the selective inhibition of Nox2 expression by
klotho in rat aorta smooth muscle cells may be mediated by the cAMP-protein kinase A pathway.37

Mouse klotho was expressed in kidneys and livers in SHRs after gene delivery. In kidneys, klotho protein expression was localized in the tubule epithelial cells. Only the transmembrane form of the klotho protein was found in the kidneys, whereas both transmembrane (130 kDa) and secreted (65 kDa) forms of klotho were identified in the circulation. Klotho gene delivery increased klotho protein levels in both kidneys and the circulation. The major sources of circulating klotho protein are alternative RNA splicing (klotho gene directly generates secreted form of klotho, which is liberated into the circulation) and proteolytic cleavage (transmembrane form of klotho protein is cleaved by enzymes and released into the circulation).1 The circulating klotho is important in cardiovascular regulation because it has direct access to the vascular endothelial and smooth muscle cells.

This study demonstrated, for the first time, that klotho gene delivery may upregulate anti-inflammatory cytokine IL-10 in SHRs, although the underlying mechanism remains to be discovered. The increased level of circulating IL-10 may contribute to the antihypertensive effect of klotho, because an increase in IL-10 expression attenuated pulmonary and systemic hypertension.38,39 It was reported that IL-10 inhibits vascular smooth muscle cell proliferation, macrophage activation, T-cell proliferation, and inflammation38,40–43 that play important roles in the pathogenesis of hypertension and end-organ damage.44 Importantly, klotho gene delivery abolished tubular atrophy and dilation and tubular deposition of proteinaceous material in SHRs, the signs of the end-stage kidneys. The tubular deposition of proteinaceous material reflects impaired renal function (unable to reabsorb small proteins). It is even more interesting that klotho gene delivery attenuated glomerular collapse and interstitial collagen staining in SHRs, which could eventually result in a loss of glomerular filtration. These findings are unexpected, because the implication of klotho in kidney protection has never been reported. These results suggest that AAV delivery of klotho may offer a new approach for prolonged and effective renoprotection.

Hypertension could contribute to the worsening of kidney function and accelerate kidney damage.45 However, the renoprotective effect of AAV.mKL cannot be fully explained by its antihypertensive effect, because klotho gene delivery did not decrease BP compared with the pretreatment level. Therefore, the renoprotective effect of AAV.mKL may be partially attributed to the direct effect of the increased klotho expression in the kidneys. The kidney is a target organ for klotho action, as well as a major site of klotho production.5 SHRs would undergo vascular changes and develop cortical ischemia that are accompanied by glomerular lesions and tubular changes.12,16 The present data suggest that klotho deficiency may contribute to renal damage in SHRs and that klotho gene delivery could provide renoprotection. The renoprotective effect of klotho may be attributed, at least in part, to its suppressing effect on Nox2 and superoxide production, although the precise mechanism remains to be found.

Perspectives
This is the first study showing that AAV delivery of the klotho gene prevented the progression of spontaneous hypertension and renal damage in SHRs. A single dose of AAV.mKL controlled hypertension and renal damage for ≤12 weeks. This finding may offer a new and effective approach for long-term control of hypertension and renal damage. The antihypertensive and renoprotective effects of klotho may be attributed, at least in part, to the downregulation of Nox2 expression and superoxide production and the upregulation of IL-10. The present finding also suggests that the suppressed klotho expression may be involved in the progression of spontaneous hypertension and renal damage in SHRs. Additional studies are required to determine the receptors that mediate the effects of klotho.
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Disclosures
None.

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Online Supplemental Methods and Data

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Online Supplemental Methods and Data

1. Supplemental Materials and Methods

Construction of Recombinant Adeno-Associated Virus with Klotho Gene

A plasmid pEFmKLcFT (pEF1/Myc-His expression vector with into full-length mouse klotho cDNA and C-terminal Flag tag) was kindly provided by Dr. M. Kuro-o (Univ. Texas Southwestern Medical Center). The full length klotho cDNA was cloned into AAV serotype-2 (AAV2) (Startagen, La Jolla, CA, USA) (Supplementary Figure S1). LacZ and GFP reporter gene were cloned into AAV2 vector as the control constructs. The constructs of AAV vector pAAV-mKL, pAAV-LacZ, and pAAV-GFP were then packaged with pHelper and pAAV.RC to produce recombinant viral vectors as described previously. The results indicated that the constructs were packaged into AAV2 and that AAV.mKL expressed in AAV/293 cells and rat aortic smooth muscle cells (Supplemental Figures S2 & S3). Virus purification and titration were performed according to the instruction manual of AAV Helper-Free System (Stratagen, La Jolla, CA, USA).

Cell Culture and Transfection with Plasmid DNA

Rat aorta smooth muscle (RASM) cells (ATCC, Manassas, VA, USA) were cultured in DMEM (Cell Signaling, Danvers, MA, USA) supplemented with 10% fetal bovine serum (ATCC), 100ug/ml of streptomycin (Sigma-Aldrich, Atlanta, GA, USA) and 100U/ml of penicillin (Sigma-Aldrich, Atlanta, GA, USA) at 37°C, 5% CO₂. RASM cells cultured in 6-cm dishes were transfected with 8µg/well of pAAV-mKL or pAAV-lacZ using Optifect™ Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol, followed by 48h of incubation in DMEM medium at 37°C in a CO₂ incubator.

Western Blot Analysis

Western blot was performed as described previously. Briefly, cells and 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 a protease inhibitor and centrifuged for 5 min at 10,000 g. The supernatants was collected and immediately mixed with an equal volume of electrophoresis loading buffer for western blot analysis of klotho, Nox1, Nox2, Nox4, or eNOS protein expression. The equal amount of protein was loaded in a 4-20% gradient SDS–PAGE gel, the protein was transferred onto nitrocellulose filters after separated in the gel. Blots were blocked in 2% BSA in TBST for 1 hour, the membranes were incubated overnight (4°C) with antibodies against klotho (dilution 1:300, R&D Systems, Inc. Minneapolis, MN, USA), NOX1, NOX4 (dilution 1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), NOX2, eNOS (dilution 1:1000, BD Transduction Laboratories Inc., Mississauga, ON, Canada), and β-actin (dilution 1:10000, Abcam Inc., Cambridge, MA, USA)
or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as internal controls. The membranes were incubated with HRP conjugated secondary anti-goat, anti-mouse or anti-rabbit antibodies (dilution 1:2000-1:5000), respectively, for 1 hour 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 protein band intensities were quantified as described previously.\(^1\)\(^\text{-}^4\) Protein expression was normalized with the expression of β-actin or GAPDH.

**Identification of Mouse Klotho mRNA Expression by RT-PCR**

RT-PCR was performed as described in our previous study.\(^4\)\(^,\)\(^5\) Briefly, total RNA of cells or tissues was extracted using a Trizol Reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The concentration of total RNA was determined by optical density reading at 260 and 280nm and verified by ethidium bromide staining. The RNA samples selected showed 2 clear bands of 18 S and 28 S corresponding to ribosomal RNAs for further RT-PCR analysis. Two μg of total RNA were mixed with OligoT20 (Invitrogen, Carlsbad, CA, USA) in the presence of 10ul dNTP for 1h at 50°C. Three μl of the complementary DNA (cDNA) obtained from RT were amplified by PCR (Invitrogen, 2.5XPCR mix) in a thermal cycler (Eppendorf, Hamburg, Germany). The specific primers for mouse klotho (Accession number: NM_013823) 5'-GGGTCACTGGGTCAATCT-3', and Anti-sense 5-GCAAAGTAGCCACAAGG-3') were used. The parameters used are as follows: denature at 94°C for 45 seconds, primer annealing at 55°C for 1min, and extension at 68°C for 1 min. Amplification was allowed to proceed for 30 cycles. PCR products were analyzed by 2% agarose gel electrophoresis stained with ethidium bromide. The PCR product of mouse klotho is 710bp. Rat β-actin (Accession number: NM_031144) primers were used as an internal control (sense 5’GAG GGAGTGGGCTGAC-3’, anti-sense 5’CTGGAAAGGTGGACAGT-3’). The PCR product for β-actin is 444bp.

**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. Four groups of SHRs (5 rats/group) and 1 group of age-matched WKY rats (5 rats/group) were used (all males, 12 weeks). All rats were housed individually in wire-mesh cages and were provided with Purina laboratory chow (No. 5001) and tap water *ad libitum* throughout the experiment. The animals were handled daily to minimize handling stress; the animals did not appear stressed during BP measurement. Resting systolic BP was measured at room temperature (RT) from the tail of each unanesthetized rat using the tail-cuff method.\(^5\)\(^,\)\(^7\) The tail-cuff procedure is a common method used by us\(^1\)\(^,\)\(^8\) and others to monitor BP.\(^9\)\(^,\)\(^10\) It has been confirmed by the intra-arterial cannulation that the noninvasive tail-cuff method is effective and reliable in monitoring systolic BP in rats.\(^11\)\(^,\)\(^12\) Blood pressure (BP) and body weight (BW) were measured 2 times in all animals before gene delivery. Following the control period, 4 groups of SHR received intravenous (IV) injection of AAV.mKL, AAV.LacZ, AAV.GFP, and phosphate buffer solution (PBS), respectively. The WKY group received PBS and served as a control. AAV.mKL (2×10^8 particles/rat, 0.5 ml), LacZ, AAV.GFP (2×10^8 particles/rat, 0.5 ml) and PBS (0.5 ml) were administered *via* the tail vein. Following gene delivery, BP and BW
were measured at least once a week throughout the experiment. Food intake, water intake, and urine output were measured using metabolic cages during weeks 5 and 8. The experiment lasted for 12 weeks after AAV delivery. At the end of week 12, all animals were perfused transcardially with heparinized saline under deep anesthesia with sodium pentobarbital (100 mg/kg, IP). Before perfusion, blood was collected for measuring plasma levels of klotho and IL-10. Serum creatinine was measured using a creatinine assay kit (BioAssay Systems, Hayward, CA) according to the manufacturer’s instruction. A small part of aorta and kidney was excised and processed for immediate determination of the in situ superoxide production. A part of aorta and kidneys were fixed in paraformaldehyde (4% PFA) overnight and embedded with OCT for immunohistochemical or histological analysis. 

Kidney collagen deposition was assayed using a HT15-trichrome Stain (Masson) kit (Sigma-Aldrich, St Louis, MO) according to the manufacturer’s instruction. The remaining tissues were snapped in liquid nitrogen for western blot and RT-PCR analysis.

**Immunohistochemical Analysis of Klotho Expression in Kidneys and Aortas and Histological Examination of Kidneys**

The immunohistochemical procedure was described in our previous studies. Briefly, following perfusion, kidneys and aortas were fixed overnight with 4% paraformaldehyde (PFA) (4°C) and then embedded with paraffin. Tissue sections (5 µm) were incubated with peroxidase-blocking solution (Dako North America, Inc. Carpinteria, CA, USA) for 5 min followed by protein blocker (Biocare Medical, Concord, CA, USA) for 15 min. The sections were then incubated with monoclonal Anti-mouse Klotho Antibody (dilution 1:50 R&D Systems, Inc., Minneapolis, MN, USA) for 1 hour followed by a donkey anti-goat secondary antibody (dilution 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 30 min. For histological examination, kidney tissue sections were counterstained with hematoxylin (HE). Images were visualized and digital photographs were taken using a Zeiss microscope.

**Identification of GFP Gene Expression**

Unfixed livers and kidneys were placed in OCT, frozen at −80°C, and cut at 20 µm. Sections were incubated in PBS in a humidified chamber (37°C) for 2 min, stained with the nuclear stain 4, 6-diamidin-2-phenylindol dichlorohydrate (DAPI, 3×10^-7 M) at 37°C for 5 min, and mounted on slides. GFP expression in liver, aorta, and kidneys were visualized with FITC filter using Leica TCS NT Confocal fluorescence microscopy to determine the localization of the transgene.

**Measurement of In Situ Superoxide Production**

The in situ superoxide production was measured in aortas and kidneys using the oxidation sensitive dye dihydroethidium (DHE, Sigma-Aldrich, Atlanta, GA, USA). Dihydroethidium enters the cells and is oxidized by O2¬ to yield ethidium bromide (EB) which binds to DNA to produce bright red fluorescence. EB emits red fluorescence (610 nm) when excited at 488 nm. Briefly, unfixed abdominal aortic rings or kidneys were embedded in OCT, frozen at −80°C, and cut at 10 µm 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. The preparations were counterstained with the nuclear stain 4,
6-diamidin-2-phenylindol dichlorohydrate (DAPI, 3×10^{-7} M, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 37°C for 5 min and mounted on slides. The images were captured with FITC filter using Leica TCS NT Confocal fluorescence microscopy, the average intensity was measured at 400 X magnification in three randomly chosen fields (15 cells each) from three independent experiments. EB and DAPI fluorescence were quantified using Image J.\textsuperscript{13,14}

Quantification of NADPH Oxidase Activity

NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activity in aortas was assessed by the lucigenin-enhanced chemiluminescence method.\textsuperscript{13,15,16} Briefly, the tissue was homogenated for quantification of NADPH oxidase activity. The homogenate was incubated with lucigenin in the dark for 15 min. To prevent auto-oxidation of lucigenin, a low concentration of lucigenin was used (5µmol/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 light emission was recorded and the basal value was stable, the homogenate was treated with 10 µM enzyme substrate NADPH (Sigma-Aldrich, Atlanta, GA, USA). The light emission was recorded for 4 min. Background counts (with lucigenin) were subtracted from each value. Lucigenin chemiluminescent counts were adjusted on the basis of tissue weight. Activity was expressed as relative light units (RLU)/mg tissue.

References


Supplementary Results

S1. Construction of Recombinant Adeno-Associated Virus with Klotho Gene. The plasmid pAAV-mKlotho was constructed by inserting the mouse klotho full-length cDNA into the EcoRI and Xba I sites of the pAAV-MCS expression vector, the restriction endonuclease digestion pattern and sequencing results showed that the klotho gene was inserted correctly into the AAV serotype-2 vector (data not shown).
S2. pAAV.GFP and pAAV.mKL Expressed in AAV/293 Cells.

(A) AAV/293 cells without transfection. (B) AAV/293 cells transfected with pAAV.GFP, pHelper and pAAV.RC showed GFP expression, indicating that GFP was packaged into AAV2. Arrows indicated 293 cells expressing AAV.GFP. (C) AAV/293 cells transfected with pAAV.mKL, pHelper and pAAV.RC showed viral plaques, indicating that mouse klotho was packaged into AAV2. Arrows indicated 293 cells expressing AAV.mKL. (D) The high magnification of (C). (40x).

A.

\[
\begin{array}{c}
\text{mKL 130KD} \\
\text{β-actin 42KD}
\end{array}
\]

RASM LacZ mKL

B.

\[
\begin{array}{c}
\text{mKL 710bp} \\
\text{β-actin 444bp}
\end{array}
\]

RASM LacZ mKL

S3. AAV.mKL Expressed in Rat Aorta Smooth Muscle (RASM) Cells. The expression of mouse klotho protein (A) and mRNA (B) in RASM cells (secondary cell line) indicated that AAV.mKL expressed in cell cultures.
S4. Food Intake (A), Water Intake (B), Urine Output (C) and Feces Output (D). Food intake, water intake, urine output and feces output were not statistically different between any two groups, suggesting that klotho gene delivery or viral vector did not affect animals’ intakes and outputs measured during weeks 5 and 8. Klotho gene delivery did not affect body weight gain (E). Data=Means±SE. N = 5 animals/group.

S5. Effect of Klotho Gene Delivery on Urine Levels of Klotho The urine level of klotho was measured using western blot. The urine level of klotho was decreased in the SHR-GFP, SHR-LacZ, and SHR-PBS groups during weeks 5 (A) and 8 (B). Klotho gene delivery increased the urine level of klotho of SHRs to that of the WKY group at both time points. Data=Means±SE. *p<0.05, **p<0.01 ***p<0.001 vs the SHR-PBS group. N = 5 animals/group.
**S6. AAV.mKL Did Not Affect eNOS, Nox1 and Nox4 in Kidneys** Klotho gene delivery did not affect expression of eNOS, Nox1, and Nox4 in kidneys.

**S7. Klotho Gene Delivery Decreased Urinary Output of Protein** Daily urinary output of protein was increased in SHR-GFP, SHR-LacZ, and SHR-PBS groups during weeks 5 (A) and 8 (B), suggesting an impaired renal function. Klotho gene delivery significantly decreased urinary output of protein, suggesting improved renal...
function. Data=Means±SE.  *p<0.05, **p<0.01 vs the SHR-PBS group.  N = 5 animals/group.

S8.  X-gal staining of LacZ in kidneys of rats treated with AAV.LacZ.  Strong X-gal staining of LacZ was found in kidneys of AAV.LacZ-treated rats, indicating that LacZ gene expression is still active at the time of animal sacrifice.  Arrows indicate X-gal staining.  X-gal staining was not found in PBS-treated rats (negative staining).  X-gal staining was processed as described in our previous study (5).

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