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MINIREVIEWS

Implications of Klotho in vascular health and disease

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Abstract

Cardiovascular disease (CVD) is a prevalent condition in general population and the first cause of death overall. Klotho, a pleiotropic protein related to longevity that acts as a co-receptor of the fibroblast growth factor 23, has been proposed as a key regulator of the development of CVD. In the few clinical studies made, it has been observed a relationship between low levels of soluble Klotho and the occurrence and severity of CVD, as well as a reduction of cardiovascular risk when they are high. Also, different polymorphisms of human Klotho gene have been related to the incidence of cardiovascular events. Moreover, several experimental studies indicate that this protein acts in the maintenance of vascular homeostasis. Klotho improves endothelial dysfunction through promotion of NO production and mediates anti-inflammatory and anti-aging effects such as suppression of adhesion molecules expression, attenuation of nuclear factor-kappa B or inhibition of Wnt signaling. Furthermore, this protein is related to the attenuation of vascular calcification as well as prevention of cardiac hypertrophy. The expression of this protein in the vascular wall implies a new scenario for the treatment of vascular disorders. The purpose of this review is to provide an overview of the relationship between the Klotho protein and CVD, in addition to its role in the maintenance of functional vascular integrity.

Key words: Klotho; Cardiovascular disease; Vascular health; Aging; Endothelial dysfunction; Vascular calcification

Core tip: Cardiovascular disease (CVD) is the first cause of death worldwide. The anti-aging factor Klotho has been linked to the development of CVD since clinical studies relate circulating levels of Klotho with the appearance of vascular disease and different *Klotho* gene variants are associated with increased cardiovascular risk. Furthermore, Klotho is involved in promotion of vascular health through different mechanisms. The recent description of its expression in vascular tissue opens up new options for the treatment of cardiovascular diseases.

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INTRODUCTION

The cardiovascular disease (CVD) is highly prevalent in the general population and the leading cause of death worldwide[1], maintaining these projections in the future[2]. CVD broadly comprises coronary artery disease (CAD),

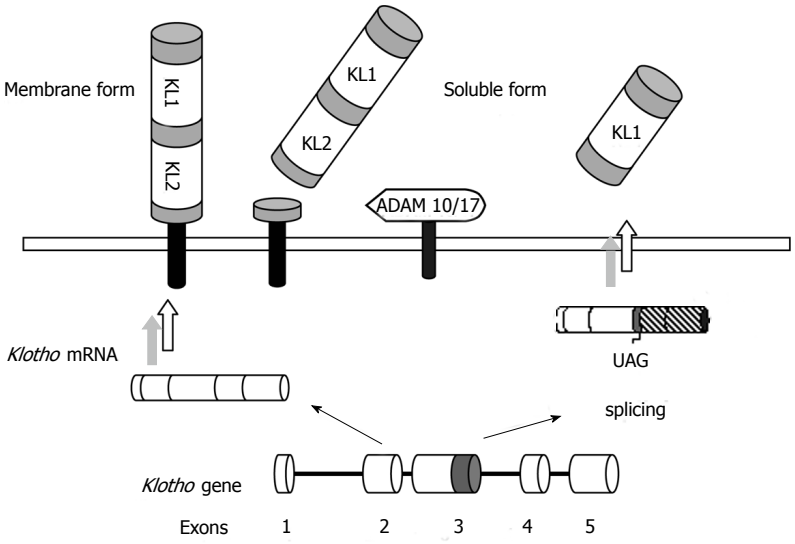

Figure 1 Mechanisms of generation of the different forms of Klotho. ADAM: Membrane-anchored A Desintegrin and metalloproteinase.

myocardial infarction, vascular stiffening and left ventricular hypertrophy[3].

Klotho, a gene originally identified in 1997 codifying for a novel anti-aging protein, has been implicated in a multitude of biological processes, most of them related to human longevity[4]. Mice lacking the *Klotho* gene develop a phenotype similar to premature human aging, which includes endothelial dysfunction, vascular calcification, progressive atherosclerosis and shortened lifespan[5]. A reduction in Klotho levels is observed in chronic kidney disease (CKD) patients, similar to other premature vascular aging diseases, such as hypertension or diabetes mellitus. Even normal aging is associated with a reduction in serum and urine concentration of Klotho[6-8].

The first function described for Klotho is its role in the metabolism of phosphorus as the obligatory co-receptor of fibroblast growth factor 23 (FGF23), a bone-derived hormone responsible of the phosphate balance in the body through promotion of renal phosphate excretion. Klotho directly binds to FGF receptors (FGFRs) constituting a high affinity complex for FGF23 which mediates the intracellular effects of this phosphatonin[9]. More recently, the involvement of Klotho in vascular protection through different mechanisms has been demonstrated. These mechanisms include inhibition of oxidative stress, modulation of inflammation or attenuation of vascular calcification[10-12]. Therefore, Klotho has been suggested as a master regulator of CVD[13]. The aim of this review is to provide an overview of what is known so far about Klotho and its relationship with CVD, besides its role in the maintenance of vascular homeostasis.

MOLECULAR CHARACTERISTICS OF KLOTHO

The human *Klotho* gene comprises 5 exons and is located in a region of approximately 50 kb on chromosome 13q12. This gene encodes for two possible transcripts: a full-length, translated into a single-pass transmembrane protein of 1012 amino acids (130 kDa), or an alternative spliced transcript, which encodes the N-terminal half of 549 amino acids (65-70 kDa) and is secreted to the extracellular space. Another form of soluble Klotho can also be generated through proteolytic cleavage of the transmembrane form by membrane-anchored A Desintegrin and metalloproteinase (ADAM) -17 and ADAM-10, so that the full-length extracellular domain is released into the circulation[14-17] (Figure 1). Soluble Klotho predominates in humans over the membrane form and is detectable in urine, serum and cerebrospinal fluid[18]. This circulating form acts as a humoral factor with multitude of functions such as anti-oxidation, modulation of renal ion channels, anti-Wnt signaling or anti-apoptosis and senescence effects[19].

The Klotho protein comprises an extracellular domain composed of two repeat sequences (KL1 and KL2), two short membrane-spanning regions (21 amino acids) and an intracellular carboxyl (11 amino acids) domain. The KL1 and KL2 sequences share 20%-40% sequence identity with the Family 1 glycosidases[4,16].

In humans, *Klotho* is mainly expressed in the kidneys, but its tissue distribution also includes brain, reproductive organs, pituitary gland, parathyroid glands, urinary bladder, skeletal muscle, placenta, thyroid gland, colon[4], and more recently described, human vascular tissue[20,21]. The membrane form mainly acts as the obligatory co-receptor for FGF23, thereby tissues expressing Klotho are potential targets for FGF23 to exert its actions[9,22,23].

CLINICAL ASSOCIATIONS OF KLOTHO AND CVD

Serum Klotho and CVD

Although the circulating levels of soluble Klotho have been initially proposed as biomarker of renal function, since some works show a decrease in serum levels during development of CKD[6], its association with cardiovascular risk has been less extensively explored.

In a first work, Semba *et al*[24] found that in community-dwelling adults higher plasma Klotho concentrations are independently associated with a lower likelihood of having CVD, defined as CAD, heart failure stroke, or

peripheral arterial disease. Likewise, in a recent study developed by our group, we observed that patients with significant CAD have lower soluble concentrations of soluble Klotho, as well as a reduced expression level of *Klotho* mRNA in the vascular wall. Besides, the reduced serum Klotho levels and decreased vascular gene expression were associated with the presence and severity of CAD independently of established cardiovascular risk factors such as age, diabetes, hypertension, smoking, dyslipidemia, and inflammation[25].

Moreover, Kitagawa *et al*[26] observed that serum Klotho level is an independent determinant of marked arterial stiffness but not of other types of vascular dysfunction such as atherosclerosis, endothelial dysfunction or vascular calcification, in CKD patients[26]. In contrast, in a very recent work, Seiler *et al*[27] found no significant relationship between soluble Klotho and cardiovascular outcomes in a CKD stages 2-4 cohort.

Taken together, these studies suggest that a reduction in the levels of soluble Klotho may promote or encourage the development and progression of CVD, while high levels of this factor prevents the risk of CVD. In any case, further studies are needed to clarify the relationship between circulating Klotho levels and cardiovascular risk.

Genetic variation of Klotho and CVD

Genetic variation studies have demonstrated that *Klotho* gene polymorphisms might be associated with longevity[28] and CAD[29-32]. In particular, the KL-VS allele, characterized by six SNPs in a region of 800 bp in exon 2 and flanking sequence, is prevalent in the population and is associated with a reduced longevity[28]. In a study where two different groups of healthy siblings were tested, Arking *et al*[29] found that this functional variant of *Klotho* gene is an independent risk factor for CAD. The risk associated with this allele is modulated by modifiable risk factors, such as hypertension, increased high-density lipoprotein cholesterol levels or smoking[29]. Likewise, in an Ashkenazi Jew group it was found that homozygous KL-VS individuals were at higher risk of stroke than wild-type subjects[33].

In the case of G-395A polymorphism, the A allele has been found to be an independent predictor of atherosclerotic CAD but not of vasospastic angina in Japanese population[30]. This polymorphism affects the promoter of the *Klotho* gene, so that the G→A substitution impairs protein binding to the region and consequently affects gene expression[34] and soluble Klotho levels. Similarly, Jo *et al*[32] observed an association of the G-395A allele with CAD but not with coronary artery calcification in Korean patients. Besides, subjects with the T allele for the C1818T polymorphism (located in exon 4) have lower prevalence of CAD than those with CC genotype[31].

MECHANISMS OF VASCULAR PROTECTION

Endothelial dysfunction (NO production)

One of the first vasculoprotective activities described for Klotho is its role in maintenance of endothelial homeostasis. $Kl^{+/-}$ mice show attenuated aortic and arteriolar vasodilatation, which can be increased after two weeks of parabiosis with wild type mice[35]. Moreover, these *Kl* heterozygous mice show a significantly reduction of urinary excretion of NO_2^- and NO_3^- (NO metabolites), suggesting a decrease in NO production[35]. In Otsuka Long-Evans Tokushima Fatty rats, an animal model which displays multiple atherogenic risk factors, adenovirus-mediated *klotho* gene delivery results in improvement of aortic relaxation and increased NO production[36]. These findings point to a direct involvement of Klotho in improving endothelial dysfunction through pathways involving NO. Consistent with this, Shimada *et al*[37] observed impaired angiogenesis, a NO-dependent process, and reduced endothelium-derived NO release in *kl/kl* mice.

This reduction of NO mediated by Klotho deficiency can be due to its accelerated degradation because of increased oxidative stress associated with aging. Klotho is able to increase resistance to oxidative stress inducing expression of manganese superoxide dismutase (Mn-SOD) through activation of FoxO forkhead transcription factor[38]. In regard of this, Klotho increases Mn-SOD activity and NO production *via* c-AMP-PKA-dependent pathway in human umbilical vascular endothelial cells (HUVECs)[10], and it also reduces H_2O_2-induced apoptosis and cellular senescence[39]. Likewise, Klotho transfection of cultured vascular smooth muscle cells (VSMCs) also reduces superoxide production and decrease angiotensin Ⅱ-induced oxidative stress[40].

Another possibility is that Klotho regulates expression levels of the endothelial NO synthase (eNOS). Six *et al*[41] recently observed that attenuation mediated by Klotho of FGF23 or phosphate-induced vasoconstriction is abolished by adding nitro-L-arginine, a competitive inhibitor of NOS. Moreover, they observed that exposure of HUVECs to Klotho increased NO production and induced eNOS phosphorylation and iNOS expression. Interestingly, Klotho was able to increase H_2O_2 production in cultured human VSMCs (HVSMCs), which suggests a more complex effect of this protein on the regulation of vascular tone through mediation of a ROS/NO balance[41].

Aging and inflammation

Inflammation is a central process in CVD[42,43] and Klotho has been suggested to play a protective role in the vessels since it mediates anti-inflammatory actions. In cultured HUVECs, incubation with Klotho results in suppression of expression of cell adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)[11]. These Klotho effects in ECs also include attenuation of the activation of NF-κB and blockade of tumor necrosis factor-α induced monocyte adhesion[11]. Likewise, the intracellular form of Klotho is capable to inhibit RIG-I-induced expression of interleukin (IL)-6 and IL-8 both *in vitro* and *in vivo*[44].

Moreover, it is known that soluble form of Klotho is able to bind to various members of Wnt family, and

thereby suppress Wnt biological activity[45,46]. Although this signal is essential for stem cells proliferation, continued activation of Wnt can contribute to cellular depletion and accelerated cellular senescence[47]. Therefore, Klotho could exert an anti-aging effect by attenuation of Wnt signaling, preventing cellular senescence[48].

Vascular calcification

Vascular calcification (VC) is one of the major complications of CKD and is associated with mineral and bone disorders. Since CKD patients, who have low levels of Klotho protein, and Klotho-deficient animals develop medial vascular calcification[4], the absence of this protein has been associated with the appearance of VC. Initially, the involvement of Klotho in the protection against VC was believed to be related to its role in the regulation of phosphate metabolism as co-receptor for FGF23. However, in recent years Klotho has shown to have direct effects on vasculature to prevent this pathology.

High levels of extracellular Pi induce mineralization of VSMCs through inorganic Pi influx mediated by cotransporters NaPi type 3 (Pit-1 and Pit-2)[49]. This process is accompanied by overexpression of osteogenic markers, such as RunX2, which leads to dedifferentiation of VSMCs[50,51]. In 2011, Hu *et al*[12] found that Klotho deficiency in mouse involved increased arterial calcification, and aortic downregulation of *SM22* (a smooth muscle cell marker) expression and upregulation of the transcripts for *Pit-1*, *Pit-2* and *RunX2*. A similar expression profile was observed in the mouse model of CKD, which was prevented by Klotho overexpression. Moreover, addition of recombinant soluble Klotho to rat VSMCs cultured in high-Pi decreased aortic calcium content and Na^+-dependent Pi uptake, confirming Klotho direct modulation of NaPi-3 activity[12]. Administration of exogenous Klotho protein to *kl/kl* mice also attenuates aortic calcification[52]. Therefore, it seems that Klotho prevents vascular calcification through mediation of NaPi-3 cotransporters activity and modulating VSMCs differentiation.

Consistent with this, Lim *et al*[21] confirmed the importance of Klotho in arterial calcification in a study where they found that silencing of Klotho in human aortic smooth muscle cells (HA-SMCs) leads to increased calcification[21]. Interestingly, treatment with vitamin D receptor activators (VDRAs), such as calcitriol or paricalcitol, restores Klotho expression in pro-calcific cultured HA-SMCs and increases serum and urine Klotho in uremic mice[21,53]. This VDRA therapy is associated with improved aortic medial calcification and increased osteopontin expression, an anticalcification factor[53].

Cardioprotection

Cardiac hypertrophy is a high prevalent pathological condition among end stage renal disease patients, which leads to cardiac dysfunction and death[54-56]. Stress signals induce abnormal growth and remodeling that progress to heart failure. Klotho is involved in cardioprotection since its deficiency produce an exaggerated cardiac hypertrophy caused by isoprotenerol (ISO) injection in mice[57]. Likewise, its administration ameliorates ISO-induced structural changes in mouse hearts, *e.g.*, disordered arrangement of myocardial fibers, fibroblastic hyperplasia, mononuclear cell infiltration or interstitial and perivascular fibrosis[58].

This cardiac protection by Klotho occurs through downregulation of TRPC6 channels, whose overexpression causes aberrant cardiac development and premature death[57]. Moreover, cardiomyocyte apoptosis is an important process in cardiac remodeling[59] and Klotho is able to suppress it by downregulation of endoplasmic reticulum stress and ROS production[58].

KLOTHO EXPRESSION IN THE VASCULAR WALL

In recent years, the detection of Klotho in human vascular tissue[20,21,60] has extended the range of putative target tissues of FGF23 actions. Coexpression of two cognate FGF23 receptors, FGFR-1 and -3 in the vascular wall, along with Klotho[21], supports this idea. Furthermore, expression of Klotho protein appears to be limited to medial layer of the vessel, since it is detected by immunohistochemistry in tunica media of healthy subjects arteries[21] or in rat aorta[61], and by western blotting in human VSMCs[21]. Likewise, *Klotho* mRNA is detected in cultured HVSMCs rather than human vascular endothelial cells[60].

However, there are conflicting data which have led to a debate about the presence of Klotho in the vascular tissue. Scialla *et al*[62] detected no expression of Klotho in human or mouse VSMCs, neither in mouse aortas. Moreover, Lindberg *et al*[63] detected only low levels of Klotho transcript in different vascular tissues (aorta, mesenteric, femoral and lung arteries) and without significant differences between wild type and *Sm22-KL*$^{-/-}$ mice (a new experimental model with targeted deletion of Klotho in VSMCs). In this study, protein expression was undetectable in vascular tissue by immunohistochemistry or western blotting, and the absence of expression of Egr-1 in aortas of mice after injection of FGF23 indicates the lack of a functional Klotho-FGF23 signaling complex in vascular tissue[63]. Conversely, Fang *et al*[64] demonstrated vascular expression of Klotho in low-density lipoprotein-deficient (*ldlr*$^{-/-}$) mice. In another study, Jimbo *et al*[61] demonstrated expression of Klotho protein in rat aortas but not in isolated VSMCs. Furthermore, they showed that extracellular signal-related kinase 1/2, an enzyme activated by FGF23 in Klotho-expressing cells[65], was phosphorylated by FGF23 in a dose-dependent manner in Klotho-overexpressing VSMCs but not in isolated VSMCs, suggesting that presence of Klotho only occurs in contractile VSMCs[61].

Some studies show a decreased Klotho vascular expression in CKD, similar to early reduction of this protein in the kidney during the disease[12]. Lim *et al*[21] observed a marked reduction of Klotho protein expression in arteries from patients with CKD. Furthermore, they showed that exposure of HA-SMC to uremic serum

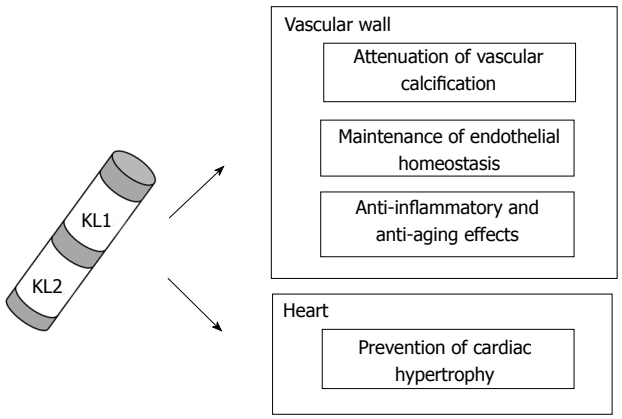

Figure 2 Mechanisms of vascular protection mediated by Klotho.

from patients with CKD, or to different conditions recalling CKD like hyperphosphatemia, hypercalcemia or proinflammatory stress, significantly reduced Klotho protein[21]. Moreover, Fang *et al*[64] also observed a reduction of Klotho activity in the aorta of a mice model of early CKD, although serum Klotho levels were increased. This decrease of vascular Klotho during disease could involve a FGF23 resistance state in the vascular bed. In contrast, Jimbo *et al*[61] showed that Klotho remained unchanged in aortas of nephrectomized rats.

As already suggested, all these discrepancies can be due to differences in experimental settings, like issues regarding specificity and sensitivity of anti-Klotho antibodies, different vasculature segments analyzed or differences in cell culture conditions, as well as, variance in CKD stage[66]. Although further studies are needed to characterize the vascular expression of Klotho in animal models, healthy subjects and CKD patients, as well as its stability under *in vitro* and *ex vivo* conditions, the set of results obtained so far seem to suggest that this tissue is sensitive to FGF23 and that CKD is a state of vascular Klotho deficiency. It is also interesting to note the relationship between the expression in human thoracic aorta tissue of vascular Klotho and ADAM-17[20], one of the metalloproteinases responsible for the shedding of Klotho from the cell surface, which suggests the possibility that vascular wall is a source of soluble Klotho, and therefore an important element in vascular protection.

CONCLUSION

Klotho is a novel factor involved in longevity and aging, which also has a central role in regulating phosphorus metabolism acting as co-receptor for FGF23[4,9]. But beyond these roles, several clinical studies have linked this protein to the development and progression of CVD. The reduction of circulating levels of Klotho is associated with the presence and severity of CAD and is also an independent marker of some forms of vascular dysfunction such as arterial stiffness[25,26]. Likewise, various genetic studies have shown the association between gene variants of human *Klotho* gene with CAD or stroke[29-32].

Klotho is involved in the protection of vasculature through various mechanisms, including prevention of endothelial dysfunction, anti-inflammatory effects, reduction of vascular calcification or attenuation of cardiac hypertrophy[11,12,35,58] (Figure 2). The disruption in the homeostasis of this factor seems to be a key element in the development of CVD. Furthermore, Klotho expression in the vessel wall, along with the enzymes responsible for generating its soluble form[20,21], makes the vascular context a new scenario to be considered for the treatment of vascular diseases.

The central role of Klotho in the development of CVD makes its possible use promising as a diagnostic biomarker or as a therapeutic factor for treatment of vascular diseases. However, further studies are needed to clarify the relationship between this factor and promotion of vascular health.

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