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Klotho: a new therapeutic target in diabetic retinopathy?

Role of Klotho in Diabetic Retinopathy

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Abstract

Klotho (Kl) is considered an antiaging gene, mainly for the inhibition of the Insulin-like Growth Factor-1 signaling. Klotho exists as full-length transmembrane (mKl), which acts as co-receptor for FGFR, and in soluble forms (sKl). The sKl may exert pleiotropic effects on organs and tissues by regulating several pathways involved in the pathogenesis of diseases associated with oxidative and inflammatory state. In diabetic Patients, serum levels of Klotho are significantly decreased compared to healthy subjects, and are related to duration of diabetes. In Diabetic retinopathy (DR), one of the most common microvascular complications of type 2 diabetes, serum Klotho levels are negatively correlated with progression of the disease. A lot of evidences showed that Klotho regulates several mechanisms involved in maintaining homeostasis and functions of retinal cells, including phagocytosis, calcium signaling, secretion of Vascular Endothelial Growth Factor A (VEGF-A), maintenance of redox status, and melanin biosynthesis. Experimental data have been shown that Klotho exerts positive effects on several mechanisms involved in onset and progression of DR. In particular, treatment with Klotho: 1) prevents apoptosis induced by oxidative stress in human retinal endothelial cells and in Retinal Pigment Epithelium (RPE) cells; 2) reduces secretion of VEGF-A by RPE cells; 3) decreases subretinal fibrosis and preserves autophagic activity. Therefore, Klotho may become a novel biomarker and a good candidate for the treatment of DR.

Key Words: Klotho; Diabetic Retinopathy; Retinal Pigment Epithelium; Vascular Endothelial Growth Factor A; Epithelial to Mesenchimal Transition; Ocular neovascularization.

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Core Tip: In diabetic Patients, serum levels of Klotho are significantly decreased compared to healthy subjects. Moreover, serum Klotho levels are negatively correlated with worsening of DR. Several evidence suggest that retina homeostasis may be affected by altered expression of membrane Klotho, as well by reduced levels of soluble Klotho. In this review we focused on the role of Klotho in DR, highlighting the importance of Klotho in maintaining retinal homeostasis and its positive effects on several mechanisms involved in DR onset and progression. Therefore, Klotho could be a novel biomarker and a good candidate for the treatment of DR.

INTRODUCTION

KLOTHO

The name Klotho (Kl) derives from that of the youngest of the Three Fates who spins the thread of human life [1]. Indeed, it is considered an antiaging gene, since phenotypes of mice with mutation in this gene are similar to those of patients with premature-ageing syndromes. Klotho shares sequence similarity with members of the glycosidase family 1 and it has been reported to function as a novel β-glucuronidase [2, 3]. It encodes for 3 proteins: α-Klotho, β-Klotho and Klotho-related protein (Klrp) [4]. β-Klotho is mainly expressed in liver and adipose tissue and is involved in metabolic processes [4]; whereas Klrp is a cytosolic β-glucocerebrosidase [5]. α-Klotho, generally simply referred as Klotho, is a type I single-pass transmembrane glycoprotein mainly expressed in the kidneys, liver, brain, and at lower level in the pituitary, skeletal muscle, urinary bladder, pancreas, testis, ovary, colon, thyroid gland, placenta and vascular tissue [1]. Both the intracellular and the transmembrane domains of α-Klotho are very short, whereas the extracellular domain is longer and contains two repeated sequences (KL1 and KL2) [4, 6]. After association with Fibroblast Growth Factor Receptors (FGFRs), the full-length transmembrane Klotho (mKl) acts as coreceptor for the bone-derived phosphaturic hormone FGF23, thus taking part to phosphate excretion and calcium homeostasis by regulating the expression and activity of the calcium channel transient receptor potential vanilloid 5 (TRPV5) [7]. Besides mKl, there are 2

isoforms of α-Klotho: a shed soluble form (sKl), which derives from the cleavage of the extracellular domain of Klotho from the cell surface by the metalloproteinases ADAM10 and ADAM17, and a secreted form that is produced by alternative splicing of Klotho mRNA [4]. The shed soluble form of Klotho seems to be dominant on both the secreted and the membrane forms in humans [8]. It has been proposed that the soluble forms of Klotho function as a hormone [9]. Moreover, since circulating levels of sKl increase following exercise training, it has been also hypothesized that klotho may be related to the antiaging effects of physical activity [10]. The sKl has pleiotropic effects on a lot of organs and tissues, thus regulating several pathways.[8]. Indeed, after the release in blood, urine and cerebrospinal fluid, sKl exerts biological effects involved in preservation of endothelial integrity and permeability, and affect intracellular signaling pathways including those related to insulin, Insulin-like Growth Factor-1 (IGF-1), PI3K, NF-kB, p53/p21, cAMP, protein kinase C and Wnt [8, 11-13]. In particular, a lot of evidence demonstrated that the anti-ageing effects of sKl have been associated with the inhibition of IGF-1 signaling and its downstream actions especially by enhancing resistance to oxidative stress [14, 15]. Indeed, inhibition of the IGF-1 signaling by sKl results in increased production of antioxidant enzymes [16]. Therefore, activity of sKl may regulate several pathways involved in the pathogenesis of diseases associated with oxidative and inflammatory state.

It is not yet clear whether intracellular signaling of circulating Klotho is mediated by a membrane receptor. Recent hypothesis suggests that sKL may act as a circulating co-receptor for membrane-bound FGFRs, thus allowing the interaction with FGF23 and regulating FGFR-mediated signaling also in cells lacking the full length form of Klotho [17]. Moreover, it has been demonstrated that sKl is able to bind membrane lipid rafts, alter their organization, and affect caveolae-mediated TRPV5 endocytosis [18], suggesting that the intracellular signaling of sKl may occur at the level of caveoale.

KLOTHO AND DIABETES

In diabetic patients, serum levels of Klotho have been found significantly decreased compared with those of healthy subjects, [19]. In addition, the amount of sKl is related to duration of diabetes and is negatively correlated to HbA1c. Kidneys are considered the main source of sKl [17], and are also the principal organ involved in the clearance of sKl from the circulation into the urine, thus playing a dual role in the homeostasis of Klotho [9]. Therefore, altered kidney function may affect the systemic effects of Klotho. Consequently, the anti-aging effects of Klotho have been extensively investigated in kidneys, reporting that increased levels of Klotho inhibit the progression of various kidney diseases [20, 21]. In animal models of diabetes, Klotho counteracts podocytic and glomerular albumin permeability induced by hyperglycemia [22], and prevents epithelial-mesenchymal transition in diabetic kidneys [21]. Interestingly, expression of Klotho has been found decreased in the renal cortices of mice with diabetes [22]. Moreover, Typiac *et al* showed that decreased levels of membrane-bound Klotho are associated to increased shedding of Klotho, to higher levels in serum of diabetic rats and a to reduced urinary excretion [23]. In diabetic patients, the amount of soluble Klotho is reduced in the early stage of chronic kidney disease (CKD), but increased with disease progression and the decrease of glomerular filtration rate [24]. A recent meta-analysis of data on sKl amount in patients with Diabetic nephropathy (DN) confirms that levels of sKl are further lowered in the early stage of DN [25], suggesting that Klotho might be considered as an early biomarker of diabetic nephropathy [23, 26]. However, although levels of sKl still remain lower in patients with DN, they seem to increase during the worsening of diabetic CKD probably linked to the decline in glomerular filtration rate that leads to reduced urinary excretion of Klotho [23, 27].

Expression of Klotho has been detected also in mouse pancreatic islets and in beta-cell line, [28, 29]. It has been showed that Klotho is involved in regulation of glucose-induced insulin secretion, probably, through regulation of TRPV2 expression [28, 29]. Indeed, overexpression of Klotho increases both insulin secretion and plasma membrane levels of TRPV2; whereas silencing of Klotho negatively affects plasma membrane levels of TRPV2, glucose-induced calcium entry and insulin secretion [28].

Moreover, treatment with α- or β-Klotho protects human beta-cells by cytokine-induced apoptosis and improved insulin secretion [30, 31].

DIABETIC RETINOPATHY

Diabetic retinopathy (DR) is a common microvascular complications of type 2 diabetes and represents the primary cause of blindness in working age adults [32]. Actually, retinal neurodegenerative lesions may occur earlier than microvascular ones, therefore DR has been defined as a highly tissue-specific neurovascular complication of diabetes by the American Diabetes Association [33]. The early manifestations of DR involves damages to both microcirculation and retinal neurons and are associated with oxidative stress [34]. The resulting sustained proinflammatory environment, in turns, increases oxidative stress, due to the reduced levels of antioxidant enzymes in the retina. Photoreceptors and the retinal pigment epithelial (RPE) cells are highly susceptible to oxidative stress in the early stage of DR and their dysfunction lead to progression of retinal degeneration [34]. Furthermore, chronic inflammation causes vasoregression and alters vascular permeability, leading to formation of microaneurysms and exudates. Then, hypoxia and the release of proangiogenic factors, such as vascular endothelial growth factor-A (VEGF-A), may promote pathological ocular neovascularization [34]. In the retina, VEGF-A is mainly produced by RPE cells, a monolayer of highly specialized cells located between the choroid and photoreceptors that forms the outer blood-retinal barrier (BRB) [35]. Due to their localization, RPE cells may affect retinal homeostasis by altering the function and maintenance of both the photoreceptors and capillary endothelium [36]. Indeed, under normal condition, VEGF-A is released at low concentrations from the basal side of the RPE to maintain endothelial cell function [37]. However, under pathological condition, such as chronic hyperglycemia, secretion of VEGF-A increases leading to activation of endothelial cells and altered permeability of the choroidal vessels [37, 38]. It is well known that dysfunction of RPE cells contributes to onset and progression of DR. Therefore, maintaining the function of RPE and

controlling the levels of VEGF-A are of great importance in preventing worsening of DR to the proliferative state.

KLOTHO AND RETINAL HOMEOSTASIS

It has been found that Klotho is expressed in the human retina, optic nerve, and lens [39, 40]. Several evidence showed that Klotho regulates a lot of mechanisms involved in maintaining homeostasis and functions of retinal cells [39, 41, 42]. Firstly, Klotho knockout mice display several morphological changes as compared to wild type mice: decreased pigmentation of the RPE layer, large choroidal vessels, thinner and deformed basal membrane, and signs of degeneration in the outer segment of photoreceptors (POS) [41]. Proteomics analysis reveals that proteins involved in eye development, visual perception and mitochondrial function are downregulated in Klotho knockout mice [42]. Accordingly, Klotho knockout mice have reduced retinal function, with functional deficit comparable to those observed in IGF-1 knockout mice [39]. Considering that Klotho knockout mice are hypoglycemic, it can be hypothesized that the effects observed in the retina may be attributable to increased sensitive to the insulin and IGF-1 signaling.

Kokkinaki *et al* demonstrated that Klotho is expressed in primary cultures of RPE cells, mainly in the cell membrane, and that its depletion compromises several important function of RPE cells [41]. Moreover, they demonstrated that treatment with recombinant Klotho protein has protective effects on RPE function, including phagocytosis, VEGF-A secretion, oxidative stress response, and melanogenesis.

Phagocytosis of POS is of particular importance in maintaining visual function and the visual cycle. It has been shown that transfection of RPE cells with Klotho siRNA significantly reduced phagocytosis [41], suggesting that Klotho is involved in the regulation of this important function. Evidences that treatment of RPE cells with Klotho significantly increased phagocytosis in RPE cells confirm this hypothesis [41]. POS phagocytosis is regulated by several factors, among them, the Ca2+ signaling and the expression of Mer Tyrosine Kinase (MerTK) seem to play an important role [43]. Rise in

intracellular Calcium is required for maintaining POS phagocytosis rate [44-46]. It has been reported that secreted Klotho may regulate calcium homeostasis by affecting activity of calcium channels, including TRPVs and the Ca2+ release-activated Ca2+ channel (CRAC) [28, 47, 48]. Interestingly, human RPE expresses both TRPV5 and CRAC, which regulate calcium entry in this cells [49, 50]. However, Kokkinaki *et al* showed that treatment of RPE cells with Klotho did not increase intracellular Calcium concentration [41], suggesting that Klotho increases phagocytosis through a mechanism independent to calcium. Internalization of POS requires the engagement of MerTK, a cell surface receptor member of the tyro/Axl/Mer family of receptor tyrosine kinase, therefore MerTK expression is critical for POS phagocytosis [43]. Interestingly, it has been demonstrated that Klotho regulates phagocytosis by upregulating MerTK expression, indeed treatment of RPE cells with Klotho induces intracellular signaling that leads to increased expression of MerTK and, consequently, improves phagocytosis efficiency [41].

VEGF-A is one of the main important pro-angiogenic factor and its excessive secretion is implicated in promoting the pathological neovascularization of the choroidal vasculature [51, 52]. RPE cells are the major responsible of VEGF-A production in the retina. Treatment of the RPE cell line ARPE-19 with Klotho significantly decreases VEGF-A secretion from both the apical and the basal sides [41]. Moreover, the presence of Klotho inhibits the phosphorylation of VEGFR2 induced by VEGF-A, thus affecting intracellular signaling activated by VEGF-A.

Due to its extremely active metabolism, the retina is one of the organ with major request of oxygen, therefore it may be susceptible to overproduction of reactive oxygen species (ROS). Under normal conditions, ROS take part to the retinal physiological signaling, however, when generation of ROS exceeds the natural antioxidants defenses, oxidative stress may contribute to the pathogenesis of several retinal diseases, including DR. Experimental data demonstrate that Klotho contributes to maintain the redox balance in the retina. Indeed, mRNA levels of Klotho have been found significantly decreased in ARPE-19 cells treated with hydrogen peroxide (H_2O_2) [53]. Moreover, Kokkinaki *et al*

demonstrated that down-regulation of Klotho expression leads to reduced expression of the anti-oxidant Superoxide dismutase 2 (SOD 2) in RPE cells [41]. On the contrary, pretreatment with sKl prevented rise in ROS induced by H_2O_2 enhancing the antioxidant activities of ARPE-19 [53], and decreased apoptosis induced by oxidative stress in human retinal endothelial cells [54].

Eye pigmentation is essential to maintain visual function. The RPE contribute to absorption of scattered light and to reduce retinal damage from ultraviolet light by forming a dark-brown pigmented wall [35, 55]. Studies on models in which Klotho expression has been down-regulated revealed that Klotho is involved in regulation of genes encoding for melanin biosynthesis [41]. Indeed, pigmentation of eyes from Klotho k/o mice was reduced and their RPE cells contained fewer melanin granules than normal RPE cells [41].

All these findings suggest that retina homeostasis may be affected by altered expression of Klotho, as well altered levels of soluble Klotho (Table 1).

KLOTHO AND DIABETIC RETINOPATHY

Levels of sKl has been found reduced in ocular pathologies characterized by inflammatory state [56-59], suggesting that the reduced levels of sKl may be a common feature in several ocular diseases. In particular, decreased levels of Klotho may be associated with increased risk of onset and worsening of diabetic retinopathy. Indeed, circulating levels of Klotho are lower in diabetic subject with DR than in those without this complication [54, 60]. Moreover, serum Klotho levels are negatively correlated with progression of DR [54, 60]. Following the onset of DR in diabetic patients reveals that patients with progression of retinopathy had lower levels of serum Klotho as compared to those without [60]. In addition, Ji *et al* found that levels of sKl are gradually reduced among patient with diabetes without DR, non-proliferative DR and proliferative DR (PDR), independently of DN [54]. Corcillo *et al* hypothesize that a halving of circulating Klotho levels may increase the risk of retinopathy progression by 44% [60]. On the other hand, the incidence of the functional "KL-VS" variant of the Klotho gene, which is

associated with higher longevity in humans, is lower in people with diabetic retinopathy and is associated with reduced serum levels of inflammatory markers and pro-angiogenic factors, suggesting that this genotype may be protective against retinopathy incidence [61].

As reported in the previous section, several experimental models demonstrated that depletion of Klotho negatively affects important function of retinal cells, including oxidative stress response, VEGF-A secretion, and phagocytosis, leading to activation of mechanisms that may contribute to onset and progression of DR. On the other hand, there are also several evidence that treatment with recombinant sKl or overexpression of Klotho ameliorate retinal function.

Oxidative stress and inflammation have been causative associated with DR [62, 63]. It has been reported that Klotho exerts protective effects against oxidative stress in retinal cells [13, 41, 42, 53]. Firstly, it has been observed that pretreatment with sKl prevents increment of ROS production in ARPE-19 cells exposed to H_2O_2 [41, 53]. In particular, Wen *et al* demonstrated that sKl improves redox balance in H_2O_2-treated ARPE-19 cells by increasing expression and nuclear translocation of nuclear factor E2-related factor 2 (Nrf2), thus restoring glutathione peroxidase (GPX), SOD2 and catalase (CAT) to the levels of untreated cells [53]. In addition, pretreatment with sKl prevents H_2O_2-induced apoptosis of ARPE-19 cells [42, 53], by increasing expression of Bcl-2 and decreasing the activation of caspase-3 [53].

It is well established that VEGF-A plays an important role in driving pathological neovascularization of the retina during DR, and that neovascularization due to severe hypoxia is a hallmark of PDR [34]. The expression of VEGF-A is regulated by hypoxia- inducible factor- 1α (HIF- 1α), which is a transcription factor involved in cellular response to hypoxia and hyperglycemia, [64, 65]. Interestingly, Klotho levels have been found decreased in ARPE- 19 cells exposed to hypoxia and in laser-induced CNV lesions in mice [66]. Xie *et al* demonstrated that HIF- 1α, besides directly increase VEGF-A transcription, may be responsible of down-regulation of Klotho expression during hypoxia [66]. Indeed, HIF- 1α activates p53, which, in turns, leads to the

increased levels of miRNA34, that targets Klotho thus reducing its expression [66]. Given that klotho is expressed in ocular tissues, it is possible that part of the sKl that acts in the eye derives by local shedding of mKl, therefore its contribution may be lost when expression of Klotho is down-regulated. It has been reported that treatment with Klotho reduces VEGF-A secretion from ARPE-19 cells [41]. In particular, Klotho was able to decrease VEGF-A secretion by reducing phosphorylation of both IGF-1Receptor (IGF-1R) and VEGR2. The pathogenic role of IGF-1 in the development of PDR is still debated, several studies indicate that increased activation of IGF-1 signaling may contribute to retinal neovascularization, however a strong relationship between IGF-1 and the development of proliferative retinopathy has not been still clearly demonstrated [67-69]. Several studies reported that IGF-1R signaling is regulated by lipid raft integrity and interaction with caveolin-1 [70-74]. In particular, down-regulation of caveolin-1 expression in RPE cells significantly reduces both basal and IGF-1-stimulated VEGF-A secretion [72]. These data together with the ability of Klotho to modify the lipid organization within lipid rafts/caveolae [18] suggest that Klotho may reduce the phosphorylation of IGF-1R by altering these microdomains. Hyperglycemia increases production and secretion of VEGF-A by Muller cells in the retina. In particular, Yu *et al* demonstrated that hyperglycemia increases the production of VEGF-A in Muller glial cells through the activation of FGFR1 [75]. It is well known that sKl acts as a co-receptor for FGFs at non-renal sites and activates protective pathways in several cell types [76, 77]. Interestingly, screening the potential pathogenic genes associated with DR revealed that hyperglycemia increases the expression of FGF23 [78], and of its membrane receptor FGFR1 on Muller glial cells [75, 79]. Considering that absence of Klotho may allow Klotho-independent activation of FGFRs resulting in pathological cellular changes [17, 76, 77], and that Klotho-independent action of FGF23 has been reported to contribute to endothelial dysfunction [17], these findings suggest that lower levels of Klotho together with increased production of FGF23 may contribute to the onset of DR and to progression to PDR by increasing VEGF-A production.

Autophagy is a highly conserved lysosomal pathway for the turnover of cytoplasmic organelles and long-lived proteins that acts as an adaptive response to cellular stresses and regulates homeostasis, differentiation, development and survival in several cell types [80]. In retinal cells, autophagy plays an important role by participating to POS degradation, visual pigment recycling, and lipofuscin degradation [81-83]. Altered activation of autophagy has been found in experimental models of DR and in the retina of diabetic patients [84, 85]. For instance, RPE cells exposed to high glucose concentration increase formation of autophagosome, suggesting that induction of autophagy is a cytoprotective response against HG [84, 85]. However, the excessive activation of this mechanism may lead to its impairment as occur in retinal Muller cells, where the process of degradation cannot be completed due to the lysosomal dysfunction [85]. It has been reported that autophagic activity is reduced in DM mice and human renal proximal tubule cells exposed to high glucose (HG) [86]. Recent studies showed that Klotho may act as a regulator of autophagy even in diabetic condition [87]. Specific expression of Klotho significantly improves autophagy in both pancreatic beta cells and in renal tubule cells exposed to HG [29, 86]. Moreover, Zou *et al* showed that activation of AMPK (5' adenosine monophosphate-activated protein kinase), a positive regulator of autophagy, is significantly decreased in the retina of Klotho deficient mice as compared to that of WT mice [42]. Although there is no direct evidence, these finding suggest that Klotho may affect autophagy also in retinal cells. A decreased activation of AMPK has been observed also in arterial endothelial cells of Klotho deficient mice [88], confirming that AMPK is a crucial mediator of protective effects of Klotho. Moreover, Klotho deficient mice have also reduced activity of silent information regulator (SIRT) 1 [88], another important player in autophagy [89]. Interestingly, the expression of SIRT1 is reduced in diabetic retinopathy and intravitreal administration of SIRT1 reverses DR in a mouse model of Type 2 Diabetes [90]. These results suggest that regulation of SIRT1 may be another mechanism through which Klotho improve DR.

Proliferative diabetic retinopathy is also characterized by formation of fibrous proliferative anterior membrane [91]. Subretinal fibrosis is mediated by Epithelial-mesenchymal transition (EMT), a process that leads RPE cells to the acquisition of a mesenchymal phenotype [92]. Several evidence demonstrated that HG induce EMT in RPE [93, 94]. It has been shown that Klotho expression is down-regulated in models of induced fibrosis, suggesting a protective role of Klotho [22, 95, 96]. In particular, the protective effects of Klotho have been related to inhibition of the Wnt/ß-catenin and the Egr-mediated signaling pathways. Recently, it has been reported that overexpression of Klotho decreased the expression of mesenchymal cell markers induced by hypoxia in ARPE-19 cells [66]. Moreover, overexpression of Klotho was able to reduce subretinal fibrosis in a mouse laser- induced CNV model [66]. Here, under hypoxic conditions, Klotho was able to block the axis that through HIF- 1α leads to the activation of p53 and promotes EMT in RPE cells, confirming that Klotho may be useful in preventing EMT also in RPE cells.

Besides hyperglycemia, dyslipidemia is another important actor in the progression of DR [97, 98]. Palmitic acid (PA) is involved in the onset of DR and may induce endothelial cell damage [98]. It has been demonstrated that Klotho pretreatment significantly reduces apoptosis induced by PA in human retinal endothelial cells [54]. This effect implies the activation of the PI3K and subsequent phosphorylation of AKT [54]. Moreover, Klotho affects expression of proteins involved in apoptosis leading to increased expression of the anti-apoptotic Bcl-2 and down-regulation of the pro-apoptotic Bax [54]. Consistent with these data, pretreatment with Klotho reduced the apoptosis rate in ARPE-19 cells exposed to H_2O_2 by up-regulating Bcl-2 expression and decreasing levels of Bax [53]. In addition, Klotho was able to prevent the decrease of mitochondrial membrane potential and the activation of Caspase-3 induced by H_2O_2 [53].

CONCLUSION

Diabetic retinopathy is a common complication of diabetes. The International Diabetes Federation estimated the global population with diabetes mellitus to be 463 million in 2019 and 700 million in 2045 [99]. These data require the development of strategies able to prevents the onset and the progression of DR. To date, the first line treatment for PDR is intravitreal anti-VEGF therapy. However, it is not so successful for routine treatment of non-proliferative DR [32, 100]. Therefore, new molecules in development have been designed to target other pathways involved in pathogenesis of DR [101, 102]. It has been demonstrated that klotho has protective effects in DN and that pathological mechanisms between DR and DN share similarities [19, 29], suggesting that Klotho may be a good candidate in counteracting DR. Experimental models targeting Klotho have been shown to have positive effects on several mechanisms involved in DR onset and progression (Figure 1). Therefore, Klotho may become a novel biomarker and a good candidate for the treatment of DR [60].

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