Vascular Klotho Deficiency Potentiates the Development of Human Artery Calcification and Mediates Resistance to Fibroblast Growth Factor 23

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Background—Klotho is known to function as a cofactor for the phosphatonin, fibroblast growth factor (FGF)-23 at the kidney. FGF-23 levels rise in chronic kidney disease (CKD) despite progression of accelerated vascular calcification. There are currently conflicting data on whether FGF-23 may exhibit direct vasculoprotective effects in CKD.

Methods and Results—In this study, we describe for the first time endogenous Klotho expression in human arteries and human aortic smooth muscle cells. We show that CKD is a state of vascular Klotho deficiency promoted by chronic circulating stress factors, including proinflammatory, uremic, and disordered metabolic conditions. Mechanistic studies demonstrated that Klotho knockdown potentiated the development of accelerated calcification through a Runx2 and myocardin-serum response factor–dependent pathway. Klotho knockdown studies further revealed that vascular cells are a Klotho-dependent target tissue for FGF-23. FGF-23 mediated cellular activation of p-ERK, p-AKT, and cellular proliferative effects, which were abrogated following Klotho knockdown. We next showed that vascular Klotho deficiency driven by procalcific stressors could be restored by vitamin D receptor activators, in vitro and further confirmed using human arterial organ cultures from CKD patients, in vivo. Furthermore, restoration of suppressed Klotho expression by vitamin D receptor activators conferred human aortic smooth muscle cells responsive to FGF-23 signaling and unmasked potential anticalcific effects.

Conclusions—Chronic metabolic stress factors found in CKD promote vascular Klotho deficiency. Mechanistic studies revealed a bifunctional role for local vascular Klotho, first, as an endogenous inhibitor of vascular calcification and, second, as a cofactor required for vascular FGF-23 signaling. Furthermore, vitamin D receptor activators can restore Klotho expression and unmask FGF-23 anticalcific effects. (Circulation. 2012;125:2243-2255.)

Key Words: chronic kidney disease ▪ inflammation ▪ vascular calcification ▪ vitamin D ▪ Klotho
Klotho was responsible for direct inhibition of mineralization, phosphate uptake, and dedifferentiation of cells of the vascular wall.

Accelerated vascular dysfunction and resultant calcification is highly prevalent in patients with CKD, diabetes, and premature aging and is a major contributor to premature cardiovascular death in these patients.6,9 It is currently unknown whether Klotho is expressed endogenously in the artery wall and whether it may impart similar vasculoprotective functions to its soluble, kidney-derived counterpart.

It is believed that Klotho expression in specific tissues renders them responsive to the circulating phosphatonin, fibroblast growth factor 23 (FGF-23). Klotho functions as a cofactor for FGF-23 in kidney by converting fibroblast growth factor receptor 1 (FGFR-1) into a specific receptor for FGF-23. Studies have shown that formation of a Klotho/FGFR-1 receptor complex may be the principal effector site for FGF-23 phosphaturic effects in kidney.1,10 Because FGF-23 levels begin to rise in the early stages of CKD, Klotho/FGFR-1/FGF-23 signaling in kidney is an important mechanism that functions to counteract hyperphosphatemia and resulting vascular complications. However, both clinical and basic studies have demonstrated conflicting evidence as to whether FGF-23 imparts a protective or a harmful role on vasculature under stress.11–15 Recent studies have implicated reduced Klotho tissue levels as a cause of FGF-23 resistance at the parathyroid gland in CKD rats.16,17 Whether vascular resistance to FGF-23 occurs in CKD that could mask its effects on the vasculature is an important question that needs addressing.

Vitamin D deficiency is a prevalent condition in CKD patients, and vitamin D receptor (VDR) activators are routinely used to prevent secondary hyperparathyroidism. Emerging evidence has implicated rising FGF-23 levels in CKD as the cause of declining active 1,25-dihydroxyvitamin D (1,25(OH)2D; calcitriol) levels in kidney.15 Clinical studies have demonstrated that serum calcitriol levels are inversely correlated with coronary artery calcification in the general population.16,17 These studies therefore suggest that vitamin D may be playing an anticalcific role on arteries.

In this study, we describe for the first time endogenous Klotho expression in human artery. We present data for vascular-produced Klotho as an endogenous inhibitor of calcification and as a cofactor for FGF-23 signaling. In addition, we provide evidence for the reversal of CKD-mediated, Klotho-dependent vascular resistance to FGF-23 after vascular VDR activation.

Methods

Human Samples

Human samples were collected from healthy people donating a kidney and CKD patients undergoing a renal transplant. Human artery collection was performed at the University Hospital Coventry and Warwickshire NHS Trust, United Kingdom. Ethical approval was obtained from the Coventry Research Ethics Committee (05/Q2802/26), United Kingdom.

Arterial Explants Organ Culture

Epigastric and renal arteries from patients with CKD and from healthy kidney donors) were dissected, and arterial rings were placed in DMEM (Invitrogen, Carlsbad, CA). Arteries were then treated with 100 nmol calcitriol or 300 nmol paricalcitol for 48 hours before harvesting for analysis.

Cell Culture

Commercially available human aortic smooth muscle cells (HA-SMCs) were obtained from 3 different age-matched sources (lot 0295, 0573, 3523; ScienCell Research Laboratory, Carlsbad, CA). All in vitro experiments using HA-SMCs were repeated using all 3 cell sources. The n number corresponds to the number of total repeat experiments performed collectively by using all 3 cell sources. HA-SMCs were cultured in 5% CO2/37°C incubator and grown with smooth muscle cell medium containing 0.5 mmol/L phosphate and 1.6 mmol/L calcium (catalog No. 1101; ScienCell Research Laboratory, Carlsbad, CA) for seeding.

Treatment of cells were performed using DMEM (catalog No. 1013, Invitrogen, Carlsbad, CA). Addition of 0.5% fetal bovine serum to DMEM was used as starvation medium for short-term experiments to assess the role of Ca, P, and tumor necrosis factor α (TNF-α). Charcoal-stripped serum (catalog No. 12676-011; Invitrogen, Carlsbad, CA) was used for all experiments involving vitamin D, because fetal bovine serum contains active vitamin D. Two percent charcoal-stripped serum was used for short-term experiments as starvation medium, whereas a concentration of 15% was used for calcification experiments, because higher concentrations are necessary for cell survival under long-term procalcitic stress; pretreatment of cells with calcitriol or calcidiol was performed for 24 hours.

DMEM was supplemented with calcium chloride (CaCl2; catalog No. 223506; Sigma, St Louis, MO) and β-glycerophosphate disodium (catalog No. G9422; Sigma, St Louis, MO) for calcium and phosphate experiments.

Antibodies for Western Blot and Immunoprecipitation

All antibodies were prepared in Tris-buffered saline with 0.2% Tween-20 for Western blot, concentration of antibodies used in this study: Klotho (catalog No. Ab75023; Abcam, Cambridge, MA) 1:500; Runt-related transcription factor 2 (Runx2) (catalog No. 05-1478, Millipore, Billerica, MA) 1:500; FGF-1 (catalog No. Sc-121; Santa Cruz Biotechnology, Santa Cruz, CA) 1:1000; FGF-3 (catalog No. sc-123; Santa Cruz Biotechnology, Santa Cruz, CA) 1:1000; myocardin (catalog No. MAB4028; R&D Systems, Minneapolis, MN) 1:1000; serum response factor (SRF) (catalog No. sc-56779; Santa Cruz Biotechnology, Santa Cruz, CA) 1:1000; α-smooth muscle actin (α-SMA; catalog No. 5292; Sigma-Aldrich, St Louis, MO) 1:1000; calponin (catalog No. sc-28545; Santa Cruz Biotechnology, Santa Cruz, CA) 1:1000; smoothelin (catalog No. sc-73042; Santa Cruz Biotechnology, Santa Cruz, CA) 1:1000; p-ERK (catalog No. 9107; Cell Signaling, Danvers, MA) 1:1000; p-AKT (catalog No. 4058; Cell Signaling, Danvers, MA) 1:1000; actin (catalog No. MAB1501; Millipore, Billerica, MA) 1:1000.

Proteins, Cytokines, and Assay Kits

FGF-23 (catalog No. 2604-FG; R & D Systems, Minneapolis, MN); TNF-α (catalog No. T 0157; Sigma, St Louis, MO); calcitriol (catalog No. D1530; Sigma, St Louis, MO); calcidiol (catalog No. H4014; Sigma, St Louis, MO); Paricalcitol was kindly provided by Abbott, Maidenhead, UK. Preparations for these proteins were made according to manufacturers’ recommendations. Alkaline phosphatase was measured by SensolOlyte pNPP Alkaline Phosphatase Assay Kit (Ana Spec, Fremont, CA). Cell proliferation was assessed by using XTT in vitro assay kit (catalog No. TOX2; Sigma-Aldrich, St Louis, MO). Further details of protocols used for alkaline phosphatase and cell proliferation assays are provided in the in online-only Data Supplement Methods.

Klotho siRNA Transfection

Klotho small interfering RNA (siRNA) was purchased from Invitrogen (part No. 4392422, Invitrogen, Carlsbad, CA). Cells were
seeded until 50% confluence and placed in opti-MEM I Reduced Serum Medium (catalog No. 31985-062, Invitrogen, Carlsbad, CA). Transfection was achieved by using Lipofectamine (vector) reagent (catalog No. 15338-100, Invitrogen, Carlsbad, CA) and PLUS reagent (catalog No. 11514-015) at manufacturers’ recommended concentrations. For all experiments, 400 mmol/L Klotho siRNA was used with a transfection time of 24 hours. For longer-term calcification experiments, cells were retransfected every other day while maintaining appropriate calcium and phosphate concentrations. Validations of siRNA efficacy experiments are provided in online-only Data Supplement Methods.

Calcification
Calcification was visualized at 21 days after treatment by Alizarin red staining as previously described.\(^1\) Calcification was also quantified by using the Arsenazo III (Fisher Scientific, Pittsburgh, PA) method. Please refer to the in online-only Data Supplement Methods for detailed descriptions.

Statistical Analysis
All experiments were performed at least 3 times, and the results were expressed as the mean±standard error. Analysis was performed by using descriptive statistics, 2-tailed paired t test or 1-way ANOVA followed by Bonferonni post hoc analysis as stated in the representative figure legends (Figures 1 through 6). Probability values <0.05 were considered as statistically significant.

Supplementary Methods
Detailed description for immunohistochemistry, immunocytochemistry, immunoprecipitation, Western blotting, and polymerase chain reaction protocols used is provided in the online-only Data Supplement Methods.

Results

**CKD Is a State of Vascular Klotho, FGFR-1, and FGFR-3 Deficiency**
We show, for the first time, Klotho protein expression in human arteries (Figure 1A) and have investigated the expression profile of Klotho in human arteries from healthy individuals and patients with CKD. Immunohistochemistry analysis demonstrated Klotho protein expression in the medial layer of arteries from healthy individuals, but marked reduction in arteries from patients with CKD. Artery Klotho protein quantification with Western blot confirmed this observation (Figure 1B; demographic patient data in online-only Data Supplement Table I). Reduced vascular Klotho protein expression in CKD patients was associated with extensive medial calcification assessed by Alizarin red staining. We found that 0 of 10 (0%) of arteries from healthy individuals and 6 of 10 (60%) of CKD arteries showed calcification (data not shown). In addition, we have shown that arteries from CKD patients exhibited increased levels of Runx2, suggesting osteogenic transformation of vascular cells in comparison with healthy controls (\(P<0.01\)) (Figure 1B). Our results suggest that Klotho may be involved in the vascular dysfunction of CKD. Concomitant FGFR-1 and FGFR-3 protein expression mirrored Klotho expression in human arteries (Figure 1A).

**The Uremic Environment Suppresses Endogenous HA-SMC Klotho, FGFR-1, and FGFR-3 Expression Associated With Osteo/Chondrocytic Transformation In Vitro**
To determine whether uremia suppressed endogenous Klotho expression, we used pooled serum from healthy individuals and patients with CKD (biochemical analysis in online-only Data Supplement Table II). HA-SMCs cultured with 10% uremic serum showed a significant decline in Klotho, FGFR-1, and FGFR-3 expression at 72 hours in comparison with the healthy treatment group (Figure 1C). Note that we did not find FGFR-2 and -4 expression in HA-SMCs (data not shown).

Hyperphosphatemic or hypercalcemic conditions have been shown to induce osteogenic transformation of vascular smooth muscle cells (VSMCs), an important process that orchestrates the development of calcification.\(^1\)\(^2\) We therefore evaluated whether hyperphosphatemia and/or hypercalcemia downregulate Klotho, FGFR-1, or FGFR-3 expression. HA-SMCs were treated with calcium, phosphate, or calcium and phosphate at concentrations arteries from patients with CKD are exposed to. Klotho, FGFR-1, and FGFR-3 expression was significantly reduced after treatment with calcium concentrations of 2.7 mmol/L or 5.0 mmol/L at 12 hours and remained reduced at 24 hours (Figure 2A). Combined treatment with increased calcium and phosphate concentrations had similar suppressive effects (Figure 2C). Treatment with phosphate concentrations of 2.0 mmol/L or 5.0 mmol/L suppressed Klotho expression only at 24 hours, whereas FGFR-1 and FGFR-3 suppression was already observed at 12 hours (Figure 2B). Accumulating evidence suggests that inflammatory stress such as TNF-\(\alpha\) seen in CKD and diabetes mellitus is an important risk factor for VC.\(^2\)\(^3\) Therefore, to determine whether proinflammatory stress may itself downregulate Klotho, FGFR-1, or FGFR-3, HA-SMCs were treated with TNF-\(\alpha\). All 3 proteins were suppressed gradually after 12 hours of TNF-\(\alpha\) treatment at 20 ng/mL, with further suppression over 36 hours (Figure 2D). In parallel with Klotho, FGFR-1, and FGFR-3 suppression, Runx-related transcription factor 2 (Runx2) was upregulated, indicating HA-SMC transformation to the calcifying phenotype (Figure 2A through 2D).

**Klotho Deficiency Potentiates the Development of HA-SMC Calcification**
To determine the roles of local HA-SMC–expressed Klotho in vascular dysfunction, we investigated whether endogenous HA-SMC Klotho deficiency is involved in the development of accelerated VC. We first generated a HA-SMC Klotho knockdown model by using Klotho siRNA (online-only Data Supplement Figures I and II). To determine the role of Klotho deficiency in the development of calcification, HA-SMCs were retransfected with Klotho siRNA every other day. We have validated our Klotho siRNA transfection efficiency over long-term 21-day cultures stressed with calcification medium (online-only Data Supplement Figure III).

Klotho siRNA transfected and control cells were cultured with high calcium and phosphate concentrations for 21 days. The Klotho siRNA-transfected group exhibited significant accelerated calcification in comparison with the empty vector and nontransfected groups (Figure 3A). Klotho, FGFR-1, and FGFR-3 protein expression were already suppressed in HA-SMCs cultured under calcifying stress and declined further with its cognate siRNA construct with the consequence of further extracellular calcium deposition (Figure 3B).
Figure 1. Klotho, FGFR-1, and FGFR-3 protein expression in human arteries from healthy individuals and patients with chronic kidney disease (CKD). 

A, Immunohistochemistry. Klotho protein is expressed in the tunica media, the smooth muscle cell layer of medium-sized muscular human arteries. Note high protein expression in arteries from healthy individuals in comparison with patients with CKD. FGFR-1 and FGFR-3 protein expression was also found in the media of human arteries, with reduced expression in arteries from patients with CKD in comparison with healthy individuals. Normal human kidney was used as a positive control for Klotho, FGFR-1, and FGFR-3 (positive staining in brown, counterstained with hematoxylin-eosin). Alizarin red staining confirmed significant deposition of calcium in the media layer of arteries from 60% of patients with CKD; no calcification was observed in arteries from healthy individuals. Representative examples from 10 arteries each from healthy individuals and patients with CKD are shown.

B, Western blot analysis of homogenized arteries confirmed Klotho protein expression at 116 kDa. Vascular Klotho protein expression of healthy individuals (n=10) and patients with CKD (n=11) demonstrated significantly lower, almost undetectable protein levels in arteries from CKD patients. Arteries from CKD patients also showed increased Runx2 expression, suggesting osteogenic transformation (P<0.01) (demographic patient data are provided in online-only Data Supplement Table I). 

C, Klotho, FGFR-1, and FGFR-3 protein are expressed in human aortic smooth muscle cells (HA-SMCs). When HA-SMCs were exposed to 10% pooled uremic serum from patients with CKD, expression of Klotho, FGFR-1, and FGFR-3 protein decreased significantly at 72 hours, compared with the treatment group exposed to 10% pooled serum from healthy individuals (P<0.05, n=6) (composition of serum used for treatment is described in online-only Data Supplement Table II). One-way ANOVA followed by Bonferroni post hoc analysis was for analysis in B and C. FGFR indicates fibroblast growth factor receptor.

* A – adventitia, M – media, L – lumen
Figure 2. Components of uremic serum, high calcium, high phosphate, and TNF-α suppress Klotho, FGFR-1, and FGFR-3 expression in HA-SMCs, a change associated with osteogenic transformation of HA-SMCs. A, HA-SMCs were treated with borderline high calcium (Ca-BH; 2.7 mmol/L CaCl₂) and high calcium (Ca-H; 5 mmol/L CaCl₂) for 12 and 24 hours in culture medium, and cellular protein was analyzed with Western blotting. Klotho, FGFR-1, and FGFR-3 protein expression was suppressed at 12 hours and remained suppressed after 24 hours of treatment. In parallel, Runx2 protein expression, a marker of HA-SMC transformation into osteo/chondrocytic phenotype, increased dramatically. B, Under borderline high (Pi-BH; 2 mmol/L β-glycerophosphate) and high (Pi-H, 5 mmol/L β-glycerophosphate) phosphate in vitro culture conditions, Klotho protein expression was suppressed at 24 hours, but FGFR-1 and FGFR-3 protein expression levels declined by 12 hours with concomitant upregulation of Runx2 protein. C, HA-SMCs were treated with borderline high calcification medium (CM-BH; 2.7 mmol/L calcium, 2.0 mmol/L β-glycerophosphate) or high calcification medium concentration (CM-H; 5.0 mmol/L CaCl₂, 5.0 mmol/L β-glycerophosphate). Klotho, FGFR-1, and FGFR-3 expression declined at 12 and 24 hours under both CM-BH and CM-H with significant upregulation of Runx2. D, Treatment of HA-SMCs with TNF-α (20 ng/mL) for 48 hours demonstrated a significant reduction of Klotho expression at 36 hours, whereas FGFR-1 and FGFR-3 expression declined already after 12 hours.
Mechanistic studies confirmed HA-SMC change to a calcifying phenotype. Key cellular regulatory proteins mirrored this transformation with upregulation of Runx2, indicating osteogenic transformation and suppression of myocardin and SRF (Figure 3C). Myocardin and the associated SRF are key regulators to maintain HA-SMC in a contractile, SMC phenotype. This regulation is particularly important in the pathogenesis of calcification, because SMCs convert from a contractile to a secretory phenotype after artery injury. This is paralleled with increased Runx2 expression, the master regulator toward a calcifying osteogenic phenotype. Increased alkaline phosphatase activity, a key bone-forming enzyme, in the supernatant of cells exposed to calcifying stress further mirrors these intracellular signal changes (Figure 3D). Our results, using Klotho siRNA pretreatment accelerates HA-SMC transformation to a calcifying phenotype, confirm the importance of SMC.

Loss of Smooth Muscle Cell Contractile Phenotype is an Early Event in Klotho Deficiency

We next sought to further characterize the role of Klotho in regulating the smooth muscle cell contractile phenotype. HA-SMCs were cultured in high calcium and high phosphate for 48 hours. Klotho and the upstream smooth muscle cell markers smoothelin α-SMA and calponin were suppressed. Klotho knockdown with Klotho siRNA in HA-SMCs treated with calcifying stress further suppressed calponin expression. Smoothelin and probably α-SMA were already suppressed maximally with high calcium and high phosphate, and no further suppression could be achieved after Klotho knockdown (Figure 3E). It is noteworthy that Runx2 expression was elevated in the nontransfected and empty vector groups with further elevation in the Klotho knockdown group confirming our observations in our long-term 21-day model.

Reduced HA-SMC Klotho, FGFR-1, and FGFR-3 Expression and Their Physical Dissociation Under Calcifying Stress Mediates Resistance to FGF-23

Immunocytochemistry analysis revealed coexpression of Klotho with FGFR-1 and FGFR-3 in HA-SMCs with a markedly reduced expression under extracellular high calcium and phosphate stress at 6 hours (Figure 4A). Coimmunoprecipitation studies revealed physical association between Klotho-FGFR-1 and Klotho-FGFR-3 (Figure 4B). Physical association ratio, calculated by optical density of target protein/immunoprecipitated protein, revealed significant dissociation of Klotho-FGFR-1 and Klotho-FGFR-3 in HA-SMCs under high calcium stress. These results suggest that there is a concomitant decline of Klotho, FGFR-1, and FGFR-3 expression and physical dissociation contributing to progression of VSMC dysfunctions.

Previous studies have shown that FGF-23 upregulates p-ERK and p-AKT signaling pathways in renal proximal tubule epithelial cells. We report that HA-SMCs treated with FGF-23 at 5 ng/mL for 12 hours upregulated p-ERK and p-AKT; however, Klotho siRNA mitigated these effects, rendering VSMCs FGF-23 resistant (Figure 4C). p-ERK and p-AKT have been previously associated with mitogenic and cell survival pathways, our results showed that HA-SMCs cultured in 10% serum from healthy individuals after adding FGF-23 5 ng/mL significantly stimulated proliferation of HA-SMCs. Klotho knockdown by Klotho siRNA transfection for 24 hours before addition of 10% serum from healthy individuals, however, mitigated these effects (Figure 4D). Furthermore, HA-SMCs cultured in 10% uremic serum containing high FGF-23 concentrations from CKD patients significantly stimulated cell proliferation, and these effects were also reduced by Klotho knockdown (Figure 4E). Klotho-FGF-23 signaling may play a pivotal role in increased cell proliferation. These results suggest that endogenous Klotho renders VSMCs a target for FGF-23 signaling and that Klotho deficiency may mediate vascular resistance in CKD.

Restoration of Klotho by VDR Activation Confers HA-SMCs FGF-23 Responsive and Unmasks FGF-23 Calcification Inhibitory Effects

We show that HA-SMCs pretreatment with active calcitriol could restore Klotho expression in procalcific environments and with the response at much higher concentrations with inactive 25-hydroxyvitamin D (25(OH)D; calcidiol), postulating local activation. Calcitriol restored FGFR-1 expression, but little effect was found with calcidiol at 24 hours. FGFR-3 expression was restored only by 50 nmol/L calcitriol treatment (Figure 5A).

In addition, HA-SMCs treated with calcification medium (CM), FGF-23, or calcitriol alone stimulated cell proliferation, with FGF-23 being the most potent stimulator. Treatment with FGF-23 together with CM did not increase cell proliferation in comparison with CM treatment alone. This suggests cellular Klotho deficiency under procalcific stress, resulting in FGF-23 resistance. However, combination treatment of FGF-23 and CM following pretreatment with calcitriol caused significant stimulation of cell proliferation. This proliferative effect was mitigated after Klotho siRNA pretreatment (Figure 5B). Furthermore, we demonstrate for the first time that FGF-23 was able to significantly inhibit extracellular calcium deposition following restoration of Klotho after pretreatment with calcitriol. These beneficial effects were again reversed after suppressing Klotho protein synthesis with Klotho siRNA (Figure 5C). This confirms the importance of vascular VDR activation to reverse stress-induced suppression of vascular Klotho to restore FGF-23 responsiveness.

Figure 2 (Continued). Treatment. Runx2 protein expression was detectable at 12 hours, with increasing expression over the following treatment period. Target protein compared with its control: n = 6. One-way ANOVA was used to compare a target protein among treatment groups within a time point. Probability values from 1-way ANOVA are < 0.001 in A through D. Bonferroni post hoc analysis was then performed, and the probability values are provided in the figure. FGFR indicates fibroblast growth factor receptor; TNF, tumor necrosis factor; HA-SMC, human aortic smooth muscle cells.
Organ cultures of human arteries from patients with CKD exhibited significant upregulation of Klotho mRNA levels following 48 hours of calcitriol or paricalcitol (a synthetic VDR activator) treatment. This treatment effect was not observed in arteries from healthy individuals (Figure 6). FGFR-1 responded in parallel, with restoration of mRNA expression in arteries exposed to uremia after treatment with VDR activators calcitriol or paricalcitol to levels observed in arteries from healthy individuals. FGFR-3 mRNA levels in arteries from patients with CKD remained suppressed even after VDR activator treatment. Basal Klotho RNA level in arteries was quantified immediately after removal from the patient. The same levels of Klotho RNA were expressed as already described after 48 hours, as shown in Figure 6.

Discussion

We show that high Klotho expression in human arteries from healthy individuals and from patients with CKD are severely Klotho protein deficient. We provide evidence for functional Klotho protein expression in human VSMCs. Published data show no Klotho expression in animal (mouse or rat) vasculature, which was confirmed in our laboratory. Further in vitro studies using HA-SMCs demonstrated that chronic stress factors including proinflammatory TNF-α, disordered mineral levels, and uremia drives Klotho deficiency. These results may explain in part the relationship between inflammation and diseases characterized by accelerated aging of organs, including CKD. A recent study has shown that inflammatory cytokines, such as TNF-α and TNF-like weak inducer of apoptosis, downregulate Klotho expression through an nuclear factor κB-dependent mechanism in kidney.27 A similar pathway could contribute to suppression of Klotho in vascular cells and unmask arteries to chronic metabolic and inflammatory stress, resulting in vascular calcification. Further studies are needed to determine whether such inflammatory cytokines in circulation or produced locally are the main drivers of Klotho suppression.

Previous studies have shown that osteogenic transformation of VMSCs renders them mineralization competent to secrete an osteoid-like extracellular matrix that calcifies over a defined time course.22 Our results show that suppression of Klotho was associated with upregulation of Runx2, with VSMC phenotype transformation. Previous studies have also shown that uremic and similar metabolic stressors can suppress Klotho at the parathyroid gland.17,28 Our results support these findings and provide the first evidence that CKD is a state of local, vascular Klotho deficiency.

Upregulation of Runx2 orchestrates osteoblastic differentiation of VSMCs by regulating downstream bone-related proteins, including alkaline phosphatase and osteocalcin. The process of VSMCs dedifferentiation from a contractile to a secretory phenotype is co-ordinated by Myocardin, which functions as a cofactor for the transcription factor, SRF, that binds to CArG-box motif, to regulate transcription of smooth muscle contractile genes, including α-SMA.24,29,30 Our data suggest that phenotypic adaptation of VSMCs to osteo/chondrocytic-like cells may be involved in the pathogenesis of endogenous smooth muscle Klotho loss. We speculate that Klotho may be a critical gene involved in maintaining smooth muscle cell integrity, and its suppression may be co-ordinated by regulators of osteo/chondrocytic transformation and loss of smooth muscle contractile phenotype in a procalcific milieu.

Previous animal studies have shown that Klotho overexpression imparts longevity31 and can exert renoprotective effects following ischemia-reperfusion injury,7 angiotensin II-induced renal failure32 and glomerulonephritis.33 Emerging evidence now suggests that Klotho may exert direct cardiovascular protective effects; studies using the Otsuka Long-Evans Tokushima Fatty rat that demonstrates multiple atherogenic risk factors have shown that Klotho gene and protein delivery protects against endothelial dysfunction.6 Several studies have shown that Klotho may protect against vascular damage through the regulation of nitric oxide release.6,34 Furthermore, Klotho can confer resistance against oxidative stress involved in the patho-

Figure 3 (Continued). Klotho deficiency accelerates the development of calcification, promotes osteo/chondrocytic transformation and loss of smooth muscle phenotype in HA-SMCs exposed to calcifying stress, in vitro. A, Qualitative assessment of calcification by Alizarin red staining and quantitative assessment of calcification by using the Aresenazo III method in HA-SMCs cultured in calcification medium (CM) for 21 days, revealed calcium deposition. HA-SMCs transfected with klotho siRNA developed a significantly accelerated calcification with marble red staining when compared to the empty vector and nontransfected treatment groups. B, Western blot analysis of cellular phenotype master regulator showed increased Runx2 protein expression in the nontransfected and empty vector groups following treatment with CM compared with the control group. However, Klotho, FGFR-1, and FGFR-3 expression was further suppressed in the Klotho siRNA group under calcification medium treatment. C, Western blot analysis of cellular phenotype master regulator showed increased Runx2 protein expression in the nontransfected and empty vector groups following treatment with CM. After further suppression of Klotho protein expression with Klotho siRNA together with CM treatment, even higher Runx2 protein levels were detected in comparison with the CM treatment groups. Myocardin and serum response factor (SRF) protein levels declined opposite to increasing Runx2 protein expression levels, indicating phenotypic transformation of HA-SMCs. This process intensified after suppressing Klotho synthesis with its siRNA construct. D, Alkaline phosphatase measured in the supernatant of the treatment groups showed increased activity under CM treatment, with a further significant raise in activity after knocking down the Klotho protein with its siRNA construct, mirroring Runx2 protein changes. E, HA-SMCs were cultured in CM for 48 hours following Klotho knockdown by Klotho siRNA transfection. Western blot analysis confirmed reduced Klotho in the nontransfected and empty vector groups under CM exposure. Further reduction of Klotho was observed in the Klotho siRNA group. These results were mirrored by calponin, whereas smooth muscle and α-SMA expression was already significantly decreased after calcification stress alone. This made it difficult to see further reduction after additional Klotho siRNA knockdown. Runx2 protein expression was increased in the empty vector and nontransfected groups and further increased in the Klotho siRNA group. CM: 2.7 mmol/L CaCl₂, 2 mmol/L β-glycerophosphate. Empty vector=lipofectamine alone; n=3. One-way ANOVA was used to compare a target protein among treatment groups. Probability values from one-way ANOVA are <0.001 in A through E. Bonferroni post hoc analysis was then performed, and the probability values are provided in the figure. FGFR indicates fibroblast growth factor receptor; siRNA, small interfering RNA; SMA, smooth muscle actin; HA-SMC, human aortic smooth muscle cells.
genesis of various vascular diseases. Klotho has also been shown to suppress retinoid-inducible gene 1-mediated inflammation involved in its antiaging effects. Of note, Hu et al has shown that circulating Klotho can suppress vascular calcification through inhibition of Na\(^{+}\)/H\(^{+}\)-dependent uptake of phosphate. Our study demonstrates for the first time that local vascular-derived Klotho deficiency in HA-SMCs develops severe calcification when grown under procalcific stress conditions. This supports the notion that Klotho deficiency renders arteries vulnerable to dysfunction and that this protein may function as a critical endogenous inhibitor of calcification. We speculate that it may function through the stabilization of VSMC contractile phenotype. Furthermore, our results uncover a new mechanism by which Klotho may exert antiaging and cardiovasculoprotective effects in the arterial system, since

Figure 4. Reduced HA-SMC Klotho, FGFR-1, and FGFR-3 expression and their physical dissociation under procalcific stress mediates resistance to FGF-23 in vitro. A, Fluorescence immunocytochemistry double-staining of HA-SMC was performed after 6 hours of treatment with control or calcification medium (CM; 2.7 mmol/L CaCl\(_2\), 2 mmol/L β-glycerophosphate) treatment. Visualization of cells by confocal microscopy and analysis of merged images revealed cellular Klotho/FGFR-1 and Klotho/FGFR-3 colocalization. Their expression was markedly reduced under CM treatment conditions. B, Coimmunoprecipitation studies with FGFR-1 and FGFR-3 pulldown experiments demonstrated physical association with Klotho protein. Klotho/FGFR-1 and Klotho/FGFR-3 exhibited significant dissociation under calcification medium treatment. C, FGF-23 treatment of HA-SMCs at 5 ng/mL in DMEM containing 0.5% fetal bovine serum induced p-AKT and p-ERK1/2 expression. However, these effects were mitigated by Klotho siRNA, confirming a Klotho-dependent cellular FGF-23 effect. D and E, HA-SMCs were cultured in 10% pooled human serum (HS) in DMEM for 24 and 48 hours. D, FGF-23 treatment at high, 5 ng/mL concentration, significantly stimulated cell proliferation in HA-SMCs cultured in serum from healthy individuals (10% normal HS). These effects were mitigated by Klotho siRNA. E, HA-SMCs cultured in serum from CKD patients (10% CKD HS) already containing high FGF-23 levels exhibited increased cell proliferation, an effect partially mitigated by Klotho siRNA. Empty vector: Lipofectamine alone. For IP experiments, n = 3, for all other experiments, n = 6. Student t test was used for analysis in B. One-way ANOVA was used to compare a target protein among treatment groups within a time point in D and E. Probability values from 1-way ANOVA are < 0.001 in D and E. Bonferonni post hoc analysis was then performed and the probability values are provided in the figure. FGFR indicates fibroblast growth factor receptor; siRNA, small interfering RNA; HA-SMC, human aortic smooth muscle cells; IP, immunoprecipitation; FGF-23, fibroblast growth factor 23; CKD, chronic kidney disease; FBS, fetal bovine serum.
osteo/chondrocytic transformation and calcific-hardening of vasculature is characteristic of aged blood vessels.\textsuperscript{36,37} Rising FGF-23 levels in CKD have been associated with a variety of cardiovascular pathologies, including hand artery calcification,)\textsuperscript{11} atherosclerosis,\textsuperscript{12} and left ventricular hypertrophy.\textsuperscript{13} Gutierrez et al\textsuperscript{38} showed that rising FGF-23 levels were independently associated with mortality among patients beginning hemodialysis. The recent Heart and Soul study demonstrated that FGF-23 is also independently associated with mortality and cardiovascular disease events in patients without CKD.\textsuperscript{14} On the contrary, there is a body of evidence that supports a

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Restoration of Klotho by vitamin D receptor activation confers HA-SMCs FGF-23 responsive and unmasks FGF-23 calcification inhibitory effects. A, HA-SMCs were pretreated with active 1,25-dihydroxyvitamin D\textsubscript{3} (1,25(OH\textsubscript{2})D\textsubscript{3}, calcitriol) and inactive 25-hydroxyvitamin D\textsubscript{3} (25(OH)D\textsubscript{3}, calcidiol) for 24 hours. HA-SMCs were then exposed to high calcium and phosphate (CM). Our results showed that pretreatment with calcitriol could restore Klotho expression most significantly at 50 nmol/L and at the much higher doses of 500 and 1000nmol/L with calcidiol. Calcitriol restored FGFR-1 expression most significantly at 50 nmol/L with no significant effects with calcidiol at these conditions, FGFR-3 expression was restored only by calcitriol at 50nmol/L with no significant effects with calcidiol at these conditions, FGFR-3 expression was restored only by calcitriol at 50nmol/L with no significant effects with calcidiol at these conditions. Calcitriol restored FGFR-1 expression most significantly at 50 nmol/L with no significant effects with calcidiol at these conditions. Calcitriol restored FGFR-1 expression most significantly at 50 nmol/L with no significant effects with calcidiol at these conditions. FGFR indicates fibroblast growth factor receptor; siRNA, small interfering RNA; HA-SMC, human aortic smooth muscle cells; FGF-23, fibroblast growth factor 23; CM, calcification medium.

FGF-23 levels were independently associated with mortality among patients beginning hemodialysis. The recent Heart and Soul study demonstrated that FGF-23 is also independently associated with mortality and cardiovascular disease events in patients without CKD. On the contrary, there is a body of evidence that supports a
Because pathological processes underlying VC resemble bone mineralization, later studies have shown that FGF-23 is able to directly decrease the mineralization of osteoblasts and calvarial cells in vitro.41,42 Furthermore, inactivating mutations of FGF-23 in human diseases such as familial tumoral calcinosis manifest with severe ectopic calcification.15 Because Klotho/FGFR-1 complex forms a specific receptor for FGF-23 signaling in kidney, we hypothesized that VSMCs and the arteries may also be a target for FGF-23 function. In addition, we speculated that vascular Klotho deficiency may mediate FGF-23 resistance, providing an explanation for discrepant findings discussed above.1,43

We showed that Klotho colocalizes and physically associates with FGFR-1 and FGFR-3 with suppression and physical dissociation under procalcific stress. FGF-23 upregulates p-ERK and p-AKT, induces proliferation and inhibits HA-SMC-mediated extracellular, calcifying matrix deposition, in vitro. However, these effects were abolished following Klotho knockdown providing first evidence of Klotho-dependent function of FGF-23 in VSMCs. Our results therefore suggest that rising FGF-23 levels in CKD, correlated with increased cardiovascular mortality, are in part a consequence of vascular resistance to FGF-23 because of uremia-mediated VSMC Klotho deficiency. Rising serum FGF-23 levels may themselves be a positive-feedback response as a result of end-organ resistance. This observation is supported by recent studies that have also implicated reduced parathyroid cell Klotho levels as the cause of FGF-23 resistance in uremic CKD rats.16,17 Furthermore, our study has shown that deficiency of local vascular Klotho occurs together with declining circulating levels in patients with CKD.7 Further studies are needed to investigate the interplay between local vascular and circulating Klotho systems.

Large observational clinical studies in patients with CKD have demonstrated that VDR activators impart cardiovascular survival benefits.44,45 However, the mechanisms are not well understood. A recent animal study has shown that, at therapeutic dosages sufficient to correct secondary hyperparathyroidism, VDR activator treatment protected the vasculature from calcifying, but higher doses stimulated aortic calcification.46 The latter was probably caused by indirect, endocrine VDR activator effects resulting in hyperphosphatemia and hypercalcemia, suppressors of vascular Klotho expression. Therapeutic dosages of VDR activator were also found to reduce VSMC phenotype transformation in the aorta. Interestingly, animal studies have shown that administration of calcitriol also increases FGF-23 levels and represents a possible intended protective effect against hyperphosphatemic conditions and also of arteries against raised local calcium and phosphate.47

In our study, we provide evidence that VDR activation can restore Klotho levels in procalcific environments. This effect appeared to plateau at midrange doses of VDR activator treatment, in vitro. Our results also show that inactive calcitriol can restore Klotho expression, suggesting that the VSMC 1α-hydroxylase (CYP27B1) enzyme48 is involved in mediating supportive autocrine/paracrine effects in the regulation of Klotho. Furthermore, we show for the first time that restoration of Klotho with VDR activator renders VSMCs again FGF-23 responsive, with proliferation and calcification inhibitory effects. These results demonstrate that both the endocrine and autocrine/paracrine vitamin D systems are involved in regulating cardiovasculoprotective role for FGF-23: FGF-23 knock-out mice present with extensive vascular and soft-tissue calcification, together with severe hyperphosphatemia beginning in early life.39,40
local vascular Klotho. VDR activator alone had no modulating effect on VSMC calcification in our in vitro model. This would support the concept of a complex interplay on a local tissue level between the vitamin D system-Klotho-FGF-23 beyond mineral homeostasis, as proposed by Haussler et al. on an endocrine level for this system, resulting in protection of the vasculature. Human arterial organ cultures confirmed that arteries from CKD patients retained their ability to restore Klotho and FGFR-1 following VDR activator treatment. We therefore propose that use of VDR activator as a therapeutic strategy in CKD for the restoration of vascular Klotho deficiency and the vascular FGF-23 response.

In conclusion, we report for the first time endogenous Klotho expression in human arteries, in vivo, and in HA-SMCs, in vitro. We provide evidence that CKD is a state of vascular Klotho deficiency, a likely explanation for accelerated vascular aging with calcification experienced by these patients. We have also shown that high levels of FGF-23 in CKD cannot be vasculoprotective because Klotho/FGFR deficiency mediates resistance. And we confirm that VDR activator therapy mediates vascular protection by increasing vascular Klotho expression and rendering vascular cells FGF-23 responsive. Our study furthers our understanding of the role of vascular Klotho in accelerated vascular disease and the interplay it mediates between FGF-23 and vascular cells.

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We thank our surgical colleagues H. Kashi, L.C. Tan, C. Imray, P. Roberts, and T. Fernando for providing us with human artery samples.

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Disclosures
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References


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Kenneth Lim, Tzong-Shi Lu, Guerman Molostvov, Christina Lee, F.T. Lam, Daniel Zehnder and Li-Li Hsiao

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SUPPLEMENTAL MATERIAL

SUPPLEMENTARY METHODS

Calcification
Calcification was visualized at 21 days after treatment by Alizarin red staining as previously described\textsuperscript{20}. Cells were washed with 0.9% NaCl isotonic solution at 37°C. Alizarin red stain (Sigma, MO, USA) was prepared at 1% concentration and pH adjusted to 4.2 using acetic acid. Cells were then stained with 1% Alizarin red stain for 1 minute and after rinsed with 0.9% NaCl isotonic solution. The results were observed under light-microscopy (Olympus IX70) and photographed by digital camera (Olympus America). Calcification was quantified using the Arsenazo III (Fisher Scientific, USA) method. Briefly, cells were decalcified in 0.1M HCl, and the supernatant removed for colorimetric assay with Aresenao III at 650nm. Cells were harvested for protein quantification by the Lowry method (BioRad, USA) and calcium concentration normalized to the cellular protein content.

Alkaline Phosphatase (ALP) Assays
Alkaline phosphatase was measured by SensoLyte® pNPP Alkaline Phosphatase Assay Kit (Ana Spec, CA). Medium from our \textit{in vitro} studies were collected for colorimetric assay using alkaline phosphatase conjugated secondary antibody and pNPP (p-Nitrophenyl phosphate) as a phosphatase substrate. The reaction was incubated at 37°C for 30 minutes. Upon dephosphorylation by phosphatases, pNPP turns yellow and was detected at absorbance of 405 nm. ALP activity was normalized to cellular protein content.
Cell proliferation assay

Cell proliferation was assessed using XTT *in vitro* assay kit (Cat. No. TOX2; Sigma-Aldrich, MO, USA). Briefly, cells were plated at 50,000 cells/well and seeded until 50% confluent in SMCM using 6-well plates. Cells were then placed in opti-medium with or without transfection reagents for 24 hours. FGF-23 treatment was then performed in DMEM containing 0.5% FBS. XTT stock solution equal to 20% of the culture medium volume was added. 1ml of media containing XTT was then transferred onto a 96-well plate and absorbance measured at 450nm. Background absorbance readings at 690nm were subtracted from readings taken at 450nm. Absorbance readings were normalized to readings at 690nm and cellular protein content to account for loss of cell numbers following transfection.

Immunohistochemistry

Tissues were stored in formalin and paraffin-embedded sections were cut using a microtome. Paraffin was removed by placing slides first in two changes of xylene, followed by 100% alcohol, 90% alcohol and then in distilled water. Antigen retrieval of sections was achieved using a pressure cooker. Following antigen retrieval, slides were blocked for endogenous peroxidase activity with hydrogen peroxide in PBS (1.5%) (1 H$_2$O$_2$ : 1 PBS) for 20 minutes and then washed with TBS-T. Sections were incubated in serum for 20 minutes. The slide was washed with TBS-T between and after primary and secondary antibody incubations. Sections stained with Vectastain Universal Elite ABC (Avidin and Biotinylated horseradish peroxidase macromolecular Complex solution) kit (Catalog No. PK-6200; Vector Laboratories, CA, USA). They were then incubated with
DAB solution, rinsed with distilled water and washed with TBS-T before counterstaining with hematoxylin solution.

**SDS-PAGE Gel Electrophoresis and Western Blot**

Sample media was mixed with 4X loading (sample) buffer containing 5% β-mercaptoethanol (Sigma, MO, USA) and Radio-Immuno Precipitation Assay (RIPA) buffer, pH 7.4 (Catalog No. BP-115, Boston BioProducts, MA, USA). The samples were then heated for 5 minutes at 95°C. 10-30μg of sample was loaded onto SDS-PAGE, NuPAGE Bis-Tris pre-cast polyacrylamide gels using the mini-cell system (Invitrogen, CA, USA). NuPAGE MOPS SDS running buffer (Invitrogen, CA, USA) was used. 500μl of antioxidant was added to the running buffer. Electrophoresis was performed at 140V-200V until adequate spread of the protein molecular marker was achieved. Following SDS-PAGE gel electrophoresis, proteins were then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). Transfer was achieved using a wet-blot (Bio-Rad) transfer system. Standard Towbin transfer buffer was used containing 25mM Tris, pH 8.3, 192 mM glycine, 20% (v/v) methanol. Proteins were then visualized with an enhanced chemiluminescence detection system.

**Immunoprecipitation (IP)**

HA-SMCs lysates or serum was pre-cleaned using protein A/G for 1 hour. 5 μg of primary antibody was incubated with HA-SMC lysates or serum in RIPA buffer pH 7.4 (Catalog No. BP-115, Boston BioProducts, MA, USA) for 1 hour. Immune complexes were captured on immobilized protein A/G agarose gel (Invitrogen, CA, USA) for 2 hours. After washing with lysis buffer, protein sample buffer was added and loaded onto
a SDS-PAGE gel for electrophoresis. Western blot analysis was then conducted as described above for detection of our protein complex of interest. To detect interaction with other proteins, co-IP was performed using antibodies against our proteins of interest.

**Immunocytochemistry**

Cells were cultured on glass slides for immunostaining. Briefly, cells were fixed with 2% ice cold paraformaldehyde for 10 minutes at room temperature. Blocking was achieved with 0.5% Saponin-PBS with 5% horse serum solution for 30 minutes at 37°C. Primary antibody incubation was performed overnight at 4°C, washed with PBS and then incubated in secondary antibody for 2 hours at room temperature. Target proteins were then visualized using confocal microscopy.

**Analysis of gene expression using real-time PCR**

Total RNA was isolated from arterial ring lysates using an RNeasy kit (Qiagen, UK) following the manufacturer's protocol. Arterial samples from patients were homogenized in liquid nitrogen and solubilized using the lysis buffer from the same kit. Reverse transcription of total RNA (100-200ng) was carried out using Superscript 3 reverse transcriptase (Invitrogen, UK) with random hexamers (Bioline, UK). Real-time PCR amplification was performed using TaqMan universal PCR master mix and TaqMan gene expression assay probes for Klotho (Hs00183100_m1), FGFR-1 (Hs00915142_m1) and FGFR-3 (Hs00179829_m1) (Applied Biosystems). 18S rRNA probe was used as an internal control.
SUPPLEMENTAL TABLES

Supplemental Table 1: Summary of demographic and clinical data of patients included for arterial Klotho expression analysis.

<table>
<thead>
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<td>11</td>
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<tr>
<td>Age (mean±SD, range; years)</td>
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<td>On active Vitamin D therapy (n)</td>
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Supplemental Table 2: Biochemical analysis of pooled serum from healthy individual and patients with CKD.

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<td>357</td>
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Supplemental Figure 1: Klotho knockdown is most optimally achieved using 400mM Klotho siRNA in HA-SMCs. Dose-dependent studies were performed using Klotho siRNA. Klotho siRNA transfection was performed for 24 hours and cells were then harvested for western blot analysis. No significant suppression of Klotho was observed using 200mM of Klotho siRNA. Klotho suppression occurred most significantly at a dose of 400mM. n=3.
Supplemental Figure 2: Klotho knockdown is achieved using Klotho siRNA but not with scrambled siRNA or empty vector. To validate our Klotho knockdown using Klotho siRNA, scrambled siRNA and Vector (lipofectamine alone) was used. HA-SMCs were transfected with 400mM Klotho siRNA or 400mM scrambled siRNA for 24 hours and then harvested for western blot analysis of Klotho expression. Significant suppression of Klotho was noted only in the Klotho siRNA group. n=3.
Supplemental Figure 3: Klotho knockdown potentiates the development of calcification at 7, 14 and 21 days. HA-SMCs were cultured in calcification medium (CM; 2.7mM CaCl₂, 2mM β-glycerophosphate) for 7, 14 and 21 days. Cells were re-transfected every other day. A) HA-SMCs calcification was quantified with the arsenazo III method. HA-SMCs transfected with Klotho siRNA developed significantly higher calcification compared to non-transfected group at all three time points, confirming effective Klotho siRNA transfection. n =3. B) Western blots confirmed reduced Klotho levels in the CM group and a further reduction in the Klotho siRNA group at each of the three time points.
Supplemental Figure 4: Full Western blot for Klotho protein. A) This shows a representative result for Klotho protein in human arteries with Klotho antibody at a dilution of 1:1000 (Catalogue No Ab69208; Abcam, UK). B) This shows a representative result Klotho protein from an *in vitro* human aortic smooth muscle cell experiment with Klotho antibody at a dilution of 1:500 (Catalogue No Ab75023; Abcam, USA).