The Role of Klotho on Vascular Calcification and Endothelial Function in Chronic Kidney Disease

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Summary: Recent insights into novel roles of klotho in vascular biology make this primarily kidney-derived protein a possible candidate to form a link between chronic kidney disease and cardiovascular morbidity and mortality. Typical features of vascular dysfunction or structural abnormalities in the arterial wall are exacerbated in klotho-deficient states. Reported klotho functions include inhibition of local phosphate transport in vascular cells, phenotypic switches of vascular cellular elements into bone-forming cells, attenuation of matrix mineralization and calcification, and also preservation of endothelial functional properties and viability. To a large extent these insights rely on animal models of kidney or cardiovascular diseases. In this review the current state of knowledge on these issues is summarized, and we aim to provide a possible new perspective on cardiovascular disease in chronic kidney disease.

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Chronic kidney disease (CKD) is associated with high cardiovascular morbidity and mortality. This increased risk of cardiovascular disease (CVD) only partially is explained by the high prevalence of traditional risk factors in patients with CKD, suggesting that other, CKD-specific factors must play a role.

α-Klotho (in this article referred to as klotho) was identified as an anti-ageing protein more than a decade ago. Defective klotho gene expression results in premature aging and a shortened life span, whereas transgenic mice overexpressing klotho have an extended life span compared with wild-type mice. As CKD progresses, klotho levels decrease. Loss of klotho appears to be a pathologic factor for the development of vasculopathy. The extracellular domain of membrane-bound klotho can be cleaved and shed into the circulation where it appears to be involved in CVD with a presumably protective role in the development of endothelial dysfunction and arteriosclerosis. In this review we describe the mechanisms by which klotho plays a role in the endothelial function and development of vascular calcification (VC).

The klotho gene encodes a single-pass transmembrane protein with a large extracellular domain, which is synthesized predominantly in the kidneys, but also is expressed in several endocrine organs including the parathyroid glands, pituitary gland, testis, ovary, placenta, and pancreas. The extracellular domain of klotho is cleaved by the membrane-anchored proteases A Disintegrin And Metalloproreinase 10 (ADAM10) and A Disintegrin And Metalloproreinase 17 (ADAM17), and secreted into extracellular fluids, including blood, urine, and cerebrospinal fluid, as shown in Figure 1. The tissue, or membrane-bound, klotho and secreted klotho have different functions. The membrane-bound form in the kidney acts as an obligatory cofactor for fibroblast growth factor (FGF)23 to activate the FGF receptors, although subsequent studies have shown that in the absence of klotho, FGF23 signal transduction in the renal tubular cells does not occur. This ectodomain can be cleaved and released into the circulation (shedding), where its enzymatic glucosidase activity. In the shed part of klotho this enzymatic function is contained and this probably explains its effects on distant calcium and phosphate channels and as such represents a novel mode of mineral homeostasis and mineral-mediated biological functions. Uremic toxin accumulation, such as indoxyl sulphate, can induce DNA methyltransferase protein expression, which is involved in the silencing of klotho through hypermethylation.

In human kidney biopsies and animal models, transmembrane klotho already is decreasing in CKD
Vascular calcification is a complex, regulated, cell-driven process that also involves numerous inhibitory and inducer proteins. Pathologically changed cells and a disrupted balance between local regulatory proteins leads to calcification. This in turn induces thickening and loss of elasticity of the large elastic and muscular arterial walls. Cellular mechanisms involved in vascular calcification consist of apoptosis of vascular smooth muscle cells (VSMCs), which is an early event, preceding actual calcification. In addition, VSMCs can undergo a phenotypic change to osteochondrogenic cells, secreting matrix vesicles, and collagen fibrils form along with apoptotic bodies, a starting nidus for early crystals containing hydroxyapatite deposits. These processes can occur in the tunica intima, the tunica media, or both. Intima calcification is seen mostly in the large arteriosclerotic plaques in the larger arteries. Media calcification (also known as Monckeberg sclerosis) can occur in arteries of any size and is characterized by diffuse mineral deposition along elastic fibers. Both forms can be seen in patients with CKD, however, media calcification is more typical and is encountered frequently. VC appears early in the course of CKD, but becomes much more prevalent as kidney function deteriorates. Interestingly, both calcium and phosphate play a prominent role in both the initiation of the phenotypic switch of cellular elements in the arterial wall, a proximal event in the process of vascular calcification, and also in the final common pathway of ectopic bone formation, which is the mineralization of primed matrix containing early nanocrystals.

Klotho deficiency in mice results in hyperphosphatemia, hypervitaminosis D, hypercalcemia, arteriosclerosis, and ectopic calcification including VC. Transgenic CKD mice overexpressing Klotho have better kidney function, enhanced phosphaturia, and less soft-tissue calcification as compared with wild-type mice with CKD. However, interpretation of the vascular consequences of animal models that modulate klotho expression is complicated by the fact that inevitably homeostasis of minerals, and possibly several endocrine axes, are disturbed dramatically. This precludes definitive proof from these models of direct effects of changed klotho expression. Keeping this caveat in mind, studies point to a key role for klotho in vascular calcification. Hu et al showed that this effect likely is owing to both direct and indirect actions of Klotho. The indirect effect comprises the interaction of klotho with the FGF23 receptor to retrieve the sodium-dependent phosphate cotransporters type IIa from the brush-border membrane of proximal renal tubular cells, leading to phosphaturia. Independent
from FGF23, soluble urinary klotho has been found also to regulate directly the phosphate transport, in the proximal tubule of the kidney by deglycosylation of sodium-dependent phosphate cotransporters type IIa, promoting its internalization.31 These effects of klotho on urinary phosphate excretion probably contribute to its inhibitory effects on calcification. Different from its effects on renal phosphate handling, klotho delivery in animal models appears to protect renal function, which might also contribute to lessening the burden of VC.21 Most intriguingly, recent data point to direct effects of klotho on VSMCs, a central player in the initiation and propagation of VC. In studies with rodents and CKD patients it has been shown that hyperphosphatemia induces VC.20,32,33 The type III sodium-dependent phosphate cotransporters, also known as PiT-1 and PiT-2, are expressed in a wide variety of tissues and are the major phosphate transporters found in VSMCs.34,35 They are induced by aldosterone and are blocked by spironolactone and shown to be involved in vascular calcification.36 Runx-related transcription factor 2 (RUNX2), also known as core-binding factor subunit α-1, is a key proximal transcription factor expressed in osteoblasts and involved in matrix mineralization. Its expression can be induced in other cells such as VSMCs, and this may lead to ectopic osteogenesis.37,38 In vitro studies with human smooth muscle cells showed that blockade or knock-down of the PiT-1 inhibits phosphate-induced calcium deposition and expression of the RUNX2 gene.39,40 In an experimental model of CKD, mice lacking klotho had increased PiT1 and PiT2 messenger RNA, while overexpressing klotho led to decreased PiT1 and PiT2 messenger RNA compared to wild-type mice. In addition, RUNX2 increased, and the smooth muscle marker 22 decreased in Klotho deficiency. The opposite effect occurred in Klotho overexpression: reduced phosphate entrance into VSMCs and reduced up-regulation of RUNX2.8 The same results were found in an in vitro study using human aortic smooth muscle cells.9

Interestingly, some studies have suggested the presence of klotho in the human arterial wall, where it also appeared to be protective against VC, in part mediated by FGF23.9 This possibility was supported by the notion that in FGF23 knock-out mice, aortic wall vitamin D–regulating enzymes were manipulated as in the kidney, also suggesting klotho-dependent FGF23 signal transduction.40 Other studies, however, excluded the presence of klotho in the vessel wall42 or showed that kidney-specific klotho KO completely duplicated the phenotype of the nonspecific KO model.43

ENDOTHELIAL DYSFUNCTION

The endothelium is a monolayer of cells lining the entire vascular system. It acts as a main regulator of vital functions of the cardiovascular system. The endothelium responds to mechanical stimuli from changes in blood flow and hormonal stimuli related to vasomotor regulation and, in turn, sends chemical signals to regulate blood flow, fluid and solute exchange, coagulation, inflammatory responses, vasculogenesis, and angiogenesis.44–47 A relaxed vascular tone and low levels of oxidative stress are maintained by releasing nitric oxide (NO), prostacyclin, and endothelin-1, and by controlling local angiotensin II activity. The endothelium also regulates vascular permeability, platelet and leukocyte adhesion and aggregation, and thrombosis.48 Endothelial dysfunction arises if the ability of the endothelial cells to maintain this delicate balance is compromised.44,48 This results in the production of reactive oxygen species, causing a decrease in the vascular bioavailability of NO and impaired flow-mediated dilatation of the vessels, increased intracellular Ca2+ concentrations promoting apoptosis, increased endothelial permeability, and expression of adhesion molecules, cytokines, and chemokines facilitating leukocyte attachment to the endothelium and transmigration into the extracellular matrix underneath.44,48,49 Endothelial dysfunction is an early event in atherosclerosis,50 and also is associated with kidney disease.

CHRONIC KIDNEY DISEASE AND ENDOTHELIAL DYSFUNCTION

Cardiovascular disease is the most common cause of death for patients with CKD or end-stage renal disease.1 In addition to hypertension, diabetes, and other classic risk factors, this risk arises from multiple pathogenic processes affecting the cardiovascular system, including endothelial dysfunction and vascular calcification.51,52 Endothelial dysfunction has emerged as an important link between CKD and the increased risk of cardiovascular events.53,54 In CKD, an important role in the development of endothelial dysfunction exists for asymmetric dimethylarginine (ADMA). ADMA is an endogenous and competitive inhibitor of NO synthase (NOS) because of its similarity with its natural substrate L-arginine, the source of NO. In vitro studies have shown that the administration of ADMA inhibits NO production.55 In addition, very recent animal studies have shown that ADMA can modulate endothelial NOS itself directly, providing another mechanism of decreased NO production.56 In CKD patients, circulating ADMA levels are increased and increase even further when kidney function deteriorates.57 This results in reduced NO production and a reduced flow-mediated dilatation of the vessels (FMD), which is a vascular motor function marker that is dependent on endothelium-derived NO.52

Phosphate, besides its well-recognized role in media calcification as described earlier, also influences
endothelial function. In a human study with 11 healthy volunteers, FMD was related inversely to serum phosphorus level. An animal study showed that in rats with CKD, hyperphosphatemia attenuated FMD, which was reversed after a low-phosphate diet. In another randomized study, 100 nondiabetic patients with stage 4 CKD were treated with sevelamer for 8 weeks, which led to increased FMD and this was associated with a decrease in serum phosphate concentration. This suggests that endothelial dysfunction and damage may be induced directly by hyperphosphatemia and may be restored partially with phosphate-binding therapy.

Deteriorating kidney function also is associated with increased oxidative stress and an inflammatory response, shown by an increase in inflammatory markers such as high sensitive C-reactive protein level, interleukin-6, and tumor necrosis factor-α. Oxidative stress and low-grade inflammation are implicated as well in the development of endothelial dysfunction in patients with CKD. Taken together, in patients with CKD, a decreased capability of producing NO together with increased serum phosphate levels, oxidative stress, and chronic low-grade inflammation results in endothelial dysfunction. This has been suggested as the initiating process in the development and progression of atherosclerosis and VC in CKD.

**KLOTHO AND ENDOTHELIAL FUNCTION**

Compared with the rapidly expanding data pointing to the potential role of klotho in the homeostasis of minerals and the process of medial layer calcification, its role in endothelial cells has not gained that much attention. However, shortly after its discovery it was shown that klotho exerts pleiotropic effects on the endothelium in maintaining endothelial wall integrity. Klotho protein has been found to regulate NO availability in the endothelium and suppress oxidative stress. The elegant study of Saito et al showed that an endothelium-dependent vasodilatory response to acetylcholine is attenuated in klotho-deficient mice and the excretion of urinary NO2 and NO3, end products of NO and indicators of the quantity produced, is reduced. This dysfunction can be restored by parabiosis experiments between wild-type mice and heterozygously Klotho-deficient mice, strongly suggesting that sKlotho is involved. In addition, in a completely different model of CVD in another species the role of klotho (a widely evolutionary preserved protein) in in vivo adenovirus-mediated Klotho gene delivery in Otsuka Long-Evans Tokushima Fatty rats (an atherosclerotic rodent model), NO production was increased and endothelial dysfunction improved. An increase in NO production also was shown after injection of Klotho plasmid in the tail vein of mice; this also reduced oxidative stress. The role of klotho as an anti-oxidant also may benefit endothelial cells. Ikushima et al showed that overexpression of Klotho in human umbilical vascular endothelial cells decreased oxidative stress–induced apoptosis and senescence. In addition, the addition of Klotho to cultured VSMCs of rats also reduced oxidative stress by decreasing intracellular superoxide and angiotensin II production, although these cells do not represent the endothelium. Most recently, it was shown that klotho activates endothelial NOS and also upregulated iNOS. This was accompanied by increased NO production in human umbilical vascular endothelial cells. In addition, these researchers showed that exogenous klotho applied to an ex vivo model of mouse aorta diminished phosphate-induced vasoconstriction, and that this effect was quick (requiring just over 3 minutes) and completely dependent on the endothelium (Fig. 2).

Beside its effects on functional properties of endothelial cells, klotho also is involved in structural properties such as endothelium integrity, viability,

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**Figure 2.** Ex vivo study of mouse arterial rings showing vasoconstriction with exposure to phosphate concentrations of 2 mmol/L. Increasing concentration of klotho not only prevented phosphate-induced constriction, but led to enhanced dose-dependent vasodilation. These effects of klotho are completely blunted by L-NMA, a competitive inhibitor of NO production, which suggests that these klotho effects are completely dependent on endothelial cells. Based on Six et al.
and subsequent vascular permeability or endothelial barrier function. In klotho-deficient mice, the endothelial wall is hyperpermeable with increased apoptosis and down-regulation of vascular endothelial cadherin surface expression. Calcium signals regulate the production of NO, permeability, proliferation, and apoptosis in endothelial cells. The transient receptor potential channel-1 (TRPC-1) is involved in vascular endothelial growth factor (VEGF)-mediated calcium entry. Klotho limits endothelial permeability by binding directly to both VEGF receptor -2 (VEGFR-2) and TRPC-1, and internalizing this complex in response to VEGF stimulation, thus limiting VEGFR/TRPC-1-mediated calcium influx, preventing the apoptosis induction. The possible importance of klotho deficiency was shown in that study by a deep penetration of Evan’s blue dye into the vessel wall after injection in klotho-deficient mice, as compared with klotho-replete mice. This loss of protection of sKlotho on the endothelial wall also might apply to patients with CKD because it has been shown that human klotho gene polymorphism is associated with arterial wall pathologic changes in hypertension. Klotho protein has been capable of ameliorating indoxyl sulfate–induced endothelial dysfunction, which may be partly through inhibiting the reactive oxygen species/p38 mitogen-activated protein kinase and downstream nuclear factor-κB signaling pathways.

CONCLUSIONS

The parallel discovery of both klotho deficiency in CKD and its fundamental and upstream role in key pathologic changes in the arterial wall has placed this protein in the center of interest and research in the nephrology community and beyond. Although currently based mainly on mechanistic animal experiments and in vitro studies, and although inconsistent epidemiologic data still exist, the concept of klotho deficiency in CKD as a prime culprit for cardiovascular complications is appealing. Currently, only indirect evidence points to a relevant role of low concentrations of soluble klotho in clinical mechanisms, both for functional endothelium-dependent properties and vascular calcification (Fig. 3). However, much additional research is required, that ranges from the development of improved assays and attempts to influence klotho expression and shedding, to interventional trials that

![Figure 3](https://example.com/Figure3.png)

**Figure 3.** Higher serum klotho levels in CKD patients are associated with (A) enhanced flow-mediated vasodilatation, (B) slower pulse-wave velocity (baPWV) as a marker of less-stiffened arteries, (C) smaller intimal-medial thickness (IMT), and (D) more frequent occurrence of the absence of the aortic calcification index (ACI). Derived from Kitigawa et al.**
modulate klotho expression and circulating levels with clinical end points.\(^1\)

**REFERENCES**


