The potential roles of FGF23 and Klotho in the prognosis of renal and cardiovascular diseases

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Abstract
Fibroblast growth factor (FGF) 23 and Klotho are two factors associated with several metabolic disorders. Similar to humans, accelerated aging processes characterized by chronic vascular disease, bone demineralization, skin atrophy and emphysema have been recognized in FGF23-null mice and Klotho-deficient mice.

The role of these factors in the control of mineral metabolism homeostasis have been shown recently, particularly at the level of parathyroid cells and also in modulating active vitamin D production, two phenomena which are relevant in the presence of chronic kidney disease. In addition, the hormonal affect of circulating FGF23 and Klotho proteins on vascular reactivity, either directly on endothelial cell functions or indirectly by modulating the brain endothelin-1-dependent sympathetic nervous system activity, has contributed to understanding their role in the pathophysiology of hypertension and atherosclerotic vasculopathies. Consequently, very recent clinical investigations seem to confirm the involvement of Klotho in modulating the severity and prognosis of human cardiovascular (CV) disorders and longevity.

The present review reports data related to the possible interactive effects of Klotho and FGF23 on the prognosis of renal and CV diseases.

Keywords: cardiovascular; FGF23; hypertension; Klotho; renal

Numerous experimental studies over the last decade have shown the increasing importance of the interactive Klotho fibroblast growth factor (FGF) 23 endocrine systems in controlling renal phosphate absorption and renal calcitriol production [1]. In addition, it was demonstrated that these new biological partners might play a role in cardiovascular (CV) system activities through direct and indirect effects on cardiac activity, cardiac structures, endothelial cell functions and blood pressure control [2, 3].

Klotho and FGF23 may function in a common single transduction pathway. The Klotho–FGF receptor complex binds to FGF23 with higher affinity than either one alone.

The clinical, pathological and laboratory observations in Klotho-null mice and FGF23-deficient mice showing a shortened life span, soft tissue calcifications, abnormal iron and vitamin D homeostasis, early arteriolosclerosis, atherosclerosis, vascular calcifications, impaired angiogenesis and vasculogenesis, [4] suggest that this pairing has a large impact on the pathophysiology of renal and CV disorders.

FGF23, a phosphatonin, is a 251 amino acid peptide with a molecular weight (MW) of 32 kDa. It belongs to the family of FGF and is phylogenetically close to FGF19 and FGF21. Bone is the main source of FGF23. In addition to osteocytes and osteoblasts, FGF23 messenger RNA (mRNA) is expressed in cells of different organs, such as hepatocytes, cardiac cells and thyroid–parathyroid cells. The Klotho gene encodes a 1014 amino acid protein with a long extracellular NH2 extremity consisting of ~980 amino acids, a single-pass transmembrane domain, and a very short (11 amino acids) cytoplasmic residue at the carboxy terminal region [5].

Klotho is expressed predominantly by the kidneys in humans, mouse and rats, although it is also detectable in the brain, pituitary and parathyroid glands, testes and ovaries, placenta, small intestine and colon, prostate, urinary bladder, skeletal muscles and pancreas [6, 7]. Levels decline with age in humans [8].

FGF23 in chronic kidney and CV diseases

FGF23 levels are elevated in the serum of patients with chronic kidney disease (CKD) and may contribute to the control of blood phosphate concentrations which tend to be elevated when glomerular filtration rate (GFR) declines [9–11]. In addition, it may serve as a negative regulator of parathyroid hormone (PTH) synthesis and secretion, which may delay the development of secondary hyperparathyroidism [12]. In fact, FGF23 increases in the blood of CKD patients, even at the early stage of the disease, before changes in phosphate and calcium blood concentrations are detectable [10, 11]. In a cohort
of 227 white patients 18–25 years of age, with non-diabetic CKD, 53% with Stages 1–2 CKD, FGF23 was identified as a risk marker for the progression of CKD. FGF23 levels were estimated by measuring either C-terminal fragment or intact FGF23 [9]. The results were highly significant after adjusting for GFR. Therefore, FGF23 may serve as an indicator for early undetected alterations of calcium–phosphate metabolism and as a predictor of CKD progression.

In dialysis patients, a marked elevation of FGF23 serum concentrations may predict the occurrence of refractory secondary hyperparathyroidism. A recent study using sera obtained from 99 chronically hemodialyzed patients showed that FGF23 levels increased during dialysis, even after correction (hemocentration observed at the end of the treatment) and this was even in the presence of lower phosphate blood levels. FGF23 was undetectable in the dialysis fluid [13]. The prehemodialysis FGF23 serum level did not correlate with dialysis efficacy, measured by Kt/V, and no correlation was found between FGF23 and bone mineral density (absolute values, T or Z scores) at different bone sites. There were no differences when the analysis was performed by gender. Markers (laboratory tests) of bone remodeling correlated with PTH concentrations in serum, but not with FGF23. The high levels of FGF23, frequently 100- to 10 000-fold above the values measured in the serum of healthy controls, could not be explained by hyperphosphatemia. The fact that renal function decreased in these patients may explain why high FGF23 levels are found in the blood of hemodialyzed patients considering that there is no FGF23 dialysance [13]. Most circulating FGF23 in CKD patients is biologically active, even at dramatically elevated concentrations. The level of immuno-reactive FGF23 determined by standard assays, particularly that measuring intact FGF23, may be considered as an accurate predictor of circulating bioactive FGF23 [14]. The more severe cases of secondary hyperparathyroidism (and/or hyperphosphatemia) in dialysis patients are associated with extremely elevated FGF23 levels [14]. A recent report has mentioned that depressed expression of Klotho and FGFR1 proteins and mRNA were decreased in the parathyroid after 6 weeks. These parathyroid glands were nonreactive FGF23 determined by standard assays, particularly that measuring intact FGF23 [9]. The results were highly significant after adjusting for GFR. Therefore, FGF23 may serve as an indicator for early undetected alterations of calcium–phosphate metabolism and as a predictor of CKD progression.

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The possible impact of FGF23 on the CV system has also been evaluated in nonuremic populations. Two studies performed in Uppsala revealed an association between higher serum concentrations of FGF23, atherosclerosis and impaired vasoreactivity [23, 24]. This was observed with normal renal function and mineral metabolism [23]. In addition, an association between higher FGF23 and arterial stiffness was detected in a group of subjects with estimated GFR <60 mL/min/1.73m². This correlation was noted even when the FGF23 blood levels were in the normal range. In this population who were at least 70 years of age, FGF23 also correlated with left ventricular mass index [2].

In fact, the associations between FGF23 blood levels, left ventricular mass index and left ventricular hypertrophy (LVH) were found to be independent of renal function and mineral status. The relationship between FGF23 and LVH was independent of other LVH risk factors such as hypertension [25].

The effects of Klotho on endothelial function

Klotho mRNA and protein are predominantly expressed in the kidney at the level of the distal convoluted tubules, and in the brain at the level of the choroid plexus. Extracellular Klotho is detected in the blood and cerebral fluid in mice and humans, showing the presence of a circulating protein. Klotho protein has been found to increase nitric oxide (NO) availability and to protect against endothelial dysfunction even when not expressed in blood vessels [3]. The vascular impact of circulating Klotho has been well demonstrated in heterozygous Klotho-deficient mice, which are characterized by attenuated arteriolar vasodilation and aortic relaxation responses to acetylcholine and by lower urinary excretion of NO₂ and NO₃. These dysfunctions were normalized by successful parabiosis between...
wild-type and heterozygous Klotho-deficient mice, suggesting the existence of a humoral Klotho-related protein present in wild-type mice, which improved the deficient responses observed in Klotho-deficient mice [3]. The normalization observed by parabiosis may be NO dependent, but improved NO production does not contribute to the antioxidative action of Klotho observed in male mice and in spontaneous hypertensive rats (SHR) rats overexpressing Klotho. Klotho gene delivery by plasmid infusion modified the animal oxygen stress status as shown in Klotho-treated mice, where aortic superoxide dismutase (SOD) protein expression and activity were increased, circulating Klotho protein elevated and lipid peroxide production decreased. Similar findings were detected in SHR rats overexpressing Klotho. Lipid peroxide concentrations were depressed in the kidneys and liver. This effect was not modified by l-arginine methyl ester administration, a phenomenon which confirms that it was not NO dependent [26]. This antioxidative action may in fact be related to a blunting effect of Klotho on the insulin/insulin growth factor-1 pathway and through stimulation of manganese SOD [27].

**The effects of Klotho on kidney injury and repair**

The positive effect of Klotho on diseased kidneys has been reported in experimental studies [28–30], where overexpression of Klotho improved the renal injury observed in mice with glomerulonephritis or ischemic acute kidney injury [29, 30]. In SHR, a genetic model of hypertension with many features of human essential hypertension (characterized by age-related hypertension and end-organ damage), the renal expression and production of Klotho is markedly suppressed. Klotho gene delivery leading to Klotho overexpression, improved SHR renal injury and decreased proteinuria and blood creatinine levels, at Week 12 [28].

This improvement in renal structures and functions was not only due to Klotho-related antihypertensive action, the blood pressure being incompletely controlled, but also to improved renal SOD production and activity [28]. Decreasing blood levels of Klotho protein detected in humans after age 40 is comparable to the depressed expression of Klotho in aged SHR rats that develop pathological hypertension and kidney changes. This period of human life is characterized by an increasing prevalence of hypertension and CV diseases, which may affect life span [8]. Interestingly, Klotho gene delivery in SHR rats was found to prevent the progression of hypertension and to depress vascular superoxide production and aerobic nicotinamide adenine dinucleotide phosphate oxidase activity. In addition, plasma interleukin-10 was upregulated. This interleukin is active in modulating blood pressure [31].

As angiotensin-2 remains a prominently recognized factor in the development of hypertension, an experimental study using a continuous infusion of angiotensin-2 for 14 days in male Sprague–Dawley rats was performed to evaluate the effect of angiotensin-2 on Klotho. The results showed that the renal expression of Klotho mRNA and protein was down regulated in the angiotensin-2-treated animals [25]. This effect was reversed completely using losartan, an angiotensin-2 receptor blocker. When hydralazine, an antihypertensive agent that does not affect the angiotensin system, was prescribed, Klotho expression remained depressed. When norepinephrine was infused in Sprague–Dawley rats to induce similar elevations of blood pressure, no changes in the tubular expressions of Klotho were detected, but the administration of nonhypertensive doses of angiotensin-2 depressed Klotho expression, confirming the particular impact of angiotensin-2 on Klotho.

In rats infused continuously with hypertensive doses of angiotensin-2, there was an elevation in urinary protein excretion and a decrease in creatinine clearance within 7 days. After an in vivo Klotho gene transfer, daily proteinuria was normalized and the histopathological kidney changes observed in the angiotensin-2-treated animals improved [25].

A down regulation of renal Klotho expression was observed in animal models of low-renin hypertension such as found in deoxycorticosterone acetate salt-hypertensive rats and of normal renin hypertension as found in SHR rats, as mentioned previously [32]. Other mechanisms may therefore down regulate Klotho expression. The NO-dependent effect of Klotho has been confirmed in different rat models. An adenviral Klotho expression plasmid may improve vascular endothelial dysfunction, thereby stimulating NO synthesis and reducing blood pressure in a model with multiple atherogenic risk factors [4]. Klotho recombinant protein induced higher endothelial cell viability and less apoptosis and decreased caspase 3 and caspase 9 activities, which are involved in apoptosis processes [33]. Activation of Mn-SOD is also protective by diminishing the impact of H2O2 on apoptosis [27]. Recent studies showed that the expression of p53/p21, a pathway involved in cellular senescence, is depressed in Klotho-treated endothelial cells. In summary, Klotho protein has anti-apoptotic and anti-senescent effects on endothelial cells [33].

**The effects of Klotho on vascular calcifications**

Vascular dysfunction may be also due to vascular calcifications. Hypertension, diabetes and hyperlipidemia are some of the various risk factors for vascular calcifications. However, chronic renal failure is the main risk factor for increased vascular calcifications. This has been observed in CKD patients and more frequently in those chronically dialyzed [34]. Recent studies have shown that CKD may be a state of Klotho deficiency. In humans, urinary Klotho protein decreases when renal function declines [35]. Lower values are detected very early in the process, even at an early stage such as CKD-1 [36]. When the GFR decreases, there is a progressive drop in Klotho excretion, the lowest values being detected in CKD-5 patients [36]. The decreasing Klotho production may partially explain the more frequent and severe vascular calcification processes observed in CKD patients [35]. This concept has been reinforced by results obtained recently in experimental studies. In heterozygous Klotho-deficient mice with CKD induced by uninephrectomy and ischemia–reperfusion injury of the remaining kidney, the pathological CKD-related
laboratory and clinical manifestations characterized by overt proteinuria, low creatinine clearance, high creatinine blood levels, increased organ calcium deposition and high blood pressure, were more severe compared to that observed in CKD wild-type animals. In Klotho-overexpressing transgenic mice, the CKD-related changes clearly improved, particularly the calcifications observed in organs and vessels [35]. In fact, in Klotho-overexpressing CKD mice, almost no aortic calcifications could be detected compared to the obvious calcifications found in CKD Klotho-deficient and CKD wild-type mice with similar renal dysfunction. In addition, Klotho overexpression decreased the rate of CKD progression in parallel to the decrease in vascular calcifications. In vitro studies demonstrated a direct effect of Klotho protein on vascular smooth muscle cells, suppressing calcification and maintaining differentiation [35]; Masuda et al. created a model of Klotho-deficient mice carrying inducible transgene depending on zinc water feeding for Klotho expression. Untreated animals developed the phenotype of Klotho−/− (KL−/−) including vascular calcifications and were rescued when Klotho expression was induced by zinc feeding. In this model, supplementation with Klotho reversed the age-associated established medial calcification of the aorta [37]. More generally, Klotho may have a direct protective effect on soft tissue calcification.

The effects of Klotho on blood pressure regulation

Klotho may also have indirect effects on vasculature. A recent study suggested a possible role of brain Klotho in controlling blood pressure [38]. As the prevalence of hypertension and related CV diseases is more frequent in cold regions and during winter, chronic exposure to moderate cold may also cause hypertension in rats, activating the sympathetic nervous system (SNS). An experimental research protocol was performed in male Sprague–Dawley rats maintained in a cold environment. Chronic infusion of endothelin-1 (ET1) in the cerebral lateral ventricles of conscious rats was able to provoke a progressive increase in blood pressure and to increase the urinary excretion of epinephrine and norepinephrine, suggesting activation of autonomic vasomotor centers. The effect of ET1 could therefore be mediated by stimulating efferent sympathetic nerve activity. Intracerebroventricular injection of adeno-associated virus with Klotho-shRNA and/or ET1-sh RNA induced silencing of brain Klotho and/or of brain ET1. Silencing brain Klotho resulted in an earlier elevation of blood pressure in cold-exposed animals [38]. Silencing brain ET1 negated this effect. Silencing brain Klotho was characterized by decreased choroid plexus Klotho mRNA and protein expression and enhanced ET1 expression. When silencing ET1 also, these phenomena were eliminated. Silencing Klotho induced upregulated expression of ET1, which may be at least in part the cause of SNS activation, as suggested by the consequent elevation of norepinephrine plasma levels. After silencing ET1, these changes did not occur. In summary, silencing brain Klotho did not alter blood pressure at room temperature, but potentiated cold-induced hypertension. The role of brain ET1 in regulating SNS activity is clinically relevant and important in facilitating the development of hypertension in cold conditions.

Klotho may be involved in the pathogenesis of hypertension as a circulating hormone, affecting endothelial functions and as a cerebral-acting agent modulating ET1 expression and consequently controlling SNS activity. In addition, Klotho, a beta-glucuronidase capable of hydrolyzing steroid beta-gluronides [5], may also alter the gene expressions of angiotensin-converting enzyme and of plasminogen activator inhibitor type 1 [39].

The effects of Klotho on clinical outcomes

In humans, >10 single-nucleotide polymorphisms (SNP) in the Klotho gene have been associated with arteriosclerotic diseases, including hypertension. A human Klotho gene variant named KL-VS, which contains six SNP sites, was found to be functional; the homozygous carriers of KL-VS were associated with CV risk factors, including low high-density lipoprotein cholesterol, high systolic blood pressure [40], early-onset coronary artery disease [40] and stroke. The impact of the KL-VS allele on longevity, CV risk factors and CV events has also been evaluated in a cohort of 525 elderly American Jews. A heterozygous advantage for survival was noted at later ages, its role in human longevity being strongly evident in the 216 subjects >95 years of age [39]. Recent investigations focused on the G-395A polymorphism in the promoter region of Klotho. The association of the Klotho gene with hypertension, coronary artery disease, cardioembolic stroke and even with vascular access dysfunction in male hemodialyzed patients was confirmed [41, 42]. Vascular access dysfunction occurred earlier and was more common in the A-allele carriers. These studies focused on the Klotho haplotype KL-VS, which contains two coding polymorphisms. SNP rs9536282 tags these coding polymorphisms. A recent study tested the association between 12 SNPs in the Klotho gene and mortality, in a cohort of hemodialyzed patients followed for 1 year. Blood samples from 1307 white and Asian incident dialysis patients were obtained. After 1 year of follow-up, 202 patients died (15.5%). SNP rs9536282 did not show a relationship to hemodialysis mortality, but the CC genotype of one tag SNP, rs577912, was associated with higher risk of death compared with the AA/AC genotypes. Testing lymphoblast cell lines from HapMap subjects genotyped at rs577912, the cell lines from subjects who had AA or AC at rs577912, expressed higher levels of Klotho mRNA compared to cell lines from subjects with CC at rs577912 (27% higher Klotho expression) [43]. In patients with essential hypertension, the frequency of A-allele carriers was increased in patients <60 years of age compared to that found in patients >60 years. In fact, the 395A variant of the G-395A SNP may be protective against essential hypertension by upregulating Klotho expression. Patients with the 395 A-allele carrier have lower brachial ankle pulse wave velocity. The conclusion may be that the G-395A polymorphism of the human Klotho gene may affect vascular function and therefore contribute to the susceptibility to
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