Activation of SIRT1 Attenuates Klotho Deficiency–Induced Arterial Stiffness and Hypertension by Enhancing AMP-Activated Protein Kinase Activity

Diansa Gao, Zhong Zuo, Jing Tian,* Quaisar Ali,* Yi Lin, Han Lei, Zhongjie Sun

Abstract—Arterial stiffness is an independent risk factor for stroke and myocardial infarction. This study was designed to investigate the role of SIRT1, an important deacetylase, and its relationship with Klotho, a kidney-derived aging-suppressor protein, in the pathogenesis of arterial stiffness and hypertension. We found that the serum level of Klotho was decreased by ≈45% in patients with arterial stiffness and hypertension. Interestingly, Klotho haplodeficiency caused arterial stiffening and hypertension, as evidenced by significant increases in pulse wave velocity and blood pressure in Klotho-haplodeficient (KL+/−) mice. Notably, the expression and activity of SIRT1 were decreased significantly in aortic endothelial and smooth muscle cells in KL+/− mice, suggesting that Klotho deficiency downregulates SIRT1. Treatment with SRT1720 (15 mg/kg/d, IP), a specific SIRT1 activator, abolished Klotho deficiency–induced arterial stiffness and hypertension in KL+/− mice. Klotho deficiency was associated with significant decreases in activities of AMP-activated protein kinase α (AMPKα) and endothelial NO synthase (eNOS) in aortas, which were abolished by SRT1720. Furthermore, Klotho deficiency upregulated NADPH oxidase activity and superoxide production, increased collagen expression, and enhanced elastin fragmentation in the media of aortas. These Klotho deficiency–associated changes were blocked by SRT1720. In conclusion, this study provides the first evidence that Klotho deficiency downregulates SIRT1 activity in arterial endothelial and smooth muscle cells. Pharmacological activation of SIRT1 may be an effective therapeutic strategy for arterial stiffness and hypertension. (Hypertension. 2016;68:1191-1199. DOI: 10.1161/HYPERTENSIONAHA.116.07709.)

Key Words: angiotensin II ■ arteries ■ blood pressure ■ elastin ■ hypertension

Arterial stiffness and hypertension are generally regarded as common aging-related disorders because the prevalences of these disorders increase with age.1,2 According to the Seventh Report of the Joint National Committee,3 more than two thirds of individuals aged >65 years experience hypertension. Arterial stiffening reflects gradual fragmentation and loss of elastin fibers and accumulation of stiffer collagen fibers in the media of large arteries. It occurs independent of atherosclerosis4 and is an independent predictor of cardiovascular outcomes.5 It has been reported that arterial stiffness predicts an increase in systolic blood pressure (BP) and incident hypertension,6 indicating a close relationship between large-artery stiffness and the development of hypertension.4 Our recent study revealed that haplodeficiency of the Klotho gene causes arterial stiffness before the elevation of BP.7 However, the underlying mechanism is not fully understood.

Of the human sirtuins, SIRT1 is the closest homologue to yeast silent information regulator 2 (Sir2) protein and functions as a NAD+-dependent protein and histone deacetylase. An accumulation of evidence has shown that SIRT1 has anti-inflammatory, antioxidant, and antiapoptotic effects in the endothelium, thus preventing endothelial senescence and dysfunction.8,9 Since the first report showing that SIRT1 activates endothelial NO synthase (eNOS),10 several studies using gene mutant mouse models have demonstrated that SIRT1 has arteroprotective effects.8–11 Angiotensin II–induced vascular remodeling and hypertension in mice were attenuated by overexpression of SIRT1.12 However, whether SIRT1 plays a role in the pathogenesis of arterial stiffening is not clear.

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that serves as an energy sensor in the regulation of cellular metabolism. AMPKα is a catalytic subunit of AMPK, and its activity is regulated via phosphorylation.13,14 Activation of AMPKα improves endothelial function by suppressing oxidative stress.15,16 Some reports have indicated that SIRT1 regulates AMPKα activity,17,18 whereas others have

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shown that AMPK can also function as a SIRT1 activator by increasing the NAD+/NADH ratio.\textsuperscript{17,19} eNOS is a major source of NO in the vascular system\textsuperscript{20,21} and plays a critical role in regulating NO bioavailability and endothelial function. AMPK may mediate eNOS activity by regulating phosphorylation of eNOS.\textsuperscript{22,23} Nevertheless, the regulation of SIRT1, AMPK\textalpha{}, and eNOS in the development of Klotho deficiency–induced aortic stiffening has not yet been assessed.

Klotho is an aging-suppressor gene that extends life span when overexpressed and shortens life span when disrupted.\textsuperscript{24,25} Klotho is predominately expressed in kidneys. In humans, the circulatory Klotho level declines with age.\textsuperscript{26} By age 70, the level of circulatory Klotho is ≈50% of what it was at age 40.\textsuperscript{26} In this study, we used a Klotho-haplodeficient mouse model for studying the underlying mechanism of arterial stiffness and hypertension. Specifically, we investigated the relationship of Klotho and SIRT1 in the pathogenesis of arterial stiffness and hypertension.

**Methods**

A detailed Methods section is available in the online-only Data Supplement.

**Clinical and Laboratory Data Collection**

We prospectively enrolled 14 essential hypertensive patients during their routine health screening. All patients were in a nontherapy situation (never having received any antihypertensive drug or had been under drug withdrawal for at least 1 week). Normotensive healthy control participants (systolic BP<130 and diastolic BP<80 mm Hg) were also prospectively recruited and matched with hypertensive patients according to age, sex, and body mass index. Clinical data collected by physical examination included age, sex, height, weight, history of hypertension, systolic BP, and diastolic BP. Total cholesterol, low-density lipoprotein, high-density lipoprotein, total triglyceride, fasting blood glucose, and serum creatinine were determined by standard procedures. All clinical data were obtained from the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). This study was approved by the Ethics Committee of The First Affiliated Hospital of Chongqing Medical University (Chongqing, China).

**Circulatory Klotho Measurement in Human Blood Samples**

Serum Klotho was measured using a commercial ELISA kit (Shanghai yuanye Bio-Technology Co, Ltd) by following the manufacturer’s protocol.

**Animal Study Protocols**

Briefly, 24 $KL^{+/−}$ mice and 24 wild-type (WT) mice (12 months old) in the 129Sv background were used. BP was measured biweekly at the age of 12 to 15 months by the tail-cuff method. At the age of 15 months, each group was divided into 2 subgroups. One subgroup received SRT1720 (15 mg/kg per day IP; Medchem Express HY-15145), whereas the other group received an equal dose of vehicle (normal saline) and served as a control. During treatment, BP was measured twice a week using the tail-cuff method, and pulse wave velocity (PWV) was measured once a week by Doppler. On the 19th day of treatment, direct BP was assessed by direct intra-arterial cannulation, and plasma was collected for measuring circulatory Klotho. After perfusion, the aorta was collected and separated into 3 parts. One part was saved at −80 °C for molecular assays, and the others were embedded in paraffin or optimal cutting temperature compound for histological and immunohistochemical analyses.

**Measurements of BP**

BP was monitored by the volume–pressure recording tail-cuff method as we described previously.\textsuperscript{7,23–29} At the end of the experiment, BP was assessed from the arcus aortae via direct cannulation of a carotid artery under anesthesia (1% isoflurane) before euthanasia.

**Measurement of PWV**

Brachial-ankle pulse wave velocity was obtained in hypertensive patients and normotensive control participants in the supine position using an automated device (BP-203RPE III BP Monitor; Omron Healthcare, Inc, Japan).

Aortic PWV was assessed in mice as described previously.\textsuperscript{7,27}

**Immunohistochemical Analysis and Histological Examination of Aortas**

The procedures were described in our previous studies.\textsuperscript{30–34} For details, refer to the online-only Data Supplement.

**Western Blots**

Standardized protocols were used as we described recently,\textsuperscript{35–42} with details available in the online-only Data Supplement.

**Measurement of In Situ Superoxide Production**

In situ superoxide production was measured in aortas using the oxidation-sensitive dye dihydroethidium.\textsuperscript{34,39,43} For details, see the online-only Data Supplement.

**Statistical Analyses**

BP was analyzed using 1-way ANOVA repeated over time. All other data were analyzed by 1-way ANOVA. The unpaired $t$ test was used for comparisons between 2 groups. The significance was set at a 95% confidence limit.

**Results**

**Serum Klotho Was Decreased in Patients With Arterial Stiffness and Hypertension**

Serum Klotho levels were decreased by $≈45\%$ in patients with essential hypertension relative to those of healthy participants (Figure 1A). The brachial-ankle pulse wave velocity was increased significantly in hypertensive patients (Figure 1B), which is associated with a significant elevation of BP compared with normal controls (Figure 1C and 1D). This result suggests that a decrease in serum Klotho was associated with a significant increase in BP and brachial-ankle pulse wave velocity in human. There were no significant differences in blood levels of total triglyceride, total cholesterol, low-density lipoprotein, high-density lipoprotein, glucose, and creatinine between hypertensive patients and healthy controls (Table S1 in the online-only Data Supplement).

**Klotho Deficiency Caused Arterial Stiffness and Spontaneous Hypertension, Which Were Associated With Decreased Expression and Activity of SIRT1 in Aorta**

PWV, an important and reliable measure of arterial stiffness, was increased significantly in $KL^{+/−}$ mice by the age of 12 to
15 months (Figure 2A). Systolic and diastolic BP were also elevated significantly in KL+/– mice compared with WT mice (Figure 2B; Figure S1). Interestingly, SIRT1 protein expression was decreased significantly in the aortas of KL+/– mice (Figure 2C). p53 is a known downstream target of SIRT1, and the ratio of acetyl-p53 to total p53 is an index of SIRT1 activity. This ratio was increased significantly in the aortas of KL+/– mice (Figure 2D), suggesting that Klotho deficiency suppresses SIRT1 activity. Western blot analysis confirmed a significant decrease in the plasma level (by ≈45%) of Klotho protein in KL+/– mice (Figure 2E). These results reveal, for the first time, that SIRT1 expression and activity are downregulated in aorta because of Klotho deficiency during the development of arterial stiffness and hypertension.

**Activation of SIRT1 by SRT1720 Attenuated Arterial Stiffening and Hypertension in KL+/– Mice Without Affecting Circulatory Klotho Levels**

We next assessed whether pharmacological activation of SIRT1 affects arterial stiffness and hypertension in KL+/– mice. Daily IP injection of SRT1720 (15 mg/kg), an activator of SIRT1, attenuated Klotho deficiency–induced elevation of PWV and BP to the control level within 1 week (Figure 3A and 3B; Figure S2A). SRT1720 did not affect PWV and BP significantly in WT mice, and direct BP measured by intra-arterial cannulation confirmed these results (Figure 3C; Figure S2B). On the contrary, body weights were not affected by treatment with SRT1720 (data not shown). The plasma Klotho level was not altered by SRT1720 treatment in either WT or KL+/– mice (Figure 3D), indicating that Klotho activity lies upstream of SIRT1. These results suggest that downregulation of SIRT1 is involved in Klotho deficiency–induced arterial stiffness and hypertension.

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Serum Klotho was decreased in patients with arterial stiffness and hypertension. A, Serum levels of Klotho. B, Brachial-ankle pulse wave velocity in hypertensive patients (HP) and normal controls (NC). C, Systolic blood pressure. D, Diastolic blood pressure in hypertensive patients and normal controls. n=13 to 14 participants/group. Data=means±SEM. *P<0.05, **P<0.01, ***P<0.001 vs NC.

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Klotho deficiency caused arterial stiffness and spontaneous hypertension, which were associated with decreased expression and activity of SIRT1 in aorta. A, Time course of pulse wave velocity and (B) systolic blood pressure in wild-type (WT) and KL+/– mice, n=10 to 12 mice/group. C, Representative Western blot bands and quantitative analysis of SIRT1 protein expression in aortas. D, Representative Western blot bands and quantitative analysis of Acetyl-p53 and Total-p53 in aortas. SIRT1 activity is expressed as the relative ratio of Acetyl-p53/Total-p53. E, Representative western blot bands and quantitative analysis of plasma Klotho. Total protein with Ponceau staining served as loading control. Protein expression was normalized to the loading control, and the relative expression calculated as the fold change relative to the experimental control (WT). n=6 animals/group for Western blot analysis. Data=means±SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs WT.
Activation of SIRT1 by SRT1720 Rescued the Downregulation of SIRT1 Activity and Protein Expression in the Aortas of KL+/– Mice

Treatment with SRT1720 attenuated the ratio of acetyl-p53 to total p53 in the aortas of KL+/– mice to the control level, indicating that SRT1720 rescues the Klotho deficiency–induced downregulation of SIRT1 activity (Figure 4A). Unexpectedly, SRT1720 also abolished downregulation of SIRT1 protein expression in the aortas of KL+/– mice (Figure 4B), whereas SRT1720 did not alter SIRT1 expression or activity in the aortas of WT mice. To gain a better understanding of the cell types that express SIRT1, we assessed the localization of SIRT1 in the aorta using immunohistochemistry. SIRT1 was abundantly and predominantly expressed in the nuclei of both endothelial cells (ECs) and smooth muscle cells (SMCs; Figure 4C). The results show that SRT1720 effectively activated SIRT1 by increasing the abundance of SIRT1 expression in both SMCs and ECs in the aortas of Klotho-deficient mice.

Activation of SIRT1 by SRT1720 Abolished Downregulation of AMPKα and eNOS Activities in the Aortas of KL+/– Mice

We assessed protein expression and activity of AMPKα and eNOS. Protein expression of phosphorylated AMPKα and phosphorylated AMPKα proteins were decreased significantly, whereas total protein expression of AMPKα and eNOS were not altered in the aortas of KL+/– mice (Figure 5A and 5B), suggesting that Klotho deficiency downregulates AMPKα and eNOS activities. Interestingly, treatment with SRT1720 abolished the downregulation of AMPKα and eNOS activity in KL+/– mice, suggesting that the Klotho deficiency–induced downregulation of AMPKα and eNOS activities is because of suppression of SIRT1. SRT1720 treatment did not affect the expression or activity of AMPKα or eNOS in WT mice (Figure 5A and 5B).

Immunohistochemical analysis showed that AMPKα and phosphorylated AMPKα proteins were abundantly expressed in both ECs and SMCs throughout the intima and tunica media (Figure 5B). Phosphorylated AMPKα was expressed in both the cytoplasm and nucleus in ECs and mainly in the cytoplasm in SMCs. Semi-quantitative analysis further confirmed that phosphorylated AMPKα expression was downregulated in both ECs and SMCs in KL+/– mice, and this effect was eliminated by SRT1720 (Figure 5B). By contrast, eNOS and phosphorylated eNOS were found only in ECs but not in SMCs (Figure 6B). Phosphorylated eNOS was decreased in the ECs of KL+/– mice, and this effect was abolished by SRT1720 (Figure 6B). These results suggest that activation of SIRT1 by SRT1720 rescues the Klotho deficiency–induced downregulation of AMPKα and eNOS activities in aortas.

Activation of SIRT1 by SRT1720 Attenuated In Situ Superoxide Levels in the Aortas of KL+/– Mice

Arterial stiffness is associated with increased generation of reactive oxygen species in the vasculature. \(^{45}\) To determine whether SIRT1 activation affects NADPH oxidase activity and superoxide levels, we assessed NADPH oxidase activity and in situ dihydroethidium staining in aortas. Superoxide production (red fluorescence, dihydroethidium staining; Figure S3A) and NADPH oxidase activity (Figure S3B) were increased in the aortas of KL+/– mice relative to WT mice. Interestingly, NADPH oxidase activity and vascular superoxide levels were significantly decreased in the aortas of KL+/– mice treated with SRT1720, suggesting that upregulation of NADPH oxidase activity and superoxide levels are likely because of suppression of SIRT1.

Figure 3. Activation of SIRT1 attenuated arterial stiffening and hypertension in KL−/− mice without affecting levels of circulatory Klotho. A, Pulse wave velocity and B) systolic blood pressure in KL−/− mice. C, Direct BP measured by intra-arterial cannulation under anesthesia. D, Representative Western blot bands and quantitative analysis of plasma Klotho. Protein expression was normalized to the loading control, and the relative expression calculated as the fold change relative to the experimental control (wild type [WT]+saline), n=10 to 12 animals/group. Data=means±SEM. *P<0.05, **P<0.01, ***P<0.001 vs WT+saline; †P<0.05, ††P<0.01, †††P<0.001 vs KL−/−+saline.
Activation of SIRT1 by SRT1720 Attenuated Klotho-Deficiency–Induced Collagen Deposition and Elastin Fragmentation in Aortas

Passive aortic stiffness largely depends on the matrix composition within the vascular medial layer. Masson trichrome staining showed that collagen deposition (blue) was increased in the medial layer of the aortas in KL+/– mice, and this effect was abolished by SRT1720 (Figure S4A). Elastin staining showed that the number of elastic fiber breaks was increased in the aortic walls of Klotho-deficient mice, and this was significantly attenuated by the activation of SIRT1 (Figure S4D).

Western blot analysis confirmed the enhanced expression of collagen I in the aortas of KL+/– mice, which was eliminated by treatment with SRT1720 (Figure S4C). Conversely, the elastin level was significantly decreased in the aortas of Klotho-deficient mice, and this level was restored by activation of SIRT1 (Figure S4D).

Discussion

Aortic stiffness is an independent risk factor for hypertension, stroke, and myocardial infarction. However, the cause and pathogenesis of aortic stiffness remains largely unknown. Klotho was originally discovered as an aging-suppressor gene, and mutation of the Klotho gene results in multiple premature aging phenotypes and shortened life span. In this study, we provided the first evidence that aortic stiffness and hypertension are associated with a significant decrease in circulatory Klotho protein levels in humans (Figure 1). We further demonstrated in an animal model that Klotho deficiency is likely to be an important factor that contributes to the pathogenesis of arterial stiffening and hypertension, because haplodeficiency of Klotho (KL+/–) induces significant increases in PWV and BP (Figure 2). This finding is significant because it points to a new direction for understanding the pathogenesis of arterial stiffening and hypertension. A recent Framingham study showed that large-artery stiffness...
precedes the development of hypertension, suggesting that arterial stiffening may be the cause of hypertension. Several longitudinal studies have demonstrated that arterial stiffness predicts an increase in systolic blood pressure and incident hypertension.\(^48,49\)

Arterial stiffness is one of the earliest detectable manifestations of adverse structural and functional changes within the vessel wall, which eventually leads to stroke and ischemic heart disease.\(^50\) The prevalence of arterial stiffness and hypertension continues to increase, especially in the aged population.\(^1,2\) Unfortunately, there is no cure for the disease, because of its unknown cause. The currently available antihypertensive drugs were mainly designed to reduce peripheral resistance and are not adequate to alter the pathological processes of vascular remodeling or stiffening. Our study suggests that Klotho may be an important etiologic factor and a potential interventional target for arterial remodeling and stiffness. Our findings also provide a strong rationale for further investigating whether Klotho supplements can attenuate arterial stiffening and associated cardiovascular mortality.

The Klotho gene is primarily expressed in the distal tubular epithelial cells of the kidneys and the choroid plexus of the brain.\(^25,47\) One of the interesting findings of this study is that a Klotho gene haplodeficiency (\(KL^{+/–}\)) suppresses SIRT1 expression and activity in aortas (Figure 4). This result is interesting, because, although Klotho is not expressed in the vasculature,\(^25,47\) Klotho deficiency downregulated vascular SIRT1 expression and activity. This finding supports the notion that Klotho functions as a hormone.\(^30,47\) Indeed, the circulating Klotho level was decreased by \(\approx50\%\) in \(KL^{+/–}\) mice (Figure 2), which was not affected by activation of SIRT1 (Figure 3). Therefore, Klotho likely serves as an upstream

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**Figure 5.** Activation of SIRT1 by SRT1720 enhanced AMP-activated protein kinase \(\alpha\) (AMPK\(\alpha\)) activity in aortas. A, Representative Western blot bands and quantitative analysis of phosphorylated AMPK\(\alpha\) (p-AMPK\(\alpha\)) and AMPK\(\alpha\) in aortas. Protein expression was normalized to \(\beta\)-actin, and the relative expression was calculated as the fold change relative to the control (wild type [WT]+saline). B, Representative photomicrographs of immunohistochemical analysis of p-AMPK\(\alpha\) and AMPK\(\alpha\) in aortic sections. Brown staining indicates positive staining, arrows indicate positively stained endothelial cells, and arrowheads indicate positively stained smooth muscle cells. Semi-quantitative analysis of p-AMPK\(\alpha\)-positive and AMPK\(\alpha\)-positive staining in the intima and media of aortas (data were calculated as fold changes of the WT-saline group). \(n=6\) animals/group. Data=means±SEM. \(*P<0.05, **P<0.01\) vs WT+saline; \(+P<0.05, ++P<0.01\) vs KL\(^{+/–}\)+saline.

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\(A\)
regulator of vascular SIRT1 in the vasculature. The circulatory Klotho level is important in the regulation of cardiovascular function because it has direct access to vascular endothelial cells and SMCs. It is noteworthy that the Klotho deficiency–induced decrease in SIRT1 activity is proportional to that of SIRT1 protein expression in aortas (Figure 4), suggesting that Klotho regulates SIRT1 activity partially by regulating its protein expression. The limitation of this in vivo study is that it does not address how Klotho regulates SIRT1 protein expression. On the contrary, the Klotho protein entails the property of glycosylation, and this moiety may functionally interact with SIRT1 and regulate its activity. This hypothesis, however, needs to be validated. Nevertheless, this study reveals, for the first time, that Klotho regulates SIRT1 expression and activity.

Klotho deficiency led to a decreased elastin/collagen ratio, which is apparently because of an enhanced degradation of elastin and increased accumulation of collagen (Figure 4), which is stiffer than elastin, and this structural remodeling contributes to the pathogenesis of arterial stiffening. One of the major findings of this study is that activation of SIRT1 by SRT1720 reverses Klotho deficiency–induced elevation of PWV and BP (Figure 3A and 3B), suggesting for the first time that downregulation of SIRT1 activity mediates Klotho deficiency–induced arterial stiffening and hypertension. The beneficial effects of SRT1720 are manifested by a largely attenuated arterial collagen deposition and elastin degradation (Figure 4).

It is noteworthy that pharmacological activation of SIRT1 decreased arterial stiffening and hypertension within 1 week of treatment (Figure 3), which is sooner than we expected. The early and quick drop in PWV and BP may be partially attributed to the relaxation of blood vessels. Indeed, diastolic...
BP, which largely reflects vascular resistance, was effectively attenuated by SRT1720. This result suggests that there may also be a functional component of arterial stiffness, that is, increased vascular tension in $K\ell^{+/−}$ mice, which may be attributed to decreased AMPKα and eNOS activities (Figures 5 and 6). Consistent with this interpretation, the Klotho deficiency–induced downregulation of AMPKα and eNOS activities in ECs were largely rescued by treatment with SRT1720. Therefore, this study demonstrates that activation of SIRT1 functionally interacts with AMPKα and upregulates its activity (phosphorylation), which, in turn, activates eNOS.$^\text{2,23}$ leading to vasorelaxation (Figure S5). By contrast, the structural recovery of blood vessels is a relatively slow process, although it contributes to attenuation of arterial stiffening and hypertension by SRT1720. It should be mentioned that although activation of SIRT1 increases AMPKα and eNOS activity and decreases NADPH oxidase activity, we cannot exclude the possibility that changes in the downstream factors of SIRT1 may also be partially secondary to reduction of hypertension.

The recovery of structural remodeling by SRT1720 may be partially attributed to the attenuation of superoxide upregulation in the aortas of $K\ell^{+/−}$ mice (Figures S3 and S4) because increased reactive oxygen species and oxidative stress contribute to vascular remodeling. It is noteworthy that NADPH oxidase activity and superoxide levels were increased in aortas because of Klotho deficiency ($K\ell^{−/−}$; Figure S3). Interestingly, SIRT1 may be involved in Klotho deficiency–induced upregulation of NADPH oxidase activity and superoxide generation in aortas, which were both blocked by SIRT1 activation. The effects of SIRT1 activation on NADPH oxidase activity may be mediated by AMPKα (Figure S5) because AMPKα regulates NADPH oxidase activity.$^\text{15,16}$ Knockout of AMPKα upregulates NADPH oxidase activity, leading to endothelial dysfunction.$^\text{15,16}$

We used $K\ell^{+/−}$ mice that mimics a half Klotho reduction in hypertensive patients (Figure 1). $K\ell$ homozygous ($K\ell^{−/−}$) mice develop extensive aging phenotypes and die before the age of 8 weeks.$^\text{6}$ $K\ell$ homozygous mice also demonstrate severe hyperphosphatemia, emphysema, and soft tissue calcification.$^\text{25,47}$ As a result, $K\ell$ homozygous mice were not used for this study.

**Perspectives**

To our knowledge, this is the first study demonstrating that Klotho deficiency downregulates SIRT1 expression and activity in aortic endothelial and SMCs, which may mediate Klotho deficiency–induced arterial stiffness and hypertension. This finding is significant because it points to a new direction for understanding the pathogenesis of arterial stiffening. Supplementation antiaging Klotho protein or pharmacological activation of SIRT1 may be effective therapeutic approaches for arterial remodeling and hypertension.

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**Disclosures**

None.

**References**


Novelty and Significance

What Is New?

- It is new and interesting that haplodeficiency of Klotho gene downregulates SIRT1 activity in vascular endothelial and smooth muscle cells.
- This study demonstrates, for the first time, that activation of SIRT1 attenuates Klotho deficiency–induced arterial stiffness and hypertension.

What Is Relevant?

- It is significant that a decrease in plasma Klotho is associated with arterial stiffness and hypertension.
- This study demonstrates, for the first time, that activation of SIRT1 attenuates Klotho deficiency–induced arterial stiffness and hypertension.

Summary

This study provides the first evidence that Klotho deficiency downregulates SIRT1 activity in arterial endothelial and smooth muscle cells. Pharmacological activation of SIRT1 may be an effective therapeutic strategy for arterial stiffness and hypertension.
Activation of SIRT1 Attenuates Klotho Deficiency–Induced Arterial Stiffness and Hypertension by Enhancing AMP-Activated Protein Kinase Activity
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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.\textsuperscript{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.\textsuperscript{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\textsuperscript{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°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\textsuperscript{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.\textsuperscript{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. 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°C) 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, 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°C. 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°C) with antibodies against SIRT1 (diluted 1:500, Abcam Inc., Cambridge, 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., Cambridge, 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°C) 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. Briefly, unfixed thoracic aortic rings were embedded in optimal cutting temperature (OCT) buffer, frozen at −80°C, 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 was determined by measuring three continuous sections at 10X magnification and then normalizing by the total area using Image J software.

**Quantification of NADPH oxidase activity**

NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activity in aortas was measured by the lucigenin-enhanced chemiluminescence method. 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.
References


Online Supplemental Data

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NC</th>
<th>HP</th>
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<tbody>
<tr>
<td>Number(Male/Female)</td>
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<td>14(11/3)</td>
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<tr>
<td>Age(years)</td>
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<td>49.36 ± 2.41</td>
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<tr>
<td>History of hypertension(years)</td>
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<td>3.43 ± 1.17</td>
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<tr>
<td>TC(mmol/L)</td>
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<td>5.47 ± 0.86</td>
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<tr>
<td>LDL(mmol/L)</td>
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<tr>
<td>HDL(mmol/L)</td>
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<tr>
<td>TG(mmol/L)</td>
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<td>2.037 ± 0.45</td>
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<tr>
<td>Glucose(mmol/L)</td>
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<tr>
<td>Creatinine(μmol/L)</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>25.17 ± 0.80</td>
<td>25.91 ± 0.60</td>
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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 µm²). 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.