Klotho and Phosphate Are Modulators of Pathologic Uremic Cardiac Remodeling


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ABSTRACT
Cardiac dysfunction in CKD is characterized by aberrant cardiac remodeling with hypertrophy and fibrosis. CKD is a state of severe systemic Klotho deficiency, and restoration of Klotho attenuates vascular calcification associated with CKD. We examined the role of Klotho in cardiac remodeling in models of Klotho deficiency—genetic Klotho hypomorphism, high dietary phosphate intake, aging, and CKD. Klotho-deficient mice exhibited cardiac dysfunction and hypertrophy before 12 weeks of age followed by fibrosis. In wild-type mice, the induction of CKD led to severe cardiovascular changes not observed in control mice. Notably, non-CKD mice fed a high-phosphate diet had lower Klotho levels and greatly accelerated cardiac remodeling associated with normal aging compared with those on a normal diet. Chronic elevation of circulating Klotho because of global overexpression alleviated the cardiac remodeling induced by either high-phosphate diet or CKD. Regardless of the cause of Klotho deficiency, the extent of cardiac hypertrophy and fibrosis correlated tightly with plasma phosphate concentration and inversely with plasma Klotho concentration, even when adjusted for all other covariables. High-fibroblast growth factor–23 concentration positively correlated with cardiac remodeling in a Klotho-deficient state but not a Klotho-replete state. In vitro, Klotho inhibited TGF-β1–, angiotensin II–, or high phosphate–induced fibrosis and abolished TGF-β1– or angiotensin II–induced hypertrophy of cardiomyocytes. In conclusion, Klotho deficiency is a novel intermediate mediator of pathologic cardiac remodeling, and fibroblast growth factor–23 may contribute to cardiac remodeling in concert with Klotho deficiency in CKD, phosphotoxicity, and aging.


Cardiovascular disease in CKD is characterized by vascular calcification¹ and uremic cardiomyopathy.² Uremic cardiomyopathy was noted in 1943³ as a state of pathologic cardiac remodeling histologically characterized by left ventricular hypertrophy (LVH) and extensive fibrosis.⁴ ⁵ The prevalence of LVH is approximately 48% in children on chronic peritoneal dialysis.⁶ In adults, increase in left ventricular mass index is found in >60% of patients after 96 weeks of hemodialysis.⁷ After multivariable adjustment, patients with stage 3 or higher (≤<30 ml/min per 1.73 m²) CKD have 2-fold higher risk of LVH compared with patients with stage 2 or lower CKD

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Cardiac fibrosis is a prominent characteristic of uremic cardiomyopathy in biopsy and postmortem examinations in humans, animals, and the higher degree of fibrosis is associated with higher mortality. Although LVH in patients with CKD has been noted for a long time, the prevalence of cardiac fibrosis is not known. Furthermore, the origin and mechanisms of fibrosis in the heart in general are not well understood. Any improvement in understanding the pathogenesis of cardiac hypertrophy and fibrosis in CKD will help construct novel therapies for this dire complication.

Klotho was originally identified as an antiaging protein. Two paralogs were discovered subsequently, and these three members are termed α, β, and γ with diverse functions. The first member of the Klotho family (α-Klotho, which is simply called Klotho here) has multiple actions. Transmembrane Klotho functions as a coreceptor for fibroblast growth factor–23 (FGF23) to regulate external phosphate balance. The extra-cellular domain of Klotho circulates as soluble Klotho and exerts myriad effects. We and others have shown that CKD is a state of severe Klotho deficiency and that Klotho restoration can ameliorate vascular calcification. It is not known whether Klotho plays a role in uremic cardiomyopathy, although genetic Klotho deficiency impairs cardiac function. Recently, Xie et al. found that Klotho can protect the heart by inhibiting TRPC6 calcium channels. In experimental animals, FGF23 seems to induce cardiac hypertrophy independent of CKD, but whether FGF23 contributes to the cardiac fibrosis in CKD and whether Klotho protects the heart from fibrosis are unknown. This study shows five points. (1) Cardiac hypertrophy and fibrosis are in primary genetic Klotho deficiency, and secondary Klotho deficiency from phosphate loading, aging, and CKD. (2) Cardiac hypertrophy precedes cardiac fibrosis and is associated with left ventricular dysfunction. (3) Higher phosphate and lower Klotho correlate with more cardiac hypertrophy and fibrosis in all models studied. (4) Higher FGF23 is associated with more dramatic cardiac hypertrophy and fibrosis only in concert with moderate or low plasma Klotho. (5) In vitro, Klotho blocks TGF-β1– and angiotensin II (Ang II)–induced hypertrophy in cardiomyocyte and attenuates TGF-β1–, Ang II–, and high phosphate–induced upregulation of fibrosis markers in cultured cardiac fibroblasts.

RESULTS

Cardiac Dysfunction, Hypertrophy, and Fibrosis in Klotho-Deficient Mice

We first examined primary Klotho deficiency from silencing of the Klotho gene resulting in hypomorphic expression. Because of the universal mortality of kl/kl (silencing of both alleles) mice during stress, we assessed cardiac function only in heterozygous Klotho–deficient (kl/+) mice by magnetic resonance imaging (MRI). At 12 weeks, the body weights were similar in kl/+ (23.8 ± 2.2 g; n = 11), wild-type (WT) (24.1 ± 1.9 g; n = 12), and transgenic Klotho–overexpressing mice (Tg-Kl; 22.3 ± 1.9 g; n = 12), and kl/+ mice had lower ejection fraction (Supplemental Movie 1), stroke volume, and cardiac output (Figure 1, A–C) and thicker left ventricular wall compared with WT and Tg-Kl mice (Figure 1, D and E). The left ventricular free wall at diastole was slightly thicker in WT than Tg-Kl mice, but cardiac function was not different (Figure 1E). Klotho levels were clearly lower in kl/+ and higher in Tg-Kl mice compared with WT mice (Supplemental Figure 1).

In addition to the cardiac hypertrophy, which was also shown previously, there was marked increase in the fibrotic area in kl/kl mice compared with WT mice reminiscent of hearts from human CKD. Note that young kl/+ mice ( 12 weeks old) already had cardiac hypertrophy but mild or no fibrosis, indicating that cardiac hypertrophy precedes fibrosis and that fibrosis worsened with age (Figure 2, A and B). Cardiac function was already abnormal at this early stage with only modest Klotho deficiency (Supplemental Figure 1).

An increase in α-actinin and upregulation of β-MHC protein were accompanied by cardiomyocyte hypertrophy in kl/kl mice (Figure 2, B–E). Global Klotho deficiency increased, and ubiquitous Klotho overexpression suppressed phosphorylation of Smad2/3 and extracellular signal–regulated kinase.

Figure 1. Klotho deficiency impairs cardiac function. kl/+, WT, and Tg-Kl mice at 12 weeks of age fed with normal diet were subjected to cardiac MRI under anesthesia. (A) Left ventricular ejection fraction, (B) left ventricular stroke volume, (C) cardiac output, (D) left ventricular wall thickness at systole, and (E) left ventricular wall thickness at diastole. Data expressed as means ± SDs of eight mice from each group, and statistical significance was assessed by one-way ANOVA followed by Newman–Keuls test. Significant differences were accepted when *P < 0.05 or **P < 0.01 between groups.


Klotho, Pi, and Cardiac Remodeling

1291
(Erk), which are known to be involved in cardiac fibrosis (Figure 2B).33–35 These results suggest that Klotho levels affect cardiac remodeling.

Cardiac Hypertrophy and Fibrosis in CKD

We next investigated whether secondary Klotho deficiency can lead to similar cardiac lesions. We studied two CKD models: (1) unilateral nephrectomy (UNX) and contralateral ischemia-reperfusion injury (IRI; UNX-IRI) followed by high-phosphate diet (2% phosphate)26 and (2) 5/6th nephrectomy. Features of CKD included increase in plasma creatinine, inorganic phosphate (Pi), FGF23, and parathyroid hormone (PTH), reduction in plasma 1,25-dihydroxy-vitamin D₃ [1,25(OH)₂D₃], and mild but statistically significant elevation of BP as described in our previous publications (data not shown).26,30 As expected,30 there was drastic reduction of plasma, urine, and renal Klotho in UNX-IRI (data not shown).

Both CKD models showed cardiac hypertrophy and left ventricular fibrosis (Figure 3, A and B, Supplemental Figure 2). There was significant increase in α-actinin (marker of cardiomyocyte hypertrophy), α-smooth muscle actin, and collagen I protein (marker of fibrosis) (Figure 3C). The increase in phospho-Smad2/3 (P-Smad2/3) and phospho-extracellular signal regulated kinase (P-Erk) in uremic heart (Figure 3C) suggests that activation of Smad and Erk signal pathways is involved.33–35

Low Circulating and Renal Klotho, Aging, and High Dietary Phosphate Synergistically Induced Pathologic Cardiac Remodeling

Phosphotoxicity is gathering attention as a potential risk factor for cardiovascular and renal disease.17,24,36 To better define the role of Klotho in cardiac remodeling induced by high-phosphate diet and aging, we challenged kl/+, WT, and Tg-KI mice at 6 months of age with the same high-phosphate diet for 12 weeks. Klotho protein in kidney, plasma, and urine was decreased by phosphate loading (Figure 4A). The high plasma Pi and FGF23 levels were exaggerated in kl/+ mice and attenuated in Tg-KI mice (Supplemental Table 1).

To explore the contribution of aging, we fed a high-phosphate diet to older mice (12 months old) with low (kl/+), normal (WT), and high endogenous Klotho (Tg-KI) for 12 weeks until they were 15 months old. Additional reductions in plasma and kidney Klotho were observed in older kl/+ mice compared with younger kl/+ mice. Renal Klotho was nearly undetectable in 12-month-old kl/+ mice fed with high phosphate (Figure 4B). Note that downregulation of Klotho in all three lines was more pronounced in older (Figure 4 B) compared with middle-aged (Figure 4A) mice. All mineral parameters and hormones were worse with aging and high-phosphate diet (Supplemental Table 1).

Cardiac hypertrophy and fibrosis were exaggerated in kl/+ mice and lessened in Tg-KI mice compared with WT mice and more severe at age 15 months compared with 9 months (Figure 4, C–E). The histologic changes were compatible with alterations of α-actinin in the heart, and collagen I and α-smooth muscle actin were higher in hearts of kl/+ mice and lower in hearts of Tg-KI mice compared with WT mice (Figure 4, D and E, Supplemental Figure 3). Expression of genes relevant to hypertrophy and fibrosis (data not shown) further supports the notions that low Klotho and high phosphate triggered pathologic cardiac remodeling, Klotho suppressed the fibrosis triggered by high dietary phosphate, and aging exacerbated phosphate or Klotho deficiency-induced pathologic cardiac remodeling.

Increased P-Smad2/3 and P-Erk (Figure 4, D and E) signifying Smad2/3 and ERK activation in left ventricle were found in kl/+ mice, and lower phosphorylation of these molecules was seen in Tg-KI mice. Activation of Erk and Smad2/3 was more pronounced in kl/+ mice at 15 months of age compared with 9 months of age, indicating that aging amplifies pathologic cardiac remodeling with activation of Smad2/3 and Erk pathways, similar to the uremic heart (Figure 3C).

Direct Effects of Soluble Klotho Protein on Neonatal Cardiomyocytes and Cardiac Fibroblasts In Vitro

One drawback of in vivo studies is the inability to conclude whether there is a direct effect of Klotho on the heart. To address that question, we used primary culture of neonatal rat cardiac myocytes and fibroblasts to determine whether Klotho can directly confer protection against Ang II–, TGF-β1–, and phosphate-induced changes. Despite the inherent and unavoidable caveats of cultured cells, this is currently still the only way to examine direct effects of Klotho and phosphate.

TGF-β1, Ang II, and high Pi media increased connective tissue growth factor and collagen I protein in cardiac fibroblasts, and the induction was attenuated by Klotho (Figure 5A). Erk phosphorylation induced by TGF-β1, Ang II, and high Pi in cardiac fibroblasts was attenuated by Klotho (Figure 5B). However, no significant activation of Smad2/3 was detectable in cultured neonatal cardiac fibroblasts.

TGF-β1 or Ang II increased α-actinin protein (Figure 5C) in cardiomyocytes, which was blunted by soluble Klotho. However, high Pi only slightly increased α-actinin in cardiomyocytes (Figure 5C) over this short period. Connective tissue growth factor protein was appreciably increased by all Ang II, TGF-β1, or high Pi (Figure 5C). Collagen I protein was induced by Ang II but not TGF-β1 or high Pi (Figure 5D). Klotho abolished the stimulation of Smad2/3 phosphorylation by TGF-β1, Ang II, or high Pi (Figure 5D). Both TGF-β1 and high Pi but not Ang II induced activation of Erk1/2, suggesting differential signal pathways between cardiac fibroblasts and myocytes.

Correlation of Plasma Biochemistry, PTH, 1,25(OH)₂D₃, FGF23, and Klotho with Cardiac Hypertrophy and Fibrosis

To quantitatively compare changes in plasma Klotho in aging, dietary phosphate manipulation, and CKD, we arbitrarily set normal plasma Klotho as that in young (3 months old) WT mice with normal kidney function fed normal phosphate diet as 100%. Aging, phosphate loading, and CKD seem to act in
Figure 2. Klotho-deficient mice with severe cardiac remodeling. (A, left panel) Representative macrograph of sagittal sections of the hearts (Trichrome) of kl/kl, kl/+ and WT mice at 6 and 12 weeks of age. (A, right panel) Shows semiquantification of the Trichrome-positive area over the whole-heart section (Image J). (B) Representative micrographs showing cardiac fibrosis by Trichrome staining and immunohistochemistry for α/β-MHC in the left ventricle of kl/kl and WT mice at 12 weeks of age. (C) Representative immunohistochemistry
concert to lower plasma Klotho (Figure 6A). Cardiac hypertrophy and fibrosis negatively correlated with plasma Klotho (Figure 6, B and C) and positively correlated with plasma Pi (Figure 6, D and E). Univariable analysis showed positive correlation of heart weight (HW/body weight) and cardiac fibrosis index with plasma Klotho, FGF23, PTH, Pi, and creatinine and mild correlation with 1,25(OH)2D3 (Figure 6F). Importantly, plasma Klotho and Pi were significantly correlated with either HW/body weight or cardiac fibrosis (Figure 6F), even when adjusted for all other confounding factors supporting the notion that Klotho and phosphate are independent pathologic factors that induce cardiac remodeling.

We did not find a relationship between FGF23, cardiac hypertrophy, and fibrosis by multivariable analysis. We then divided plasma Klotho and FGF23 levels into tertiles and analyzed correlation of plasma FGF23 with cardiac hypertrophy and fibrosis at three plasma Klotho levels. Animals with higher FGF23 and lower Klotho had more severe cardiac hypertrophy and fibrosis (Figure 7, A and B). There was a significant relationship of log10 plasma FGF23 concentration versus HW/body weight or fibrosis index (Figure 7, C and D) only in moderate or low plasma Klotho groups, suggesting that FGF23 acts as an important contributor to pathologic cardiac remodeling in concert with lower plasma Klotho.

**DISCUSSION**

Longitudinal observation from the Chronic Renal Insufficiency Cohort revealed that, although left ventricular mass index was relatively stable during transition from CKD to ESRD, ejection fraction declined.37 Reduced kidney function is associated with abnormal cardiac structure but not initially with overt abnormal systolic or diastolic function; kl/+ mice had lower cardiac hypertrophy, ejection fraction, stroke volume, and cardiac output compared with WT or Tg-Kl mice (Figures 1 and 2) but without florid heart failure. Frailty and sudden death precluded us from doing cardiac MRI in kl/kl mice, but one can fathom that cardiac function will only be worse with the worse histology in kl/kl mice. Whether the cardiac defect in kl/kl mice starts during embryonic development is a possibility that we cannot rule out at present.

The coexistence of cardiac hypertrophy, fibrosis, and low Klotho in CKD (Figure 3), dietary phosphate loading (Figure 4), and primary Klotho deficiency (Figure 2) strongly suggests that Klotho deficiency plays a causative role in pathologic cardiac remodeling. Another cardiac phenotype previously described in Klotho deficiency was sinoatrial node dysfunction, which is in the only part of the heart that expresses Klotho endogenously.32 Moreover, cardioprotection of Klotho was proposed to act through suppression of the TRPC6 signal pathway.31 Klotho mRNA was found in the sinoatrial node,32 but membrane Klotho protein has never been shown to be expressed in cardiomyocytes or cardiac fibroblasts. We are primarily examining a different aspect of Klotho biology, which is the systemic effects of soluble Klotho. Aging, phosphotyrosinicity, and CKD could individually and synergistically contribute to the decline in plasma Klotho (Figure 6A), and compound each other to induce cardiac remodeling. Plasma Klotho levels were negatively associated with severity of pathologic cardiac remodeling, even corrected for other variables. The *in vitro* experiments with neonatal rat cardiac fibroblast and myocytes provided additional evidence to support the notion that Klotho directly suppresses cardiac hypertrophy and fibrosis.

In addition to the indirect effect on cardiac remodeling through elevation of circulating FGF23, which induces cardiac hypertrophy,30 hyperphosphatemia is also independently associated with cardiac hypertrophy and fibrosis (Figure 6, D and E). *In vitro* studies only showed effects of high Pi on fibrosis but not on cardiomyocytes hypertrophy. High Pi alone may not be a sufficient trigger for hypertrophy *in vitro* but is able to initiate fibrosis. Another possibility is that hypertrophy is a chronic process and that 1 day of Pi treatment is not sufficient. Pathologic cardiac remodeling was appreciably attenuated in Tg-Kl mice, although they were fed a high-phosphate diet, supporting that Klotho and phosphate may independently affect the heart.

Although low Klotho is detrimental, extremely high circulating Klotho is not beneficial.38,39 In both humans39 and rodents38 with a >2-fold rise in plasma Klotho, one notes hypophosphatemia, elevated FGF23, osteomalacia, and pathologic fractures.38,39 In contrast, in Tg-Kl mice, the plasma Klotho is only increased by 50% or less compared with WT mice (Supplemental Figure 1A), and Tg-Kl mice do not have severe hypophosphatemia and only minimal derangement of FGF23.

Klotho suppresses renal fibrosis in an obstructive uropathy model.40,41 The mechanism can be partially caused by inhibition of Wnt-induced cell cycle arrest and the resultant decrease in fibrogenic cytokines,41,42 suppression of TGF-β1-induced Smad activation,40,43 or blockade of Ang II signaling.44 Klotho directly suppresses Ang II– and TGF-β1–induced hypertrophy and fibrosis in cardiomyocytes *in vitro* by inhibition of phosphorylation of Smad2/3 and/or Erk (Figure 6), which are key players in cardiac and renal fibrosis.33–35,45
The heart is composed of cardiomyocytes and noncardiomyocytes; among them, cardiac fibroblast is the major cell type surrounding cardiomyocytes and contributing to cardiac development, structure, cell signaling, and electromechanic function.46 Fibroblasts can be transformed into myofibroblasts contributing to extracellular matrix accumulation. Fibrosis, originating from nonmyocytes47 and enhanced by cardiac myocytes,48 can lead to increased wall stiffness and diastolic dysfunction.49 Fibrosis also interrupts electrical signals,50 rendering the tissue more arrhythmogenic, which is highly prevalent in the CKD population.12,51,52

Phosphotoxicity is a risk factor for progression of kidney disease and cardiovascular disease in CKD/ESRD, but causality has not been established.24,53–55 In rodents with normal renal function, long-term high-phosphate intake induced cardiac remodeling and was associated with decreased renal and systemic Klotho (Figure 4) and increased plasma PTH and FGF23 (Supplemental Table 1). Epidemiologic studies showed that high plasma PTH and low plasma 1,25(OH)2D3 are associated with cardiac hypertrophy.56,57 In spontaneously hypertensive heart failure rats (hemizygous cp mutation), 1,25(OH)2D3 treatment prevents cardiac hypertrophy.58 The fact that high-dietary phosphate loading in normal animals induced moderate cardiac hypertrophy and fibrosis (Figure 4) with mild increase in vitamin D (Supplemental Table 1) does not support the pathogenic role of 1,25(OH)2D3 deficiency in phosphate-induced cardiac remodeling. Vitamin D, PTH, FGF23, phosphate, and Klotho plus other unknown uremic toxin(s) and aging itself may individually, additively, and synergistically contribute to pathologic cardiac remodeling, but their individual effects may not be of sufficient magnitude to emerge from a multivariate analysis in these complex models. Multivariate regression analysis only showed significant correlation with plasma Klotho and phosphate in cardiac remodeling in our collective experimental models (Figure 6).

FGF23 is increased in CKD,25,59 but the mechanism of the increase is not entirely known. Circulating and renal Klotho deficiency precedes the increase in plasma FGF2325,60 and is a potential mechanism of stimulating FGF23.17,36 The role of FGF23 in mediation of cardiac hypertrophy was suggested by epidemiologic data30,61–63 and strongly suggested by an animal study.30 There may be interactive effects between FGF23 and Klotho.38,39,64,65 Although plasma FGF23 is not independently associated with cardiac remodeling (Figure 6), a clear positive relation of plasma FGF23 levels with cardiac hypertrophy and fibrosis is present in moderate or severe Klotho

![Figure 3. Cardiac hypertrophy and fibrosis in CKD mice.](https://www.jasn.org/BASIC-RESEARCH)

WT mice at 12 weeks of age underwent CKD surgery (UNX-IRI) or laparotomy (sham). One month after surgery, mice were fed with high-phosphate diet for 12 weeks. (A, upper panel) Representative macrographs of sagittal sections (H&E). Scale bar, 3 mm. (A, lower panel) Summary of the ratio of HW to body weight of sham and CKD mice. (B, upper panel) Representative micrographs of left ventricular sections (Trichrome). Scale bar, 100 μm. (B, lower panel) Summary of semiquantification of the Trichrome-positive area over the whole-heart section by Image J. (C, left panel) Representative immunoblots for P/T-Smad2/3, P/T-Erk, and α-SMA. Collagen I and β-actin in left ventricular lysates from sham and CKD were induced by UNX-IRI. Summaries are shown in C, right panel. Data are means±SDs (n=4 from each group). Statistical significance was assessed by t test. Significance was accepted when **P<0.01 between groups. α-SMA, α-smooth muscle actin; H&E, hematoxylin and eosin; T, total.
deciency (Figure 7), suggesting that FGF23 can still be patho-
genic in conjunction with or perhaps, as a pathologic interme-
diate for Klotho de
ciency.

We showed that circulating Klotho de
ciency caused by
genetic disruption, CKD, dietary phosphate loading, and aging
was associated with cardiac hypertrophy and dysfunction. Low
plasma Klotho and high plasma phosphate showed robust
correlation with pathologic cardiac remodeling, even when
corrected for other covariables. High FGF23 was also correlated
with severity of cardiomyopathy but only in the presence of
low plasma Klotho levels. A direct antifibrotic effect of Klotho
was shown in cultured cells in vitro. This study shows the
pathophysiologic importance of Klotho deficiency and high
phosphate on the heart, which may be relevant for uremic
cardiomyopathy, and opens up potential novel diagnostic
and therapeutic potentials on the horizon.

CONCISE METHODS

Animal Experiments

Rodent (mice and rats) models were approved by the Institutional
Animal Care and Use Committees from the University of Texas
Southwestern Medical Center and the University of Münster. Klotho
hypomorphic13 and Tg-Kl
mice were described previously.26,66 Two
CKD rodent models were used: UNX-IRI26 and 5/6th nephrectomy.30,67
Sham animals underwent laparotomy and manual manipulation of
the kidneys. For high-phosphate diet, 2 weeks after surgery, animals

Figure 4. Synergism of high-phosphate diet, Klotho deficiency, and aging on cardiac remodeling. kl/+,
WT, and Tg-Kl mice at 6 or 12
months of age were fed normal or high-phosphate diet for 12 weeks and terminated at age 9 or 15 months, respectively. Plasma, urine,
and kidney Klotho at (A) 9 and (B) 15 months old were determined by immunoprecipitation-immunoblotting or immunoblotting. Right
panels summarize plasma, urine, and kidney Klotho protein levels. (C, upper panel) Representative macrographs of heart sections
(Trichrome); (C, lower panel) Summarizes semiquantification of the Trichrome-positive area over the whole-heart section (Image J).
(D) Representative immunohistochemistry for P-Erk, collagen I, and Alexa-Fluor–WGA in left ventricular sections of kl/+,
WT, and Tg-Kl
mice at 9 and 15 months old. (E) α-SMA, P-Smad2/3, and Alexa-Fluor–WGA in left ventricular sections of kl/+,
WT, and Tg-Kl
mice at 9
and 15 months. Data expressed as means±SDs (n=4 from each group). Statistical signifi-
cance was assessed by one-way ANOVA
followed by Newman–Keuls test. Significant differences were accepted when *P<0.05 or **P<0.01 between groups. α-SMA, α-smooth
muscle actin; HC, heavy chain; WGA, wheat germ agglutinin.
Figure 5. In vitro effect of soluble recombinant Klotho protein on neonatal cardiac fibroblasts and cardiomyocytes. (A and B) Cardiac fibroblasts were prepared from neonatal rat hearts and cultured in six-well plates. (A) After full confluence, TGF-β1, Ang II, and high Pi were added with Klotho or vehicle for 24 hours, and total cell lysate was immunoblotted for (left panel) TGF-β1, (center panel) Ang II, and (right panel) high Pi effect on CTGF and collagen I protein expression. (B) TGF-β1, Ang II, and high Pi were added with Klotho or vehicle for 30 minutes, and total cell lysate was prepared for immunoblot to examine (left panel) TGF-β1, (center panel) Ang II, and (right panel) high Pi on phosphorylation of Erk and Smad2/3 in cardiac fibroblasts. (Top panel) Representative immunoblots. (Middle and bottom panels) Summary of all experiments. (C and D) Cardiomyocytes were prepared from neonatal rat hearts and cultured in six-well plates. (C) On confluence, TGF-β1, Ang II, and high Pi were added with Klotho or vehicle for 24 hours, and total cell lysate was immunoblotted for (left panel) TGF-β1, (center panel) Ang II, and (right panel) high Pi effect on CTGF, collagen I, and α-actinin. (D) TGF-β1, Ang II, and high Pi were added with Klotho or vehicle for 30 minutes, and total cell protein lysate was immunoblotted for (left panel) TGF-β1, (center panel) Ang II, and (right panel) high Pi effect on phosphorylation of Erk and Smad2/3 in cardiomyocytes. (Top panel) Representative immunoblots. (Middle and bottom panels) Summary of all experiments. Data are expressed as means±SDs of three independent experiments for each group, and statistical significance was assessed by one-way ANOVA followed by Newman–Keuls test. Significant differences when *P<0.05 or **P<0.01 between groups. CTGF, connective tissue growth factor; T, total.
were switched from normal rodent chow to 2.0% phosphate diet (Teklad 08020; Harlan, Madison, WI) for 18 weeks.

Cardiac MRI
Cardiac function was evaluated by cardiac MRI using a 7T Small Animal MR Scanner (Varian, Inc., Palo Alto, CA) with a 38-mm birdcage RF coil as previously described.68

Primary Culture of Neonatal Rat Ventricular Myocytes and Cardiac Fibroblasts
Cells were isolated from hearts of neonatal SD rats with established methods.69,70 For immunofluorescence, 1×10^6 cells were seeded per well on glass coverslips in six-well plates. For protein and RNA isolation, 2×10^6 cells were seeded in 6-cm culture dishes.

Static Heart Morphology and Morphometry
Sections were stained with Trichrome for fibrosis and imaged with a 5× objective. To measure surface area of cardiomyocytes, paraffin-embedded sections were labeled with wheat germ agglutinin conjugated to Alexa Fluor 555 (Invitrogen, Carlsbad, CA) with standard methods.30

Immunohistochemistry in the Heart and the Kidney
For immunohistochemistry within glucose 6-phosphate dehydrogenase (G6PDH) and glucose 6-phosphate isomerase (GPI), and in the kidney, sections of the heart and kidney were incubated with monoclonal antibodies specific to G6PDH and GPI (1:250; Dako). The sections were then stained with diaminobenzidine (DAB) and counterstained with hematoxylin.

Figure 6. Summary of Klotho levels and cardiac phenotype: effect of age, high-phosphate diet, and CKD on plasma Klotho levels and pathologic cardiac remodeling. (A) Plasma Klotho levels normalized to that of 3-month-old WT mice with normal renal function on a normal Pi diet (set to 100%; z axis). Groups of animals: age (3, 9, and 15 months) and dietary phosphate (normal phosphate [N Pi], 0.9%; high phosphate [H Pi], 2.0%; high-phosphate diet plus CKD [H Pi+CKD]). (B) Double log_{10} plot of HW/body weight (BW) and (C) double log_{10} plot of percentage of cardiac fibrosis versus plasma Klotho concentration in all groups of animals. The relationships between log_{10} plasma Klotho concentration and log_{10} HW/BW or log_{10} cardiac fibrosis were determined by linear correlation analysis, and significant association was accepted when $P<0.05$. (D) Double log_{10} plot of HW/BW and (E) double log_{10} plot of percentage of cardiac fibrosis versus plasma phosphate concentration in all groups of animals. The relationships between log_{10} plasma phosphate concentration and log_{10} HW/BW or log_{10} cardiac fibrosis were determined by linear correlation analysis, and significant association was accepted when $P<0.05$. (F) Unadjusted and adjusted association of cardiac hypertrophy and fibrosis with plasma parameters. The association between cardiac variables (HW/BW and percentage of cardiac fibrosis) with each variable (Klotho, FGF23, PTH, 1,25 Vit D, Pi, and creatinine) was assessed with Pearson correlation coefficients with the SAS program (v9.3; SAS Institute, Cary, NC). For each plasma parameter, the partial correlation coefficients were computed to adjust for the potential confounding effects of the other five biomarkers in evaluating the association with cardiomyopathy. $P>0.05$ was considered statistically significant. $r$, Pearson correlation coefficient. *Adjusted for the other five plasma parameters.
(1:50; Molecular Probes, Eugene, OR) for staining β-actin filaments was applied for double staining.

Quantifying Klotho Protein and Transcript in the Kidney, Urine, and Blood of Rodents

Immunoblots in the kidney and urine were performed as described. Heparinized plasma was subjected to immunoprecipitation with rabbit anti-human Klotho and immunoblotted with anti-Klotho antibody (KM2076; 0.5 μg/ml). Lysates from cardiac myocytes, fibroblasts, and tissues were prepared for immunoblot as described. Total RNA was extracted followed by generation of cDNA. Primers used for quantitative PCR are in Supplemental Table 2, and the methods are described. Data were expressed at amplification number of $2^{-ΔΔCt}$ by normalization of cyclophilin and comparison of controls.

Additional detailed experimental methods are presented in Supplemental Material.

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See related editorial, “Klotho Deficiency and the Cardiomyopathy of Advanced CKD,” on pages 1229–1231.

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