Antiaging Gene Klotho Deficiency Promoted High-Fat Diet–Induced Arterial Stiffening via Inactivation of AMP-Activated Protein Kinase

Yi Lin, Jianglei Chen, Zhongjie Sun

Abstract—Klotho was originally discovered as an aging-suppressor gene. The objective of this study is to investigate whether klotho gene deficiency affects high-fat diet (HFD)–induced arterial stiffening. Heterozygous Klotho-deficient (KL+/−) mice and WT littersmates were fed on HFD or normal diet. HFD increased pulse wave velocity within 5 weeks in KL+/− mice but not in wild-type mice, indicating that klotho deficiency accelerates and exacerbates HFD-induced arterial stiffening. A greater increase in blood pressure was found in KL+/− mice fed on HFD. Protein expressions of phosphorylated AMP-activated protein kinase-α (AMPKα), phosphorylated endothelial nitric oxide synthase (eNOS), and manganese-dependent superoxide dismutase (Mn-SOD) were decreased, whereas protein expressions of collagen I, transforming growth factor-β1, and Runx2 were increased in aortas of KL+/− mice fed on HFD. Interestingly, daily injections of an AMPKα activator, 5-aminoimidazole-4-carboxamide-3-ribonucleoside (5-ami), abolished the increases in pulse wave velocity, blood pressure, and blood glucose in KL+/− mice fed on HFD. Treatment with 5-aminoimidazole-4-carboxamide-3-ribonucleoside for 2 weeks not only abolished the downregulation of phosphorylated AMPKα, phosphorylated eNOS, and Mn-SOD levels but also attenuated the increased levels of collagen I, transforming growth factor-β1, Runx2, superoxide, elastic lamellae breaks, and calcification in aortas of KL+/− mice fed on HFD. In cultured mouse aortic smooth muscle cells, cholesterol plus KL-deficient serum decreased phosphorylation levels of AMPKα and LKB1 (an important upstream regulator of AMPKα activity) but increased collagen I synthesis, which can be eliminated by activation of AMPKα by 5-aminoimidazole-4-carboxamide-3-ribonucleoside. In conclusions, Klotho deficiency promoted HFD-induced arterial stiffening and hypertension via downregulation of AMPKα activity. (Hypertension. 2016;67:564-573. DOI: 10.1161/HYPERTENSIONAHA.115.06825.)

Online Data Supplement

Key Words: AMP-activated protein kinases ■ cholesterol ■ collagen ■ diet, high-fat ■ elastin ■ metabolic syndrome X ■ vascular stiffness

Arterial stiffening is one of the aging-related disorders, which can be accelerated by metabolic syndromes, diabetes mellitus, and arteriosclerosis. Arterial stiffening leads to isolated systolic hypertension. Pulse wave velocity (PWV) is a noninvasive measure of arterial stiffening. Abundant epidemiological studies have demonstrated that arterial stiffening is an independent predictor of cardiovascular outcomes such as myocardial infarction, cognitive decline in aging, stroke, and chronic kidney diseases. Two longitudinal studies indicated that arterial stiffness predicts an increase in systolic blood pressure and incident hypertension.

Klotho (KL) gene was originally identified as a putative antiaging gene in mice. The Klotho gene was named after the purported Greek goddess Klotho who spins the thread of life. KL homozygous deficient mice carry a disruption in the promoter region of the KL gene, leading to extensive premature aging phenotypes including severe hyperphosphatemia, ectopic soft tissue calcification, and early death (<10 weeks). Overexpression of KL, however, extended life span in mice. The mouse full-length KL gene encodes a single-pass transmembrane domain of 1014 amino acids (130 kDa). The short-form KL (76 kDa) can be generated by alternative RNA splicing or proteolytic cleavage. KL protein is predominantly expressed in the kidney and slightly expressed in parathyroid glands and brain choroid plexus. Both human and mouse KL gene are alternatively spliced after exon 3, which encodes the KL repeat of Klotho, called secreted Klotho (sKL). Soluble KL might include the truncated extracellular domain (KL1 and KL2), the KL1 fragment, and the KL2 fragment. sKL and soluble KL circulate in the blood and regulates function in organs and cells (eg, vascular cells) that do not express KL. KL plays important roles in a variety of physiological and pathological processes including modulation of Wnt...
signal transduction, antioxidation, and renal ion channels and transporters.\textsuperscript{11,15,16} 

Aging and aging-related medical complications (metabolic syndrome, hypertension, diabetes mellitus, and chronic kidney disease) are associated with a decreased ratio of elastin/collagen (arterial remodeling) and arterial calcification (elastocalcinosis), which contribute significantly to arterial stiffening.\textsuperscript{17-20} Transforming growth factor \( \beta_1 \) (TGF-\( \beta_1 \)) via binding to its receptors induces a variety of gene expressions in vascular smooth muscle cells that may result in collagen deposition and calcification.\textsuperscript{21,22} The prevalence of arterial stiffening is increased with age, whereas angiogenesis gene klotho levels are decreased with age.\textsuperscript{1} However, whether KL deficiency plays a role in the development of arterial stiffening is not fully understood.

Adenosine monophosphate–activated protein kinase (AMPK) has been shown to be essential in regulating vascular homeostasis. AMPK\( \alpha \) is a serine/threonine kinase that regulates cellular energy homeostasis through its enzymatic activity stimulated by phosphorylation of threonine-172 in the catalytic \( \alpha \) subunit.\textsuperscript{23,24} The phosphorylation status at threonine-172 is often used as an indicator of the activation state of AMPK\( \alpha \).\textsuperscript{25} In addition, the activation of AMPK\( \alpha \) through 5-aminoimidazole-4-carboxamide-3-ribonucleoside (AICAR), an adenosine mimetic, was shown to decrease mean arterial pressure and vascular tone in hypertensive rats.\textsuperscript{26} AMPK\( \alpha \) inactivation has been found in a model of calcification in rat aortic smooth muscle cells, and metformin has been shown to inhibit calcification via the activation of the AMPK\( \alpha \) pathway.\textsuperscript{27} However, whether AMPK\( \alpha \) is involved in the pathogenesis of arterial stiffening is not clear. The purpose of the study is to investigate whether klotho deficiency plays a role in the pathogenesis of arterial stiffening in animals fed on high-fat diet (HFD). We found that KL deficiency plus HFD accelerated and exacerbated arterial stiffening, which was associated with AMPK\( \alpha \) inactivation in the arteries. In addition, we further assessed the effect of activation of AMPK\( \alpha \) by AICAR on HFD-induced arterial stiffening in KL\( ^{-/-} \) mice.

Methods

Animal Studies
Heterozygous KL\( ^{-/-} \) mutant mice with >9 generations in 129/Sv background were kindly provided Dr Kuro-o et al.\textsuperscript{28} Briefly, all mice were housed in cages at room temperatures (25±1°C) and were provided with laboratory chow (Cat 5053; Picolab) and tap water ad libitum. This project was approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center (details are available in the Methods in the online-only Data Supplement).

Statistical Analysis
Data were analyzed using a 1-way ANOVA. The Newman–Keuls procedure was used to assess differences between means. Data were expressed as mean±SEM. A \( P<0.05 \) were considered significant.

Results

KL Deficiency Accelerated and Exacerbated HFD-Induced Arterial Stiffening and Hypertension
To explore whether KL deficiency affects the development of arterial stiffening in animals with metabolic syndrome, KL heterozygous mutant mice and their wild-type (WT) littermates were fed on HFD (Figure 1A). HFD increased PWV as early as 5 weeks in KL\( ^{-/-} \)-deficient mice but not in WT mice (Figure 1B). These results suggested, for the first time, that klotho deficiency promotes HFD-induced arterial stiffening.

Interestingly, HFD induced a greater increase in systolic blood pressure (BP) by 5 weeks in KL-deficient mice than in WT mice fed on HFD (Figure 1C), indicating that KL deficiency exacerbates HFD-induced systolic hypertension. Systolic BP was elevated to a lesser extent in KL-deficient mice on normal diet (ND) and WT mice on HFD (Figure 1C). Similar changes were found in mean arterial BP (Figure S1A in the online-only Data Supplement). It is noticed, however, that diastolic BP was not elevated in KL\( ^{-/-} \) mice until 6 weeks after HFD (Figure S1B), suggesting that HFD-induced elevation of systolic BP and diastolic BP in KL\( ^{-/-} \) is isolated.

Hyperglycemia is an important feature of metabolic syndrome. HFD slightly increased blood glucose levels in both KL-deficient and WT mice (Figure 1D). Interestingly, KL-deficient mice with HFD developed an earlier increase in blood glucose levels than WT mice with HFD (Figure 1D). No significant changes in insulin sensitivity and glucose tolerance were found in these animals (Figure S1C and S1D). These data indicated that animals on HFD might be still in the early stage in the development of metabolic syndrome.

Obesity is another hallmark of metabolic syndrome. HFD tended to increase body weight in both genotypes, but no significance was found until the 11th week. KL-deficient mice fed on HFD started to demonstrate a significantly greater increase in body weight than in the WT-ND and the KL\( ^{-/-} \)-ND (Figure 1E).

KL Deficiency Exacerbated HFD-Induced Expression of Aortic Collagen I and TGF\( \beta_1 \) but Decreased Levels of Aortic Phosphorylated AMPK\( \alpha \), Phosphorylated eNOS, and Mn-SOD Levels in Mice Fed on HFD
During the sixth week of HFD, one third of the animals were euthanized for assessing the molecular changes associated with arterial stiffening. HFD increased collagen I and TGF\( \beta_1 \) expression in WT mice (Figure 2A). KL-deficient mice on ND had greater levels of TGF\( \beta_1 \) and collagen I than WT mice on ND (Figure 2A). Interestingly, KL deficiency further increased HFD-induced TGF\( \beta_1 \) and collagen I expression (Figure 2A). An increase in TGF\( \beta_1 \) and collagen I expression could contribute to arterial stiffening. Unexpectedly, there was no significant difference in tropoelastin levels among different animal groups (Figure 2A). Taken together, these results suggested that KL deficiency further promotes HFD-induced collagen synthesis and arterial stiffening.

Interestingly, KL-deficient mice on HFD displayed decreased phosphorylation levels of AMPK\( \alpha \) and eNOS (Figure 2B), suggesting that KL deficiency plus HFD down-regulates AMPK\( \alpha \) and eNOS activities. KL-deficient mice on HFD also demonstrated lower levels of manganese-dependent superoxide dismutase (Mn-SOD) (Figure 2B). We confirmed that KL\( ^{-/-} \)-deficient mice had about half of short-form KL protein present in plasma (Figure S1E and S1F).
No significant elastic fiber breaks or obvious calcification were found in cross sections of aortas at this stage of HFD feeding by 6 weeks of HFD feeding (Figure S2A–S2D). Therefore, arterial stiffening observed in KL-deficient animals on HFD preceded the noticeable elastin degradation and arterial calcification at this early stage.

**HFD Increased Plasma Total Cholesterol and Low-Density Lipoprotein-Cholesterol in Mice**

HFD increased total plasma cholesterol and low-density lipoprotein (LDL)-cholesterol in both WT and KL-deficient mice to a similar extent (Figure 2C and 2D), indicating that KL deficiency did not affect HFD-induced increases in plasma cholesterol. There was no significant change in plasma free fatty acids and triglycerides after 6-week HFD feeding in either WT or KL-deficient mice (Figure S2E and S2F).

Because hypercalcemia and hyperphosphotemia have been found in KL homozygous (−/−) mutant mice, we measured plasma levels of calcium and phosphorus in these animals. No significant changes in plasma calcium and phosphorus were found in these animals (Figure 2E and 2F), suggesting that arterial stiffening found in KL−/−-deficient mice fed on HFD might not be attributed to changes in plasma calcium and phosphorus.

Given that arterial stiffening might be accelerated by chronic kidney disease, we measured blood albumin and blood urea nitrogen levels. No significant differences in blood albumin and blood urea nitrogen levels were observed (Figure S2G and S2H), suggesting that arterial stiffening in KL-deficient mice on HFD might not be caused by kidney dysfunction.

**AICAR Alleviated Arterial Stiffening, Hypertension, and Blood Glucose Levels in KL-Deficient Mice Fed on HFD**

Because HFD decreased activation of AMPKα, we treated animals with AICAR, an analog of AMP that activates AMPK. Interestingly, AICAR treatment decreased PWV in KL-deficient mice on HFD to the control levels within 1 week (Figure 3A). These results indicated that the activation of AMPK is sufficient to eliminate arterial stiffening in KL-deficient animals on HFD. In addition, AICAR abolished the increases in systolic arterial BP in all treated groups (Figure 3B), suggesting that AMPK might be an
effective therapeutic target for hypertension. Similar results were observed for mean and diastolic arterial BP (Figure S3A and S3B). Furthermore, AICAR abolished the mild increase in blood glucose levels induce by HFD in both WT and KL-deficient mice (Figure 3C), suggesting that the activation of AMPK by AICAR might regulate glucose homeostasis in animals fed on HFD. Finally, AICAR also decreased body weight in KL-deficient mice fed on HFD (Figure 3D). Therefore, the activation of AMPKα improved not only arterial stiffening but also metabolic syndrome in KL-deficient mice.

**AICAR Slightly Decreased Blood Total Cholesterol and LDL-Cholesterol in KL-Deficient Mice Fed on HFD**

AICAR treatments slightly but significantly decreased blood total cholesterol and LDL-cholesterol in KL-deficient mice but not in WT mice fed on HFD (Figure S3C and S3D). These data suggested that KL-deficient mice might be more sensitive to AICAR in terms of downregulation of HFD-induced hypercholesterolemia, which may partially contributes to a decrease in arterial stiffening by AICAR in KL-deficient mice. Although KL homozygous mice (−/−) have hypercalcemia and hyperphosphotemia, serum levels of calcium and phosphorus were in normal range in KL heterozygous (+/−) mice (Figure S3E and S3F). No significant changes in serum calcium and phosphorus were observed after AICAR treatments (Figure S3E and S3F).

**AICAR Increased Phosphorylated AMPKα, Phosphorylated eNOS, and Mn-SOD Levels and Decreased Superoxide Production, Collagen I Levels, and TGFβ1 Expression in Aortas**

AICAR increased phosphorylation levels of AMPKα and eNOS in all treated groups although it did not alter the protein expression of AMPKα and eNOS (Figure 4A and 4B). These results suggested that AICAR treatments might decrease arterial stiffening and hypertension by activating AMPKα and eNOS. In addition, AICAR treatments also increased Mn-SOD in all 3 groups (Figure 4C). Interestingly, the urinary level of nitrite/nitrate (index of nitric oxide) was increased during weeks 1 and 2 of treatment with AICAR (Figure S4), suggesting that the activation of AMPKα enhances bioavailability of nitric oxide (NO).

Interestingly, KL deficiency plus HFD for 15 weeks significantly increased superoxide levels in aortas, which can be abolished by AICAR (Figure 5A and 5B). AICAR completely abolished Klotho deficiency- and HFD-induced upregulation of collagen I and TGFβ1 in aortas (Figure 6A–6C). These data suggested that the beneficial effects of AICAR on arterial
stiffening and hypertension may be mediated via the AMPKα-eNOS-Mn-SOD-superoxide-TGFβ1-collagen I pathway.

**AICAR Decreased Aortic Runx2 Levels and Abolished Arterial Calcification and Elastic Fiber Breaks in KL-Deficient Mice on HFD**

Arterial calcification and elastic fiber breaks have been considered as important factors contributing to the pathogenesis of arterial stiffening.28,29 AICAR significantly decreased protein expression of Runx2 (a marker of osteoblasts) but not of tropoelastin in aortas in KL-deficient mice fed on HFD (Figure 6A, 6D, and 6E). Interestingly, KL-deficient mice fed on HFD for 15 weeks developed arterial calcification and more arterial elastic fiber breaks, which can be abolished by AICAR (Figure S5A–S5D). Therefore, these results demonstrated that HFD-induced calcification and elastic fiber fragmentation in arteries of KL-deficient mice may be mediated by downregulation of AMPKα. Furthermore, the activation of AMPKα by AICAR might alleviate arterial calcification via decreasing arterial Runx2, a key transcription factor in the regulation of bone formation.

**KL Protein Deficiency Plus Cholesterol Inactivate AMPKα via Downregulation of LKB1 Activity**

To further study the molecular mechanisms of direct regulation of collagen I protein expression by KL and to avoid in vivo complications, we used a cell line of mouse vascular smooth muscle cells (MOVAS). Interestingly, we found that water-soluble cholesterol dose dependently increased precursor of collagen I in MOVAS (Figure S6A and S6B). Neither full-length KL nor short-form KL was detected in MOVAS (Figure S7A). KL gene was not detected in MOVAS (Figure S7B). Because fetal bovine serum used in cell culture contained both full-length and short-form KL, we removed ≈50% of KL in both forms from fetal bovine serum using an IP purification kit (Figure S7C–S7E). Interestingly, cholesterol plus KL deficiency indeed decreased phosphorylation of AMPKα (Figure S6C–S6E), whereas AICAR treatments increased phosphorylated AMPKα (pAMPKα) levels (Figure S6C–S6E). As shown in Figure S6F and S6G, KL-deficient fetal bovine serum potentiated the cholesterol-induced increase in collagen I. AICAR treatments abolished the increase in collagen I induced by cholesterol alone or KL-deficient fetal bovine serum plus cholesterol in MOVAS (Figure S6F and S6G). Interestingly, cholesterol plus KL deficiency also attenuated phosphorylation level of LKB1 and protein level of LKB1 in MOVAS (Figure S6H–S6K). Cholesterol plus KL deficiency did not affect calcium/calmodulin-dependent protein kinase α (CaMKKα) and CaMKKβ levels in MOVAS (Figure S7F–S7I). LKB1 and CaMKKβ are considered key activators of AMPKα in various mammalian cells.30 Taken together, these results indicated that cholesterol plus KL deficiency might inactivate AMPKα likely via decreasing LKB1 activity, resulting in collagen I accumulation in aortic smooth muscle cells.

**Discussion**

Aging is associated with a decline in klotho levels and an increase in prevalence of arterial stiffening and hypertension.1 At the age of 70 years, the serum level of klotho is about one half of what it was at 40 years.11,31 This study demonstrated, for the first time, that one half deficiency of klotho, an aging-suppressor gene, accelerated and exacerbated HFD-induced arterial stiffening and hypertension (Figure 1B and 1C). This finding advances the current understanding of aging-related arterial stiffness and hypertension.1 An increase in PWV and elevation of systolic BP occur at approximately the same time.
in KL(+/−) mice fed on HFD (eg, 5 weeks). Therefore, arterial stiffening may not be because of elevation of BP because hypertension-associated arterial remodeling is a slow and chronic process. By contrast, arterial stiffening would lead to an immediate elevation of systolic BP. Indeed, systolic BP was elevated as early as 5 weeks of HFD, whereas diastolic BP was not increased until 6 weeks of HFD in KL-deficient mice (Figure 1C; Figure S1B). The recent Framingham study showed that large artery stiffness precedes the development of hypertension.32 This report indicated that arterial stiffening may be the cause of hypertension. 32 Two longitudinal studies have demonstrated that arterial stiffness predicts an increase in systolic BP and incident hypertension.8,9 This is especially true for aging-related arterial stiffening.1 Kidney function was normal (Figure S2G and S2H), excluding the involvement of renal dysfunction in HFD-induced arterial stiffness and hypertension in KL-deficient mice.

Although HFD caused arterial stiffening only in KL(+/−) mice, it increased total plasma cholesterol levels in both KL(−/−) and WT mice to a similar extent (Figure 2). Therefore, KL deficiency makes animals more susceptible to HFD-induced arterial damage. KL is predominately expressed in kidneys.10–12 We showed that KL(−/−)-deficient mice have one half of KL in kidneys when compared with WT mice. 33 Kidneys are the major source of circulating klotho.34 Indeed, plasma levels of KL protein were decreased by 50% in KL(+/−)-deficient mice (Figure S1). These results suggest that a reduction in the circulating KL may synergize with increased plasma LDL-cholesterol levels to promote HFD-induced arterial stiffening and hypertension. Therefore, KL-deficient mice are a unique animal model for studying the mechanism of arterial stiffness associated with metabolic syndrome.

It is noticed that one half klotho deficiency plus HFD diminished activities of AMPKα (pAMPKα) in aortas (Figure 2). Interestingly, the activation of AMPKα by AICAR abolished the increases in PWV and BP in KL(−/−) mice fed on HFD (Figure 2; Figure S3). To the best of our knowledge, this is the first report demonstrating that inactivation of AMPKα may be involved in the pathogenesis of arterial stiffening and hypertension. These interesting findings suggest that AMPKα might be a potential therapeutic target for arterial stiffening and hypertension. The impaired AMPKα activity may mediate the downregulation of eNOS activity (phosphorylated eNOS) and Mn-SOD expression, which can be eliminated by the
activation of AMPKα by AICAR (Figure 4). Normalization of eNOS activity and Mn-SOD expression was associated with abolishment of the increased superoxide levels in aortas of *KL<sup>−/−</sup>* mice fed on HFD (Figure 5). The activation of AMPKα also attenuated the increased levels of TGFβ1 and collagen I in aortas from *KL<sup>−/−</sup>*-deficient mice fed on HFD. These results strongly suggest that the inactivation of AMPKα is an important upstream factor in the regulation of arterial stiffening in *KL<sup>−/−</sup>*-deficient mice, and that the activation of AMPKα is sufficient to restore arterial compliance which is associated with normalization of TGFβ1 and collagen I levels. Arterial collagen deposition has been believed to be an important factor contributing to the pathogenesis of arterial stiffening. Thus, one of ultimate goals in the treatment of arterial stiffening is to block collagen synthesis.

It is expected that eNOS activity was increased by 1-week treatment with AICAR because the activation of AMPKα by AICAR would functionally interact with eNOS and upregulate its activity (phosphorylation) via LKB1, which occurs quickly. Indeed, the nitric oxide (NO) level was increased within 1 week of treatment with AICAR (Figure S4). Therefore, the early and quick drop in PWV and BP within 1 week of treatment may be partially attributed to relaxation of blood vessels caused by increased bioavailability of NO. Thus, there may be a functional component of arterial stiffness caused by increased vascular tension in *KL<sup>−/−</sup>* mice treated with HFD. By contrast, the structural recovery of blood vessels is a relatively slow process although it contributes to the attenuation of arterial stiffening and hypertension by AICAR.

*KL* deficiency did not significantly affect metabolic parameters (cholesterol, body weight, and phosphorus) under normal or HFD diets (Figure 2). AICAR only slightly decreased the HFD-induced increase in plasma cholesterol levels (Figure S3). It has been well documented that AICAR increases fatty acid oxidation via the activation of the ACC1 and ACC2 pathways in skeletal muscles and liver. AICAR may also inhibit cholesterol synthesis in the liver. Metformin, another AMPK activator, also decreases the cholesterol levels. It is noticed, however, that the slight drop of plasma cholesterol levels cannot explain the significant attenuating effect of AICAR on HFD-induced arterial stiffening and hypertension (Figure 3). On the contrary, AICAR can directly activate AMPKα in vascular cells which, in turn, increases eNOS activity (Figure 4) leading to improvement in arterial stiffening and hypertension in *KL<sup>−/−</sup>* mice fed on HFD. Indeed, AICAR almost abolished the increase in PWV and BP, suggesting that the rescuing effect of AICAR may be primarily mediated by its vascular effect.
KL gene deficiency or mutation is associated with arterial stiffening and hypertension. A decrease in plasma Klotho is used as a biomarker of arterial stiffening in patients with chronic kidney disease. Aging-related hypertension is largely caused by arterial stiffening or remodeling. Unfortunately, the current antihypertensive drugs are mainly designed to reduce peripheral resistance and are not adequate to alter the pathological process of arterial stiffening or even selectively reduce systolic BP in isolated systolic hypertension. Thus, this study suggests that supplementation with Klotho protein or pharmacological activation of AMPK α may be a novel therapeutic strategy to alleviate arterial stiffening and hypertension. The antihypertensive effect of AICAR may be mediated by the activation of eNOS and decreased arterial stiffening.

To assess the direct effect of cholesterol in SMCs, water-soluble cholesterol was added to the cultured SMCs. Cholesterol loading by water-soluble cholesterol has been shown to induce mouse aortic SMCs into a macrophage-like state. Water-soluble cholesterol delivered cholesterol rapidly and directly to the plasma membrane. Thus, cholesterol loading to cultured aortic SMCs might recapture the effects of high LDL-cholesterol on aortic SMCs in animals fed on HFD. KL protein-deficient serum exacerbated cholesterol-induced collagen I protein expression, and AICAR abolished the promoting effects of KL deficiency on cholesterol-induced collagen I expression in SMCs (Figure S6). AMPK has been reported to inhibit TGFβ-induced fibrogenic responses (collagen I) in hepatic stellate cells by targeting transcriptional coactivator p300. Adenoviral transduction of constitutively active AMPKα was sufficient to prevent TGFβ-induced collagen I and fibronectin in cultured fibroblasts. Therefore, these data strongly suggest that AICAR decreased arterial stiffening largely via suppressing cholesterol-induced collagen I synthesis.

Interestingly, KL deficiency plus cholesterol loading also attenuated the phosphorylation level of LKB1 and protein expression of LKB1 in SMCs (Figure S6). LKB1 is one of the key activators of AMPK α in various mammalian cells. Therefore, these results suggest that KL deficiency plus cholesterol might inactivate AMPK α likely through decreasing LKB1 activity.

In this study, BP was measured using a computerized volume–pressure recording tail-cuff method, a noninvasive and high-throughput measurement technique. It facilitates long-term monitoring of BP in unanesthetized animals. This method has been confirmed to be in good agreement with the radio-telemetry measurement and recommended by the American Heart Association. The repeatable measurements of BP over a 15-week period are a strong guarantee for the reliability of the BP data. This method can reliably monitor BP and is a common method for monitoring BP in our laboratory.

KL deficiency and HFD did not affect aortic elastin protein expression levels (Figures 2 and 6) but increased elastic lamellae breaks (Figure S5). The increased elastic lamellae fragmentation, which seems to be mediated by downregulation of AMPKα, would also contribute to arterial stiffening. Another interesting finding is that KL-deficient mice fed on HFD developed arterial calcification (Figure S5). Arterial calcification could contribute to arterial stiffening. RUNX2 is a reliable marker of osteoblasts and has been used as an indicator of bone formation. Therefore, KL deficiency plus HFD leads to arterial calcification likely via inactivation...
of AMPKα because AICAR abolished the upregulation of RUNX2 and arterial calcification in KL-deficient mice fed on HFD (Figure 6; Figure S5).

The serum levels of klotho decrease with age after 40 years.31 By contrast, the prevalence of arterial stiffening and hypertension increases with age.1 KL homozygous (+/−) mice were used which mimics a half KL reduction in the aged population.31 KL homozygous mice develop extensive aging phenotypes and die before the age of 8 weeks (body weight = 8 g).10 AMPKα activity, which can be abolished by the activation of AMPKα by AICAR. Therefore, KL deficiency might promote HFD-induced arterial stiffening via downregulation of AMPKα activity, which leads to upregulation of collagen I levels in aortas. Therapeutic activation of AMPKα might be a novel strategy for alleviating arterial stiffening and hypertension. It should be mentioned that hypertension is a complicated and multifactorial disorder. The antihypertensive effect of AMPK activation may not be applied to other forms of hypertension that do not involve reduction of AMPK activity.

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Disclosures

None.

References

Novelty and Significance

**What Is New?**
- It is new and interesting that haploinsufficiency of klotho gene accelerates the development of high-fat diet-induced arterial stiffening and hypertension.
- This study demonstrates, for the first time, that klotho gene deficiency plus high-fat diet downregulates vascular AMPKα expression and activity.

**What Is Relevant?**
- It is significant that klotho deficiency promotes high-fat diet-induced arterial stiffening and hypertension which are prevalent cardiovascular disorders associated with aging.

**This study reveals that activation of AMP-activated protein kinase-α may be a new therapeutic strategy for arterial stiffening, an independent risk factor for cardiovascular mortality and morbidity.**

**Summary**
Klotho deficiency promoted high-fat diet–induced arterial stiffening and hypertension. The promoting effects of klotho deficiency on arterial stiffening might be mediated by downregulation of AMP-activated protein kinase-α activity.
Antiaging Gene Klotho Deficiency Promoted High-Fat Diet–Induced Arterial Stiffening via Inactivation of AMP-Activated Protein Kinase

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**Supplemental Methods**

Since homozygous $KL^{-/-}$ deficient mice suffer extensive premature aging phenotypes and die early (<8 weeks), heterozygous $KL^{+/-}$ deficient mice and their WT littermates at the age of 6 month old were used. Mice were fed with a high fat diet (Harlan Teklad) containing 21.2% fat (wt/wt) and 1% cholesterol (wt/wt) or normal diet containing 5% fat and 141 ppm of cholesterol (see experimental scheme Fig. 1A). After 6 week HFD feeding, one third of the animals were sacrificed for the initial pathological and biochemical analysis of the mouse artery. Based on our initial biochemical analysis, at the end of the thirteenth week of the HFD feeding, we decided to inject the animals daily with AICAR, which is an AMP analog and can activate AMPK.\(^1\) Two more weeks after treatment with AICAR, all animals were sacrificed (Fig. 1A)

**Measurements of PWV.** PWV of arterial blood flow was measured using a Doppler Signal Processing Workstation (DSPW, Indus Instruments, Houston, TX, USA).\(^2\) Briefly, all mice were anesthetized in with Ketamine (90 mg/Kg body weight) and Xylazine (10 mg/Kg body weight) via IP injection. Each mouse was taped supine to electrocardiogram (ECG) electrodes on a heated procedure board with a constant temperature at 37 °C (Indus Instruments). A 2-mm diameter, 10-MHz Doppler probe was used. The flow of aortic arch and the flow of abdominal artery were recorded. The distance between the aortic arch and the abdominal artery was measured. PWV was calculated by using the manufactory software DSPW.

**Measurements of blood glucose.** Animals were fasted for 3 hours before blood glucose measurement. Blood glucose was measured biweekly or tri-weekly from the tail vein blood during the control period using a *Reli On* Ultima glucose reader (Solartek Products, Inc; Alameda, CA). Insulin sensitivity test (IST) and glucose tolerance test were performed. Briefly, blood glucose levels were measured at 30, 60, 90 and 120 minutes after subcutaneous injections of insulin
(0.5 Unit/Kg, Sigma) or glucose (1g/Kg, Sigma), respectively. The baseline glucose level was determined before the injections of insulin or glucose.

**Measurements of Blood Pressure (BP)**
BP was measured by a computerized 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, Torrington, CT) as we described previously.\(^3\) This method has been validated by using a telemetry system.\(^4\)\(^-\)\(^5\) Briefly, mice were gently handled and trained for the VPR tail-cuff measurement to minimize handling stress. No signs of stress were observed during the measurement of BP. The operator was also strictly trained for the measurement procedure. At least 20 stable cycles were obtained for data analysis for each measurement. The VPR tail-cuff method can reliably monitor BP and is a common method for monitoring BP in our laboratory.\(^3\),\(^6\)\(^-\)\(^8\)

**Tissue collections.** Briefly, animals were euthanized with an overdose of Ketamine (180 mg/Kg body weight) and Xylazine (20 mg/Kg body weight). Blood was collected with heparin as an anticoagulant. Plasma samples were stored at -80°C. Following blood collections, animals were perfused transcardiacally using heparinized saline. The first small part of aorta (ascending aorta) was placed in 4% PBS-buffered paraformaldehyde (PFA) for 24 hours and then embedded in paraffin. The second small part of aorta (aorta arch) was incubated in OCT compound and then stored in -80°C until use. The rest of aorta was stored in -80°C for later molecular assays.

**Plasma chemical and lipid profiles.** Plasma samples were sent to the Yale University Mouse Metabolic Phenotyping Centers. Total cholesterol, cholesterol in low density lipoprotein, triglycerides, non-esterified fatty acids, blood inorganic phosphorous, calcium, blood urea nitrogen, and albumin were measured.
**Morphological Analysis.** A series of cross sections of aorta (5 µm) were cut. Cross sections of aorta were stained with H&E stain assay, NovaUltra™ Alizarin Red Stain Kit (Cat: IW-3001, IHCWorld, Woodstock, MD), and Verhoeff's Elastic Stain Kit (American MasterTech, Cat KTVEL, Lodi, CA), respectively. Images of aorta from 3-5 consecutive cross sections for each animal were collected at equal exposure conditions under Nikon Eclipse Ti microscopy (object lens at 10x and 40x). The fraction area for calcified components (red color) in aortic media was obtained using NIS-Elements BR 3.0 (Nikon). The number of elastic fiber breaks was counted from images collected with higher magnification (40x) and the area of aortic media was obtained using NIS-Elements BR 3.0 (Nikon).

**In situ measurement of superoxide.** Dihydroethidium (DHE) (D7008, Sigma) was used to measure superoxide levels in aortic cross sections as we described previously. Briefly, 6-µm-thick cross sections of frozen OCT compound-embedded ascending aorta were cut on a cryomicrotome and fixed with 4% paraformaldehyde for 10 minutes. 2.5 µM DHE was added to the sections and incubated at 37°C for 20 minutes. Fluorescence images of ethidium-stained aortic section for each sample were collected at equal exposure conditions with Leica SP2 MP Confocal. Mean fluorescence density of aorta (5 sections/mouse) was analyzed using NIH Image J.

**Cell culture.** Mouse aortic smooth muscle cells, MOVAS was purchased from ATCC (cat#). Cells were cultured in DMEM (high glucose) supplemented with 5% fetal bovine serum (FBS, ATCC), 100 µg/ml of streptomycin and 100 U/ml of penicillin (Sigma) at 37°C, 5% CO2. Briefly, confluent cells were incubated with DMEM containing 5 mM Glucose and 0.05% serum albumin for 2 hours and then cells were incubated with DMEM containing 5 mM glucose and 2.5% FBS or 2.5% KL-deficient FBS in the presence of water soluble cholesterol (C4951, Sigma) for 48 hours. Methyl-β-cyclodextrin (MBCD) (C4555, Sigma) was used as a carrier for cholesterol in water-soluble cholesterol. KL-deficient FBS was generated with an IP kit (Pierce). Cells were collected with Ripa buffer.
Western blotting. Western blot was performed as described in our previous studies. Briefly, frozen aorta or cells were lysed in Ripa buffer containing protease inhibitor cocktail, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 2 mM sodium vanadate, 1 mM EDTA, and 1 mM EGTA. The protein concentration was measured using the Pierce BCA assay (Thermo Scientific). The lysates (30 µg protein/well) under reduced conditions were directly subjected to SDS-PAGE (4-15% Tris-HCL precast gel, Bio-Rad) followed by western blotting with antibodies against Klotho (AF1819, R&D Systems), collagen I (ab765p, Millipore), elastin (ab21610, Abcam), Runx2 (ab76950, Abcam), TGFβ1 (sc-146, Santa Cruz), pAMPKα (Thr172) (2531, Cell Signaling), p-eNOS (S1177) (9571, Cell Signaling), Mn-SOD (06-984, Millipore), pLKB1 (sc-28456-R, Santa Cruz Biotechnology), LKB1 (sc-5638, Santa Cruz Biotechnology), CaMKKα (sc-11370, Santa Cruz Biotechnology), and CaMKKβ (sc-50341, Santa Cruz Biotechnology). The blot was then rinsed and reprobed with antibodies against α-tubulin (2135, Cell Signaling), β-actin (Abcam), AMPKα (2193, Cell Signaling), or eNOS (612664, BD Transduction) for the loading control.

RNA Isolation and RT-PCR. Total RNA was purified from mouse kidney and MOVAS using TRizol® Reagent, followed by Qiagen RNeasy® Mini Kit. RNA (500 ng) was reverse-transcribed using SuperScript™ III Reverse Transcriptase with OligodT20 in the presence of 10ul dNTP for 1h at 50°C. The resulting cDNAs were used as templates for PCR with oligonucleotides primers to amplify Klotho mRNA and β-actin mRNA. One specific pair of primers for mouse KL mRNA was used, which target exons 1 and 2 of mouse Klotho cDNA (F: 5’-CCTGGTCACTTTGC-3’ and R: 5’-AGCACAAGTGCACAGACTTCTGCC-3’). The primers for β-actin gene were used as the internal control. The PCR product for β-actin was 708 bps. PCR reactions (50-µL volume) contained 3 µl of above cDNA, 0.2 µM of appropriate oligonucleotide primer pair, and 1 x of New England Biolabs Taq 2X Mater Mix.
PCR amplification conditions were as follows: 5 min at 95°C followed by 30 cycles of 95°C for 1 min, optimized annealing temperature for each primer pair for 1 min, and 68°C for 1 min. The PCR products were separated on 1.5% agarose gels and stained with ethidium bromide. The bands were visualized using a ChemiDoc System BioRad Imager.

References

**Supplemental Data**

Figure S1. (A), mean arterial blood pressure for the first 13 weeks. (B), diastolic blood pressure for the first 13 weeks. (C), insulin sensitivity test in the 11th week of HFD feeding. (D), glucose tolerance test in the 9th week of HFD feeding. (E&F), plasma KL protein levels in KL deficient and WT mice. *p<0.05, **p<0.01, ***p<0.001 vs WT-normal diet (ND); *p<0.05, +++p<0.001 vs KL+/−-ND; ^p<0.05, ^^p<0.01, ^^^p<0.001 vs WT-HFD.
Figure S2. (A), H&E staining and Alizarin-red staining of mouse aorta in the 6th week of HFD feeding. (B), quantification of Alizarin-red staining in arterial medium. (C). Verhoeff’s Elastic Lamellae staining in mouse aorta in the 6th week of HFD feeding. (D), the number of ruptures of aortic elastic lamellae (per 10⁴ mm²). (E), blood free fatty acid levels. (F), blood triglyceride levels (G), blood albumin levels. (H), blood urea nitrogen levels.
Figure S3. (A), mean arterial blood pressure after AICAR treatments. (B), diastolic blood pressure after AICAR treatments. (C), serum total cholesterol levels. (D), plasma LDL-Cholesterol. (E), serum calcium levels. (F), serum phosphorus levels. *p<0.05, ** p<0.01, *** p<0.001 vs WT-ND-Saline; +p<0.05, ++p<0.01, +++ p<0.001 vs KL+/--ND-Saline; ^p<0.05, ^^ p<0.01 vs KL+/--HFD-AICAR.
Nitric oxide levels in urine (µM)

Figure S4. Urinary levels of nitrite/nitrate, an index of nitric oxide, during weeks 1 and 2 of treatment with AICAR. *p<0.05, **p<0.01, ***p<0.001 vs. WT-ND-Saline; +p<0.05, vs. KL+/--ND-Saline; ^p<0.05 vs. KL+/--HFD-Saline.
Figure S5. AICAR alleviated arterial calcification and elastic fiber breaks. (A), H&E staining and Alizarin-red staining of mouse aorta. (B), quantification of Alizarin-red staining in arterial medium. (C), Verhoeff’s Elastic Lamellae staining in mouse aorta. (D), number of ruptures of aortic elastic lamellae (per $10^4 \text{ mm}^2$). N=5-8. ***p<0.001 vs. WT-ND-Saline; +++p<0.001 vs. KL+/−-ND-Saline; ^^^p<0.001 vs. KL+/−-HFD-Saline.
Figure S6. KL protein-deficient FBS exacerbated cholesterol-induced collagen I protein expression and AICAR abolished the promoting effect of KL deficiency on cholesterol-induced collagen I expression in cultured mouse aortic smooth muscle cells. Confluent cells were incubated with various reagents for 48 hours. (A&B), effects of water-soluble cholesterol on collagen I expression. (C-E), effects of AICAR on pAMPKα(T172) and AMPKα. (F&G), effects of AICAR on collagen I synthesis induced by cholesterol plus KL deficient serum. (H-K), effects of cholesterol plus KL deficiency on pLKB1 and LKB1. N=5-8. *p<0.05, **p<0.01, ***p<0.001 vs. no treatment; +p<0.05, ++p<0.01, +++p<0.001 vs. cholesterol; ^p<0.05, ^^^p<0.001 vs. CHL-KL deficient FBS.
Figure S7. (A&B), KL protein and mRNA in MOVAS. (C-E), full length and short form KL protein levels in FBS after purification by IP kit. (F&G), effects of cholesterol plus KL deficiency on CaMKKα. (H&I), effects of cholesterol plus KL deficiency on CaMKKα. *p<0.05, ** p<0.01, ***p<0.001 vs serum from WT mice or normal FBS.