Klotho in vascular biology

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Chapter 1

General introduction

The protein Klotho was first described in 1997, after the serendipitous generation of a mouse with a disruption in the promoter of an unknown gene (1). This new knockout mouse was found to develop a systemic syndrome resembling human ageing and re-introduction of the gene reversed the ageing phenotype, serving as the inspiration for naming the gene and protein Klotho, for the eponymous mythological Greek goddess Κλωθώ who was thought to determine lifespan by spinning the thread of life. It was later determined that Klotho overexpression indeed had the opposite effect and extended lifespan in mice by 20-30% (2).

Klotho deficiency and overexpression

Deficiency of Klotho induces a premature ageing-like syndrome that includes a short lifespan (1), vascular calcification (3, 4), osteoporosis (5), pulmonary emphysema (6, 7), cardiac hypertrophy (8, 9), a decrease in renal function (10), cognitive dysfunction (11, 12), infertility (1), hearing deficits (13), decrease in retinal function (14), and general atrophy of muscles (15), skin (16), and other tissues (1). This is a remarkably extensive ageing phenotype for a single gene deficiency. Conversely, overexpression or supplementation of Klotho protects against renal disease (17-29), cardiac disease (8, 17, 30-34), pulmonary disease (35, 36), neurodegenerative disease (37-42), muscle disease (27, 43, 44), diabetes (45, 46), and various tumors (22, 47, 48).

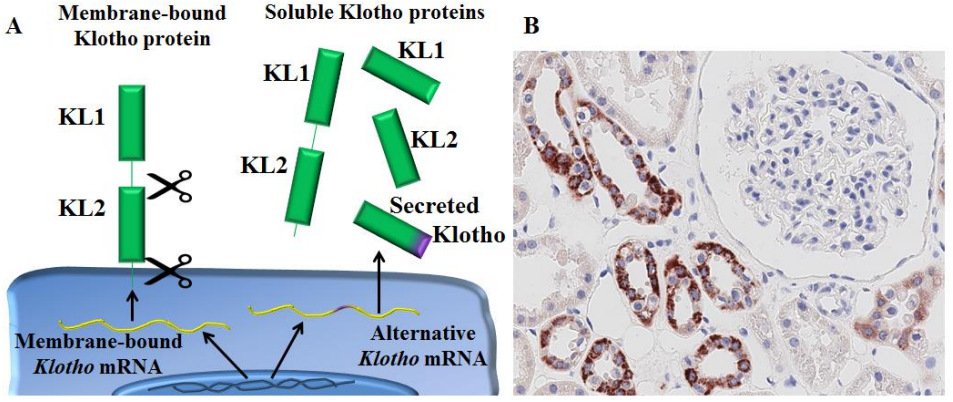

Figure 1. Paradigm of Klotho protein expression. (A) Schematic overview: mRNA for membrane-bound Klotho and an alternatively spliced Klotho mRNA transcripts are transcribed. The normal transcript is known to code for the membrane-bound Klotho protein, containing KL1 and KL2 regions and two sites for proteolytic cleavage, which generate full-length soluble Klotho and separate KL1 and KL2 domains. Secreted Klotho is thought to be translated as a splice variant. **(B)** Klotho protein expression pattern in human kidney, using antibody KM2076. Original magnification 320×.

Klotho and chronic kidney disease (CKD)

Membrane-bound Klotho is expressed primarily in the distal convoluted tubule in the kidney (with additional expression in the choroid plexus, parathyroid gland, and sinoatrial node) and contains two internally homologous regions termed KL1 and KL2 (1, 49). Klotho is also cleaved off of the membrane, generating soluble Klotho, which is found in blood, urine, and cerebrospinal fluid (50-53). It has also long been hypothesized that a putative shorter Klotho protein, termed secreted Klotho, would be the product of alternative splicing (54). Figure 1A displays the paradigm of forms of Klotho proteins. The fact that Klotho is predominantly expressed in the distal convoluted tubule in the kidney (Figure 1B) explains the early occurrence of Klotho deficiency in chronic kidney disease (CKD) (10, 55, 56). Since patients with CKD also develop a premature ageing-like phenotype (57), the question is raised to what extent lack of Klotho is responsible for that. More specifically, the extensive mineral homeostasis imbalances that develop in CKD-mineral bone disorder (MDB) are very similar to the phenotype of Klotho knockout mice. This includes hyperphosphatemia, which develops as a consequence of ablated fibroblast growth factor 23 (FGF23) signaling in the absence of Klotho as an obligate co-receptor for FGFR1c, thereby reducing phosphaturia and contributing to the development of vascular calcification (58, 59). The prominence of vascular calcification in Klotho deficiency points to Klotho being a major player in cross-talk between the kidney and the cardiovascular system. The excessive cardiovascular mortality in CKD patients (60) raises the question whether maintenance of Klotho levels would be beneficial in preventing cardiovascular disease in these patients.

Klotho and the vasculature

The vascular effects of various degrees of Klotho deficiency, which include vascular calcification (1), endothelial dysfunction (61, 62), arterial stiffening (63, 64), impaired angiogenesis (65), and hypertension (66, 67), are increasingly being recognized as potentially relevant to both ageing and vascular complications of CKD. Klotho overexpression and supplementation have so far been shown to protect against vascular calcification (4, 10), endothelial dysfunction (68), atherosclerosis (69), thrombosis (69), and hypertension (28). These effects are at least in part mediated by soluble Klotho, but there are a lot of contradictory data on whether Klotho may be expressed in the vasculature itself (70, 71). Whether the aforementioned anti-tumor effects are in part due to Klotho effects on tumor angiogenesis is also unknown. Overall, despite significant gaps in our current knowledge, Klotho appears to be a promising target in designing novel therapies for ageing-related and CKD-related vascular disease.

Aim and scope of this thesis

The aim of this thesis is to investigate the role of Klotho in vascular biology, with a particular focus on vascular Klotho expression and the role of Klotho in vascular remodeling, smooth muscle cell (SMC) de-differentiation and associated pathological SMC behavior. **Chapter 2** provides a broad overview of what was previously known about the link between Klotho and the vasculature, describing the vascular Klotho deficiency phenotype, interventions that have been performed to modulate the Klotho deficiency phenotype, and the experimental effects of increased Klotho levels. We also provide a synthesis of the data on vascular Klotho expression, and describe what is known about the mechanisms in which Klotho affects SMCs and endothelial cells (ECs).

Part I then focuses on renal and vascular Klotho expression. In **Chapter 3**, we review the current knowledge on Klotho expression, with a special emphasis on providing a comparative framework for tissues/cell types, a classification for anti-Klotho antibody validation, the controversy surrounding vascular Klotho expression, and the establishment of the kidney as the principal source for *circulating, soluble Klotho*. In **Chapter 4**, we investigate whether *membrane-bound Klotho* is expressed in human arterial tissue, with particular emphasis on Klotho protein expression and antibody validation. In **Chapter 5**, we continue our investigation of *vascular Klotho* expression, further addressing the controversy around vascular immunoreactivity of anti-Klotho antibodies. We also investigate whether artery-specific Klotho knockout mice have a vascular phenotype. **Chapter 6**, in turn, focuses more generally on the concept of *secreted Klotho*, which has long been thought to be the product of an alternatively spliced Klotho mRNA transcript and of which we study the translation and potential clinical relevance.

Part II centers around the role of Klotho in arterial remodeling with a particular focus on aberrant SMC behavior. In **Chapter 7**, we investigate the development of *vascular calcification* in Klotho deficiency using various imaging techniques and we assess whether new insights in the pathophysiology of vascular calcification in CKD with regard to calciprotein particles are also applicable to Klotho deficiency-induced vascular calcification. In **Chapter 8**, we detail that Klotho-deficient mice are also affected by *arteriolar hyalinosis*, which is also seen in the kidney in human ageing, and we investigate the phenotypic variability of Klotho deficiency using different Klotho knockout strains. **Chapter 9** then focuses on whether Klotho deficiency leads to or exacerbates *intimal hyperplasia*, which was originally thought to be the case, but which has not been investigated since. To this end, we used different experimental models of intimal injury, comparing Klotho$^{+/-}$ and WT mice. In **Chapter 10**, we study *vascular function* in Klotho deficiency *ex vivo*, both focusing on *endothelial dysfunction* and SMC contractility. Continuing our shift of focus towards the endothelium, we describe in **Chapter 11** our experiments on the effects of Klotho on *angiogenesis* and *tumor angiogenesis* in glioblastoma (GBM), as an

example of a highly-angiogenic tumor. While anti-tumor effects of Klotho are well-recorded in various tumors, this is the first study assessing the effect of Klotho on tumor angiogenesis.

Part III is a collection of clinically oriented chapters, aimed at working towards clinical applications of Klotho in diagnostics or therapy. **Chapter 12** describes broadly how Klotho has been used experimentally in animal models of fibrosis and cancer, which reveals how, so far, pre-clinical evidence towards a potential therapy has been supported by lines of evidence narrowing down how and which forms of Klotho can be used. Furthermore, we describe what we now about Klotho structure-function relationships in various pathways, which *therapeutic strategies* have been attempted in delivering Klotho experimentally in animal models, and what obstacles remain before Klotho-based therapies can be tested. In **Chapter 13**, we detail what we know about the current potential of the exploration of *clinical aspects* of Klotho-related tests (like the measurement of serum Klotho levels or the relevance of single-nucleotide polymorphisms (SNPs) in the Klotho gene) in patients with vascular disease. In **Chapter 14**, we study whether *Klotho SNPs* are associated with graft failure in kidney transplantation recipients, which is a process to which transplant vasculopathy is a contributing factor.

Finally, in **Chapter 15**, we summarize and discuss the results of this thesis and provide a context for their interpretation, as well as perspectives for future research.

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