25-Hydroxyvitamin D₃ 1-Alpha-Hydroxylase-Dependent Stimulation of Renal Klotho Expression by Spironolactone

Ioana Alesutan  Martina Feger  Tatsiana Pakladok  Sobuj Mia  Mohamed Siyabelden E. Ahmed  Jakob Voelkl  Florian Lang

Department of Physiology, University of Tübingen, Tübingen, Germany

Key Words
25-hydroxyvitamin D₃  1-alpha-hydroxylase • Aldosterone • CYP27B1 • KLOTHO • Mineralocorticoid receptor • Spironolactone • Vitamin D₃ receptor

Abstract
Background: Klotho, a transmembrane protein, protease and hormone mainly expressed in kidney, is required for the suppression of 1,25(OH)₂D₃-generating 25-hydroxyvitamin D₃ 1-alpha-hydroxylase (Cyp27b1) by FGF23. Conversely, 1,25(OH)₂D₃ stimulates, by activating the vitamin D₃ receptor (Vdr), the expression of klotho, thus establishing a negative feedback loop. Klotho protects against renal and vascular injury. Klotho deficiency accelerates aging and early death, effects at least partially due to excessive formation of 1,25(OH)₂D₃ and subsequent hyperphosphatemia. Klotho expression is inhibited by aldosterone. The present study explored the interaction of aldosterone and DOCA as well as the moderately selective mineralocorticoid receptor antagonist spironolactone on klotho expression. Methods: mRNA levels were determined utilizing quantitative RT-PCR in human embryonic kidney cells (HEK293) or in renal tissues from mice without or with prior mineralocorticoid (aldosterone or DOCA) and/or spironolactone treatment. In HEK293 cells, protein levels were determined by western blotting. The experiments in HEK293 cells were performed without or with silencing of CYP27B1, of vitamin D₃ receptor (VDR) or of mineralocorticoid receptor (NR3C2). Results: In HEK293 cells aldosterone and in mice DOCA significantly decreased KLOTHO gene expression, effects opposed by spironolactone treatment. Spironolactone treatment alone significantly increased KLOTHO and CYP27B1 transcript levels in HEK293 cells (24 hours) and mice (8 hours or 5 days). Moreover, spironolactone significantly increased klotho and CYP27B1 protein levels in HEK293 cells (48 hours). Reduced NR3C2 expression following silencing did not significantly affect KLOTHO and CYP27B1 transcript levels in presence or absence of spironolactone. Silencing of CYP27B1 and VDR significantly blunted the stimulating effect of spironolactone on KLOTHO mRNA levels in HEK293 cells. Conclusion: Besides blocking the effects of aldosterone,
spironolactone upregulates KLOTHO gene expression by upregulation of 25-hydroxyvitamin D₃ 1-alpha-hydroxylase with subsequent activation of the vitamin D₃ receptor by 1,25(OH)₂D₃, an effect possibly independent from the mineralocorticoid receptor.

Introduction

Klotho, a transmembrane protein mainly expressed in the kidney [1-4], is secreted into blood, urine and spinal fluid [5, 6]. Klotho serves a wide variety of functions including regulation of phosphate homeostasis [7, 8].

As shown in mice, klotho deficiency leads to phosphate overloading with growth deficit, severe calcifications and reduced lifespan [9, 10]. Klotho is required for the downregulation by FGF23 of 25-hydroxyvitamin D₃ 1-alpha-hydroxylase (encoded by the Cyp27b1 gene). Klotho deficiency leads to 1,25(OH)₂D₃ excess with enhanced intestinal phosphate and Ca²⁺ absorption resulting in hyperphosphatemia, subsequent calcium/phosphate precipitations as well as vascular calcification [1, 10, 11]. Klotho expression is stimulated by 1,25(OH)₂D₃ thereby closing a negative feedback loop [4]. Klotho expression is downregulated by various inflammatory mediators and aldosterone [12-14] and upregulated by angiotensin II blockade [15].

Besides its role in phosphate homeostasis, klotho regulates ion channels, influences epithelial-to-mesenchymal transition, exerts favourable effects on kidney as well as vasculature and counteracts aging [8, 10, 16, 17]. Renal failure is associated with reduced klotho levels, resulting in resistance to FGF23 [6]. The reduction of klotho expression in chronic kidney disease (CKD) is paralleled by hyperphosphatemia despite reduced 1,25(OH)₂D₃ levels [8]. Klotho is protective in kidney disease [14, 18, 19]. In CKD, klotho levels are reduced not only in kidney but as well in vascular smooth muscle cells thereby promoting vascular calcification [10, 20]. In the heart, klotho exerts favorable effects on cardiac remodelling [21].

In klotho-hypomorphic mice, aldosterone is upregulated [22]. Aldosterone excess may be harmful to renal, vascular and cardiac tissue [23-28] and aldosterone antagonists may exert beneficial effects in those tissues [29]. Aldosterone may foster vascular calcification in rats and klotho-hypomorphic mice [30, 31]. Aldosterone levels are enhanced in CKD and, even in the presence of angiotensin II blockade, additional aldosterone blockade may be beneficial [32, 33].

The present study explored how aldosterone and spironolactone influence KLOTHO gene expression. As a result, spironolactone upregulates KLOTHO mRNA expression even in the absence of exogenous aldosterone. Additional experiments addressed the mechanisms involved.

Materials and Methods

Cell culture of HEK293 cells

Human embryonic kidney cells (HEK293) were routinely cultured in Dulbecco’s Modified Eagle Medium DMEM containing 4.5 g/l glucose (PAA Laboratories GmbH, Germany), supplemented with 2 mM L-glutamine (PAA Laboratories GmbH, Germany), 10% FBS (Gibco, Life Technologies GmbH, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies GmbH, Germany). The media was changed to 10% charcoal stripped FBS media (Gibco, Life Technologies GmbH, Germany) 24 hours prior to each experiment to reduce the effects of endogenous ligands. Cells were treated for 24 or 48 hours with 100 nM aldosterone and/or 10 µM spironolactone (Sigma-Aldrich, Germany) dissolved in DMSO prior to RNA or protein isolation, respectively, as described previously [30, 34-36]. Equal amounts of vehicle were used as control.
Silencing of HEK293 cells

For silencing, HEK293 cells were cultured in the growth medium containing charcoal stripped FBS (Gibco, Life Technologies GmbH, Germany). The cells were subsequently transfected with 10 nM validated NR3C2 siRNA (ID no. s8839, Ambion, Life Technologies GmbH, Germany), with 10 nM VDR siRNA (ID no. s1477, Ambion, Life Technologies GmbH, Germany), with 10 nM CYP27B1 siRNA (ID no. s3890, Ambion, Life Technologies GmbH, Germany) or with 10 nM negative control siRNA (ID no. 4390843, Ambion, Life Technologies GmbH, Germany) using siPORT amine transfection agent (Ambion, Life Technologies GmbH, Germany) according to the manufacturer’s instructions. The cells were used 48 hours after transfection. The efficiency of silencing was verified by quantitative RT-PCR.

Animal experiments

All animal experiments were conducted according to the German law for the care and use of laboratory animals and were approved by local authorities. Experiments were performed in C57Bl6 mice under control diet and access to drinking water ad libitum. In a first study group, male and female mice were subcutaneously injected with vehicle (soybean oil), DOCA (50mg/kg BW), spironolactone (75mg/kg BW) or DOCA and spironolactone together [37]. Eight hours after injection, mice were sacrificed and kidney tissues were rapidly removed and immediately snap frozen. In another study group, male mice were treated with either spironolactone (80mg/L) [30] or with vehicle drinking water ad libitum and sacrificed after 5 days of treatment. Blood was collected by retroorbital puncture and the plasma concentration of 1,25(OH)2D3 was determined by an EIA kit (Immunodiagnostic Systems, UK) according to manufacturer’s instructions. Kidney tissues were rapidly removed after sacrificing the mice and immediately snap frozen.

Quantitative RT-PCR

Total RNA was isolated from mouse kidney tissues by using Trifast Reagent (Peqlab Biotechnologie GmbH, Germany) according to the manufacturer’s instructions. HEK293 cells were washed with PBS and total RNA was isolated using Trifast Reagent (Peqlab, Biotechnologie GmbH, Germany) according to the manufacturer’s instructions. Reverse transcription of 2 µg RNA was performed using oligo(dT)12-18 Primers (Invitrogen, Life Technologies GmbH, Germany) and SuperScriptIII Reverse Transcriptase (Invitrogen, Life Technologies GmbH, Germany). cDNA samples were treated with RNaseH (Invitrogen, Life Technologies GmbH, Germany). Quantitative real-time PCR was performed with the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, Germany) and iQ™ Sybr Green Supermix (Bio-Rad Laboratories, GmbH, Germany) according to the manufacturer’s instructions.

The following mouse primers were used (5′-3′ orientation) for quantitative RT-PCR measurements:

- Cyp24a1 fw: GTGAAGGCTGGCGCCAAGAAG; Cyp24a1 rev: TCTACCTCGGTCTCATCAGC
- Cyp27b1 fw: CAGTTTACGTTGCCGGACCTA; Cyp27b1 rev: GGAGATGACCTTTCTTGTGCC
- Gapdh fw: AGGTGCGGTTGACAGGATTTG; Gapdh rev: TGTAGACCATGTTGAGGAGTCA
- Klotho fw: CCGTGTACCTTGTGTTGG; Klotho rev: CCCACAGATACATCCTGGTG
- Vdr fw: GATGCCACCAAGACCTAC; Vdr rev: GTCTGCAAGATTTGAGGC
- CYP27B1 fw: GGAAACCTGAACACTTGAAGAATTGC; CYP27B1 rev: AGTCCGACACTTGAAGTCT
- GAPDH fw: GAGTCAACCATATCGGTGGAC
- KLOTHO fw: GGTGTCAGTTGCCATGAGC
- NR3C2 fw: AGCAAGGAAAGAGAGGAGAG
- VDR fw: TCTCCAATGCTGGAGCTGGAG

The following human primers were used (5′-3′ orientation) for quantitative RT-PCR measurements:

- CYP27B1 fw: GGAAACCTGAACACTTGAAGAATTGC; CYP27B1 rev: AGTCCGACACTTGAAGTCT
- GAPDH fw: GAGTCAACCATATCGGTGGAC
- KLOTHO fw: GGTGTCAGTTGCCATGAGC
- NR3C2 fw: AGCAAGGAAAGAGAGGAGAG
- VDR fw: TCTCCAATGCTGGAGCTGGAG

The specificity of the PCR products was confirmed by analysis of the melting curves and in addition by agarose gel electrophoresis. All PCRs were performed in duplicate, and mRNA fold changes were calculated by the 2-ΔΔCt method using GAPDH as internal reference.

Western blot analysis

HEK293 cells were washed with PBS and lysed with ice-cold RIPA lysis buffer (Cell Signaling, Danvers, MA, USA) supplemented with complete protease and phosphatase inhibitor cocktail (Thermo...
Fisher Scientific, Rockford, IL, USA). After centrifugation at 10000 rpm for 5 min, protein concentration was determined by Bradford assay (Biorad Laboratories, Hercules, CA, USA). Proteins were boiled in Roti Load 1 protein loading buffer (Carl Roth, Karlsruhe, Germany) at 100°C for 10 min, separated on SDS-polyacrylamide gels and transferred to PVDF membranes [13]. The membranes were incubated overnight at 4°C with rabbit anti-CYP27B1 antibody (dilution 1:500, Santa Cruz, Dallas, Texas, USA), rat anti-α-klotho antibody (1:1000, kindly provided by Kyowa Hakko Kirin Co. Ltd, Japan) or rabbit anti-GAPDH antibody (1:1000; Cell Signaling, Danvers, MA, USA) and then with secondary anti-rabbit HRP-conjugated antibody (1:1000; Cell Signaling, Danvers, MA) or secondary anti-rat HRP-conjugated antibody (1:1000; Cell Signaling, Danvers, MA) for 1 hour at RT. For loading controls, the membranes were stripped in stripping buffer (Thermo Fisher Scientific, Rockford, IL, USA) at RT for 10 min. Antibody binding was detected with the ECL Western Blotting Substrate (Pierce, Rockford, IL, USA). Positive and negative controls were used to determine the molecular size of klotho protein in HEK293 cells. Bands were quantified using Quantity One Software (Bio-Rad, München, Germany) and results are shown as the ratio of total protein to GAPDH normalized to the control treated group.

**Statistics**

Data are provided as means ± SEM, n represents the number of independent experiments. All data were tested by ANOVA followed by post hoc analysis, or unpaired Student t-test where appropriate. P < 0.05 was considered statistically significant.

**Results**

The present study explored the interactions of the mineralocorticoid aldosterone and the moderately selective mineralocorticoid receptor antagonist spironolactone on KLOTHO gene expression. Human embryonic kidney cells (HEK293) were treated for 24 hours with aldosterone (100 nM) without and with simultaneous spironolactone treatment (10 µM). To avoid potential effects of endogenous ligands in the medium, the experiments were performed using charcoal-stripped FBS media. As illustrated in Fig. 1A, aldosterone treatment was followed by a significant decrease of KLOTHO mRNA levels. Spironolactone did not only reverse the effect of exogenous aldosterone but significantly increased KLOTHO mRNA levels beyond the values observed in control treated HEK293 cells. Cotreatment with both, aldosterone and spironolactone and treatment with spironolactone alone was followed by a statistically significant increase of KLOTHO mRNA levels (Fig. 1A). This effect was paralleled by similar changes in CYP27B1 mRNA levels. Aldosterone tended to decrease CYP27B1 transcript levels in HEK293 cells, an effect, however, not reaching statistical significance (Fig 1B). Similar to the observed effects of spironolactone on KLOTHO expression, spironolactone treatment significantly increased CYP27B1 gene expression (Fig 1B).

Similar observations were made in vivo. Treatment of mice with subcutaneous DOCA injection significantly decreased renal Klotho mRNA levels (Fig. 2A). Injection of spironolactone without or with additional DOCA injection significantly increased Klotho mRNA levels (Fig. 2A). Similar to the in vitro experiments, DOCA treatment tended to downregulate the renal Cyp27b1 transcript levels, an effect, however, not reaching statistical significance. Spironolactone treatment alone resulted in a statistically significant increase of Cyp27b1 mRNA levels (Fig. 2B).

Another series of experiments explored, whether spironolactone similarly regulates klotho and CYP27B1 protein levels in HEK293 cells. As shown in Fig. 3A,B, spironolactone treatment for 48 hours was followed by a statistically significant increase in klotho protein expression in HEK293 cells. This effect was paralleled by similar changes in CYP27B1 protein levels. Spironolactone treatment significantly increased CYP27B1 protein expression in HEK293 cells (Fig. 3C,D).

Further experiments elucidated the effect of spironolactone on KLOTHO gene expression. In order to test, whether the increases of KLOTHO and CYP27B1 mRNA levels were mediated by
inhibition of the mineralocorticoid receptor (encoded by the NR3C2 gene), the NR3C2 gene was silenced in HEK293 cells. As illustrated in Fig. 4, silencing of NR3C2 did not significantly alter KLOTHO mRNA expression and thus did not mimic the effect of spironolactone. The subsequent treatment with spironolactone was followed by a statistically significant increase of KLOTHO gene expression to similarly high levels in HEK293 cells silenced with negative control siRNA and in HEK293 cells silenced with NR3C2 siRNA. Similar observations were made on CYP27B1 mRNA levels (Fig. 4). Silencing of NR3C2 did not significantly modify CYP27B1 gene expression. Treatment with spironolactone was followed by a statistically significant increase of CYP27B1 mRNA levels to similarly high levels in HEK293 cells silenced with negative control siRNA and in HEK293 cells silenced with NR3C2 siRNA (Fig. 4). Thus, the mineralocorticoid receptor NR3C2 may not be involved in the upregulating effect of spironolactone on KLOTHO and CYP27B1 gene expression in the absence of aldosterone.

As increased KLOTHO transcript levels were paralleled by increased CYP27B1 mRNA levels, additional experiments explored, whether the effect of spironolactone on KLOTHO mRNA
Fig. 3. Effects of spironolactone on klotho and CYP27B1 protein expression in HEK293 cells. (A) Representative original western blots showing klotho and GAPDH protein abundance in HEK293 cells after 48 hours treatment with vehicle alone (Ctr) or with 10 µM spironolactone (Spiro). (B) Arithmetic means ± SEM (n=12, arbitrary units) of normalized klotho/GAPDH protein ratio in HEK293 cells after 48 hours treatment with vehicle alone (white bar; Ctr) or with 10 µM spironolactone (black bar; Spiro). (C) Representative original western blots showing CYP27B1 and GAPDH protein abundance in HEK293 cells after 48 hours treatment with vehicle alone (Ctr) or with 10 µM spironolactone (Spiro). (D) Arithmetic means ± SEM (n=12, arbitrary units) of normalized CYP27B1/GAPDH protein ratio in HEK293 cells after 48 hours treatment with vehicle alone (white bar; Ctr) or with 10 µM spironolactone (black bar; Spiro). Results are normalized to the control treated HEK293 cells. *(p<0.05) indicates statistically significant differences from HEK293 cells treated with vehicle alone.

Fig. 4. Effects of spironolactone on KLOTHO and CYP27B1 gene expression following silencing of mineralocorticoid receptor in HEK293 cells. (A) Representative original bands of mineralocorticoid receptor NR3C2 (upper bands) and calibrator/control GAPDH (lower bands) mRNA expression in HEK293 cells after 48 hours silencing with 10 nM negative control siRNA or with 10 nM NR3C2 siRNA. (B) Arithmetic means ± SEM (n=6; arbitrary units) of NR3C2 relative mRNA levels in HEK293 cells after 48 hours silencing with 10 nM negative control siRNA (Neg. siRNA, white bar) or with 10 nM NR3C2 siRNA (NR3C2 siRNA, black bar). Arithmetic means ± SEM (n=6; arbitrary units) of KLOTHO (C) and CYP27B1 (D) relative mRNA levels in HEK293 cells after 48 hours silencing with 10 nM negative control siRNA (white bars) or with 10 nM NR3C2 siRNA (black bars), treated for 24 hours with vehicle alone (Ctr; left bars) or with 10 µM spironolactone (Spiro, right bars). ***(p<0.001) indicate statistically significant differences from HEK293 cells silenced with negative control siRNA and treated with vehicle alone.
Fig. 5. Effects of spironolactone on KLOTHO mRNA expression following silencing of 25-hydroxyvitamin D$_3$ 1-alpha-hydroxylase or vitamin D$_3$ receptor in HEK293 cells. (A) Representative original bands of 25-hydroxyvitamin D$_3$ 1-alpha-hydroxylase CYP27B1 (upper bands) and calibrator/control GAPDH (lower bands) mRNA levels in HEK293 cells after 48 hours silencing with 10 nM negative control siRNA or with 10 nM CYP27B1 siRNA. (B) Arithmetic means ± SEM (n = 6; arbitrary units) of CYP27B1 relative mRNA levels in HEK293 cells after 48 hours silencing with 10 nM negative control siRNA (Neg siRNA, white bar) or with 10 nM CYP27B1 siRNA (CYP27B1 siRNA, black bar). (C) Arithmetic means ± SEM (n = 6; arbitrary units) of KLOTHO relative mRNA levels in HEK293 cells after 48 hours silencing with 10 nM negative control siRNA (white bars) or with 10 nM CYP27B1 siRNA (black bars), treated for 24 hours with vehicle alone (Ctr, left bars) or with 10 µM spironolactone (Spiro, right bars). (D) Representative original bands of vitamin D$_3$ receptor VDR (upper bands) and calibrator/control GAPDH (lower bands) mRNA levels in HEK293 cells after 48 hours silencing with 10 nM negative control siRNA or with 10 nM VDR siRNA. (E) Arithmetic means ± SEM (n = 6; arbitrary units) of VDR relative mRNA levels in HEK293 cells after 48 hours silencing with 10 nM negative control siRNA (Neg siRNA, white bar) or with 10 nM VDR siRNA (VDR siRNA, black bar). (F) Arithmetic means ± SEM (n = 6; arbitrary units) of KLOTHO relative mRNA levels in HEK293 cells after 48 hours silencing with 10 nM negative control siRNA (white bars) or with 10 nM VDR siRNA (black bars), treated for 24 hours with vehicle alone (Ctr, left bars) or with 10 µM spironolactone (Spiro, right bars). **(p<0.01), ****(p<0.001) indicate statistically significant differences from HEK293 cells silenced with negative control siRNA and treated with vehicle alone. #(p<0.05), ##*(p<0.01) indicate statistically significant differences from HEK293 cells silenced with negative control siRNA and treated with 10 µM spironolactone.

levels in HEK293 cells was mediated by the 25-hydroxyvitamin D$_3$ 1-alpha-hydroxylase and/or the vitamin D$_3$ receptor. As illustrated in Fig. 5, silencing of CYP27B1 did not significantly alter KLOTHO mRNA levels. The subsequent treatment with spironolactone was followed by a statistically significant increase of KLOTHO gene expression in HEK293 cells silenced with negative control siRNA, an effect virtually abrogated in HEK293 cells silenced with CYP27B1 siRNA. Similar observations were made following silencing of the vitamin D$_3$ receptor (VDR). Silencing of VDR did not significantly modify KLOTHO transcript levels. However, treatment
with spironolactone was followed by a statistically significant increase of *KLOTHO* mRNA levels in HEK293 cells silenced with negative control siRNA, but not in HEK293 cells silenced with VDR siRNA (Fig. 5F).

Additional experiments were performed to elucidate the effect of spironolactone on *Klotho* gene expression in vivo (Fig. 6). Mice were fed with control drinking water or with drinking water containing spironolactone (80mg/l) for 5 days. As shown in Fig. 6A, spironolactone treatment was followed by a statistically significant increase of *Klotho* mRNA levels. This effect was again paralleled by a significant increase of *Cyp27b1* gene expression following spironolactone treatment (Fig. 6B). However, no significant differences in klotho or CYP27B1 protein expression could be observed in the kidney tissues between spironolactone treated mice and control treated mice (data not shown). Moreover, the spironolactone treatment did not significantly modify plasma 1,25(OH)_2D_3_ levels (145.3 ± 6.2 and 150.4 ± 6.2 pmol/l; n=6; in control treated and spironolactone treated mice, respectively), renal *Cyp24a1* mRNA expression (1.10 ± 0.18 and 1.41 ± 0.29 a.u.; n=8-9; in control treated and spironolactone treated mice, respectively) and renal *Vdr* mRNA expression (1.02 ± 0.07 and 1.09 ± 0.03 a.u.; n=8-9, in control treated and spironolactone treated mice, respectively).

**Discussion**

Mineralocorticoid receptor activation is associated with a variety of pathological disorders and mineralocorticoid receptor blockade has been shown to exert beneficial effects in several preclinical models and human studies [38]. The present study confirms the previous observation [13] that klotho expression is downregulated by aldosterone. The present observations demonstrate that the moderately selective mineralocorticoid receptor antagonist spironolactone [39, 40] does not only reverse the inhibitory effect of aldosterone on renal *Klotho* gene expression, but upregulates *Klotho* mRNA levels even in the absence of exogenous aldosterone. Despite low expression of *KLOTHO* in HEK293 cells due to promoter methylation, similar effects of spironolactone could be observed in those cells as in vivo [41, 42]. Spironolactone upregulated *KLOTHO* mRNA and protein levels even in charcoal-stripped PBS media, an observation pointing to an effect independent of mineralocorticoid receptor blockade. Along those lines, silencing of the mineralocorticoid receptor did not significantly modify *KLOTHO* mRNA levels and did not mimic the upregulation of *KLOTHO* mRNA levels following spironolactone treatment. Taken together, the observations suggest that spironolactone stimulates *KLOTHO* gene expression at least in part by a mechanism other than mineralocorticoid receptor blockade. As silencing did not completely abrogate NR3C2 expression, however, the present observation do not safely rule out involvement of the mineralocorticoid receptor.
The effect of spironolactone on KLOTHO gene expression is paralleled by a similar regulation of CYP27B1 mRNA and protein levels. More importantly, the effect of spironolactone on KLOTHO gene expression is significantly blunted or virtually abrogated following silencing of either the 25-hydroxyvitamin D₃, 1-alpha-hydroxylase or the vitamin D₃ receptor. Thus, spironolactone stimulates the 25-hydroxyvitamin D₃ 1-alpha-hydroxylase leading to stimulation of 1,25(OH)₂D₃ formation and subsequent activation of the vitamin D₃ receptor. Nevertheless, no significant differences in klotho or CYP27B1 protein levels could be observed between spironolactone treated mice and control treated mice. Possibly, the effect of spironolactone may have been too small to be apparent following analysis of the heterogeneous tissues of whole kidneys. Furthermore, no significant increase in plasma 1,25(OH)₂D₃ levels was observed following a 5 days treatment of mice with spironolactone, an observation possibly reflecting an auto- or paracrine effect [43].

1,25(OH)₂D₃ is a well-known stimulator of klotho expression [20, 44]. Activation of the vitamin D₃ receptor thus leads to upregulation of KLOTHO gene expression. Klotho is required for the inhibitory effect of FGF23 on 25-hydroxyvitamin D₃ 1-alpha-hydroxylase and klotho thus decreases the 1,25(OH)₂D₃ production [4, 45, 46]. The stimulation of klotho by 1,25(OH)₂D₃ thus closes a negative feedback loop limiting the formation of 1,25(OH)₂D₃ [4]. Previous observations pointed to mineralocorticoid receptor-independent effects of spironolactone [47-51]. Although aldosterone activates NFkB, spironolactone additionally inhibits NFkB in a MR-independent manner [51, 52]. NFkB represses 25-hydroxyvitamin D₃ 1-alpha-hydroxylase expression and treatment of HEK293 cells with an NFkB inhibitor increases Cyp27b1 expression [53]. In addition to blocking the effects of aldosterone, spironolactone could therefore increase Cyp27b1 expression and subsequent klotho expression by inhibiting NFkB activity independent of the mineralocorticoid receptor.

In patients with chronic kidney disease (CKD), klotho and 1,25(OH)₂D₃ levels are strongly reduced, while phosphate levels are increased [10, 54, 55]. The increased phosphate levels in these patients are correlated with vascular calcification, a main factor in the mortality of those patients [56]. Despite the stimulatory effect of 1,25(OH)₂D₃ on phosphate reabsorption, reduced levels of 1,25(OH)₂D₃ are similarly associated with increased mortality in CKD patients [54]. Although previous studies in preclinical models suggested an adverse effect of calcitriol on vascular calcification, supplementation of 1,25(OH)₂D₃ at physiological doses increased klotho levels and provided beneficial effects [57-59]. Moreover, 1,25(OH)₂D₃ stimulates nitric oxide production [60] and modifies glucose metabolism. Accordingly, 1,25(OH)₂D₃ may exert beneficial cardiovascular effects [61, 62]. The klotho protein similarly reduces vascular dysfunction, renal fibrosis and aging [17, 20, 63-65]. Klotho inhibits epithelial-to-mesenchymal transition, which was implicated in anti-cancerogenous effects of klotho [8, 16]. Spironolactone is protective in various models of renal and cardiovascular disease, an effect at least partly independent from lowering of blood pressure [66-69]. The effects of spironolactone on in vivo Klotho mRNA expression were significant, but minor and presumably cannot fully account for the beneficial effects of spironolactone in various disease models [70]. Yet, the upregulation of Klotho expression is in accordance with the various vasculo- and reno-protective effects of spironolactone [70]. Mineralocorticoid receptor blockade may provide beneficial effects independently of klotho and 1,25(OH)₂D₃ [30, 31, 38]. It is possible, however, that the upregulation of Cyp27b1 and Klotho could contribute to the protective effects of spironolactone. As especially in CKD patients, both 1,25(OH)₂D₃ and klotho are reduced, the present observations suggest a possible benefit of spironolactone in CKD patients. Even though the potassium retaining effects of spironolactone are a concern especially in CKD patients, several studies do suggest that mineralocorticoid receptor blockers may be well tolerated by CKD patients [71-73].

Conclusion

Cyp27b1 expression is upregulated by spironolactone by a mineralocorticoid receptor-independent mechanism, followed by a subsequent upregulation of klotho expression in
murine renal tissue and in HEK293 cells. This regulation could contribute to spironolactone associated organ protection.

Conflict of Interest

All authors disclose that they have no potential conflict of interest. And that the results presented in this paper have not been published previously in whole or part, except in abstract format.

Acknowledgements

The authors acknowledge the technical assistance of E. Faber and the meticulous preparation of the manuscript by Lejla Subasic and Tanja Loch. The klotho antibody was a kind gift from Kyowa Hakko Kirin Co.Ltd, Japan. This study was supported by the Deutsche Forschungsgemeinschaft.

References

1. Kuro-o M: Klotho. Pflugers Arch 2010;459:333-343.
2. Drueke TB: Klotho, FGF23, and FGF receptors in chronic kidney disease: a yin-yang situation? Kidney Int 2010;78:1057-1060.
3. Takeshita K, Fujimori T, Kurotaki Y, Honjo H, Tsujikawa H, Yasui K, Lee JK, Kamiya K, Kitaichi K, Yamamoto K, Ito M, Kondo T, Iino S, Inden Y, Hirai M, Murohara T, Kodama I, Nabeshima Y: Sinoatrial node dysfunction and early unexpected death of mice with a defect of klotho gene expression. Circulation 2004;109:1776-1782.
4. Tsujikawa H, Kurotaki Y, Fujimori T, Fukuda K, Nabeshima Y: Klotho, a gene related to a syndrome resembling human premature aging, functions in a negative regulatory circuit of vitamin D endocrine system. Mol Endocrinol 2003;17:2393-2403.
5. Imura A, Iwano A, Tohyama O, Tsuji Y, Nozaki K, Hashimoto N, Fujimori T, Nabeshima Y: Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane. FEBS Lett 2004;565:143-147.
6. Akimoto T, Yoshizawa H, Watanabe Y, Numata A, Yamazaki T, Takeshima E, Iwazu K, Komada T, Otani N, Morishita Y, Ito C, Shiizaki K, Ando Y, Muto S, Kuro O, Kusano E: Characteristics of urinary and serum soluble Klotho protein in patients with different degrees of chronic kidney disease. BMC Nephrol 2012;13:155.
7. Dermaku-Sopjani M, Sopjani M, Saxena A, ShojaiYeFard M, Bogatikov E, Alesutan I, Eichenmuller M, Lang F: Downregulation of NaPi-IIa and NaPi-IIb Na-coupled phosphate transporters by coexpression of Klotho. Cell Physiol Biochem 2011;28:251-258.
8. Kuro-o M: Klotho in health and disease. Curr Opin Nephrol Hypertens 2012;21:362-368.
9. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurbayashi M, Kaname T, Kume E, Iwasaki H, Ida A, Shiraki-Iida T, Nishikawa S, Nagai R, Nabeshima Y: Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 1997;390:45-51.
10. Hu MC, Shi M, Zhang J, Quinones H, Griffith C, Kuro O, Moe OW: Klotho Deficiency Causes Vascular Calcification in Chronic Kidney Disease. J Am Soc Nephrol 2011;22:124-136.
11. Ohnishi M, Razzaque MS: Dietary and genetic evidence for phosphate toxicity accelerating mammalian aging. FASEB J 2010;24:3562-3571.
12. Moreno JA, Izquierdo MC, Sanchez-Nino MD, Suarez-Alvarez B, Lopez-Larrea C, Jakubowski A, Blanco J, Ramirez R, Selgas R, Ruiz-Ortega M, Egido J, Ortiz A, Sanz AB: The inflammatory cytokines TWEAK and TNFalpha reduce renal klotho expression through NFkappaB. J Am Soc Nephrol 2011;22:1315-1325.
Alesutan et al: Spironolactone-Sensitive Klotho Expression

13 Tang C, Pathare G, Michael D, Fajol A, Eichenmuller M, Lang F: Downregulation of Klotho expression by dehydration. Am J Physiol Renal Physiol 2011;301:F745-F750.

14 Tang R, Zhou QL, Ao X, Peng WS, Veeraragoo F, Tang TF: Fosinopril and losartan regulate klotho gene and nicotinamide adenine dinucleotide phosphate oxidase expression in kidneys of spontaneously hypertensive rats. Kidney Blood Press Res 2011;34:350-357.

15 Yoon HE, Ghee JY, Piao S, Song JH, Han DH, Kim S, Ohashi N, Kobori H, Kuro-o M, Yang CW: Angiotensin II blockade upregulates the expression of Klotho, the anti-ageing gene, in an experimental model of chronic cyclosporine nephropathy. Nephrol Dial Transplant 2011;26:800-813.

16 Doi S, Zou Y, Togao O, Pastor JV, John GB, Wang L, Shizaki K, Gotschall R, Schaivi S, Yorioka N, Takahashi M, Boothman DA, Kuro-o M: Klotho inhibits transforming growth factor-beta1 (TGF-beta1) signaling and suppresses renal fibrosis and cancer metastasis in mice. J Biol Chem 2011;286:8655-8665.

17 Kurosu H, Yamamoto M, Clark JD, Pastor JV, Nandi A, Gurnani P, McGuinness OP, Chikuda H, Yamaguchi M, Kawaguchi H, Shimomura I, Takayama Y, Herz J, Kohn CR, Rosenblatt KP, Kuro-o M: Suppression of aging by the hormone Klotho. Science 2005;309:1829-1833.

18 Haruna Y, Kashihara N, Tomita S, Namikoshi T, Sasaki T, Fujimori T, Xie P, Kanwar YS: Amelioration of progressive renal injury by genetic manipulation of Klotho gene. Proc Natl Acad Sci U S A 2007;104:2331-2336.

19 Ko GJ, Lee EA, Jeon US, Pyo HJ, Chin HJ, Chae DW, Kim S, Kwon YJ: The Association of Klotho Polymorphism with Disease Progression and Mortality in IgA Nephropathy. Kidney Blood Press Res 2012;36:191-199.

20 Lim K, Lu TS, Molostov G, Lee C, Lam FT, Zehnder D, Hsiao LL: Vascular Klotho deficiency potentiates the development of human artery calcification and mediates resistance to fibroblast growth factor 23. Circulation 2012;125:2243-2255.

21 Xie J, Cha SK, An SW, Kuro O, Birnbaumer L, Huang CL: Cardioprotection by Klotho through downregulation of TRPC6 channels in the mouse heart. Nat Commun 2012;3:1238.

22 Fischer SS, Kempe DS, Leibrock CB, Rzepka R, Boini KM, Ackermann TF, Foller M, Hocher B, Rosenblatt KP, Kuro-o M, Lang F: Hyperaldosteronism in Klotho-deficient mice. Am J Physiol Renal Physiol 2010;299:F1171-F1177.

23 Hadchouel J, Busct C, Procino G, Valentì G, Chambrey R, Eladari D: Regulation of extracellular fluid volume and blood pressure by pendrin. Cell Physiol Biochem 2011;28:505-512.

24 Jia EZ, Xu ZX, Guo CY, Li L, Gu Y, Zhu TB, Wang LS, Cao KJ, Ma WZ, Yang ZJ: Renin-angiotensin-aldosterone system gene polymorphisms and coronary artery disease: detection of gene-gene and gene-environment interactions. Cell Physiol Biochem 2012;29:443-452.

25 Makowka A, Olejniczak-Fortak M, Nowick M: A Comparison of the Antihypertensive and Anti-Inflammatory Effects of Aliskiren and Ramipril Add-On Therapy in Peritoni Dialysis Patients - A Pilot Open Label Study Kidney Blood Press Res 2012;36:18-25.

26 Marney AM, Brown NJ: Aldosterone and end-organ damage. Clin Sci (Lond) 2007;113:267-278.

27 McCurley A, Jaffe IZ: Mineralocorticoid receptors in vascular function and disease. Mol Cell Endocrinol 2012;350:256-265.

28 Nowakowska FE, Herlitz H, Saeed A, Atttman PO, Jensen G, Alaupovic P, Gurun G: Lipoprotein abnormalities in patients with atherosclerotic renovascular disease. Kidney Blood Press Res 2011;34:311-319.

29 Jain G, Campbell RC, Warnock DG: Mineralocorticoid receptor blockers and chronic kidney disease. Clin J Am Soc Nephrol 2009;4:1685-1691.

30 Voelld A, Alesutan I, Leibrock CB, Quintanilla-Martinez L, Kuhn V, Feger M, Mia S, Ahmed MS, Rosenblatt KP, Kuro-o M, Lang F: Spironolactone ameliorates PIT1-dependent vascular osteoinduction in klotho-hypomorphic mice. J Clin Invest 2013;123:812-822.

31 Wu SY, Yu VR, Cai Y, Jia LX, Wang X, Xiao CS, Tang CS, Qi YF: Endogenous aldosterone is involved in vascular calcification in rats. Exp Biol Med (Maywood) 2012;237:31-37.

32 Guney I, Selcuk NY, Altintepe I, Atilay H, Basaralı MK, Buyukbas S: Antifibrotic effects of aldosterone receptor blocker (spironolactone) in patients with chronic kidney disease. Ren Fail 2009;31:779-784.

33 Hene RJ, Boer P, Konmans HA, Mees EJ: Plasma aldosterone concentrations in chronic renal disease. Kidney Int 1982;21:98-101.

34 Jaffe IZ, Mendelsohn ME: Angiotensin II and aldosterone regulate gene transcription via functional mineralocorticoid receptors in human coronary artery smooth muscle cells. Circ Res 2005;96:643-650.
Alesutan et al: Spironolactone-Sensitive Klotho Expression

35 Jaffe IZ, Tintut Y, Newfell BG, Demer LL, Mendelsohn ME: Mineralocorticoid receptor activation promotes vascular cell calcification. Arterioscler Thromb Vasc Biol 2007;27:799-805.

36 Kiyosue A, Nagata D, Myojo M, Sato T, Takahashi M, Satonaka H, Nagai R, Hirata Y: Aldosterone-induced osteopontin gene transcription in vascular smooth muscle cells involves glucocorticoid response element. Hypertens Res 2011;34:1283-1287.

37 Boini KM, Hennige AM, Huang DY, Friedlich B, Palmad M, Boehmer C, Grahama F, Art unc F, Ullrich S, Avram D, Oswood H, Wulf P, Kuhl D, Vallon V, Haring HU, Lang F: Serum- and glucocorticoid-inducible kinase 1 mediates salt sensitivity of glucose tolerance. Diabetes 2006;55:2059-2066.

38 Mukero P, Milan A, Williams TA, Veglio F: Mineralocorticoid receptor blockade in the protection of target organ damage. Cardiovasc Hematol Agents Med Chem 2006;4:75-91.

39 Epstein M, Calhoun DA: Aldosterone blockers (mineralocorticoid receptor antagonist) and potassium-sparing diuretics. J Clin Hypertens (Greenwich) 2011;13:644-648.

40 Lammers C, Dartsch T, Brandt MC, Rottlander D, Halbach M, Peinkofer G, Ockenpoehler S, Weigergraeb M, Schneider T, Reuter H, Zobel C: Spironolactone prevents aldosterone induced increased duration of atrial fibrillation in rat. Cell Physiol Biochem 2012;29:833-840.

41 Azuma M, Koyama D, Kikuchi J, Yoshizawa H, Thasinas D, Shiizaki K, Kuro-o M, Furukawa Y, Kusano E: Promoter methylation confers kidney-specific expression of the Klotho gene. FASEB J 2012;26:4264-4274.

42 Zhang H, Li Y, Fan Y, Wu J, Zhao B, Guan Y, Chien S, Wang N: Klotho is a target gene of PPAR-gamma. Kidney Int 2008;74:732-739.

43 Messa P, Alfieri C, Rastaldi MP: Recent insights into vitamin D and its receptor. J Nephrol 2011;24:S30-S37.

44 Haussler MR, Haussler CA, Whitfield GK, Hsieh JC, Thompson PD, Barthel TK, Bartik L, Egan JB, Wu Y, Kubicek JL, Lowmiller CL, Moffet EW, Jurutka PW: The nuclear vitamin D receptor controls the expression of genes encoding factors which feed the "Fountain of Youth" to mediate healthful aging. J Steroid Biochem Mol Biol 2010;121:88-97.

45 Yoshida T, Fujimori T, Nabeshima Y: Mediation of unusually high concentrations of 1,25-dihydroxyvitamin D in homozygous klotho mutant mice by increased expression of renal 1alpha-hydroxylase gene. Endocrinology 2002;143:683-689.

46 Razzque MS, Sitara D, Taguchi T, St Arnaud R, Lanske B: Premature aging-like phenotype in fibroblast growth factor 23 null mice is a vitamin D-mediated process. FASEB J 2006;20:720-722.

47 Grossmann C, Benesic A, Sprecher AW, Freuding R, Mildenberger S, Gassner B, Geke M: Human mineralocorticoid receptor expression renders cells responsive for nongenotropic aldosterone actions. Mol Endocrinol 2005;19:1697-1710.

48 Hansen PR, Rieneck K, Bendtzen K: Spironolactone inhibits production of proinflammatory cytokines by human mononuclear cells. Immunol Lett 2004;91:87-91.

49 Schuetz EG, Brimer C, Schuetz JD: Environmental xenobiotics and the antihormones cyproterone acetate and spironolactone use the nuclear hormone pregnenolone X receptor to activate the CYP3A23 hormone response element. Mol Pharmacol 1998;54:1113-1117.

50 Sonder SU, Mikkelson M, Rieneck K, Hedegaard CJ, Bendtzen K: Effects of spironolactone on human blood mononuclear cells: mineralocorticoid receptor independent effects on gene expression and late apoptosis induction. Br J Pharmacol 2006;148:46-53.

51 Sonder SU, Woetmann A, Odum N, Bendtzen K: Spironolactone induces apoptosis and inhibits NF-kappaB independent of the mineralocorticoid receptor. Apoptosis 2006;11:2159-2165.

52 Fukuda S, Horimai C, Harada K, Nakamatsu T, Fukasawa H, Muto S, Itai A, Hayashi M: Aldosterone-induced kidney injury is mediated by NF-kappaB activation. Clin Exp Nephrol 2011;15:41-49.

53 Ebert R, Jovanovic M, Ulmer M, Schneider D, Meissner-Weigel J, Adamski J, Jakob F: Down-regulation by nuclear factor kappaB of human 25-hydroxyvitamin D3 1alpha-hydroxylase promoter. Mol Endocrinol 2004;18:2440-2450.

54 Plt S, Tomaschitz A, Friedl C, Amrein K, Drechsler C, Ritz E, Boehm BO, Grammer TB, Marz W: Vitamin D status and mortality in chronic kidney disease. Nephrol Dial Transplant 2011;26:3603-3609.

55 Shimamura Y, Hamada K, Inoue K, Ogata K, Ishihara M, Kagawa T, Inoue M, Fujimoto S, Ilee M, Yuasa K, Yamazaki S, Sugita T, Terada Y: Serum levels of soluble secreted alpha-Klotho are decreased in the early stages of chronic kidney disease, making it a probable novel biomarker for early diagnosis. Clin Exp Nephrol 2012;16:722-729.
56 Shanahan CM, Crouthamel MH, Kapustin A, Giachelli CM: Arterial calcification in chronic kidney disease: key roles for calcium and phosphate. Circ Res 2011;109:697-711.
57 Becker LE, Koleganova N, Flecha G, Noronha IL, Zeier M, Geldyev A, Kokeny G, Ritz E, Gross ML: Effect of paricalcitol and calcitriol on aortic wall remodeling in uninephrectomized ApoE knockout mice. Am J Physiol Renal Physiol 2011;300:F772-F782.
58 Guerrero E, Montes de Oca A, Aguilera-Tejero E, Zafra R, Rodriguez M, Lopez I: The effect of vitamin D derivatives on vascular calcification associated with inflammation. Nephrol Dial Transplant 2012;27:2206-2212.
59 Lau WL, Leaf EM, Hu MC, Takeno MM, Kuro O, Moe OW, Giachelli CM: Vitamin D receptor agonists increase klotho and osteopontin while decreasing aortic calcification in mice with chronic kidney disease fed a high phosphate diet. Kidney Int 2012;82:1261-1270.
60 Molinari C, Uberti F, Grossini E, Vacca S, Cipolloni M, Ciseri M: 1alpha,25-dihydroxycholecalciferol induces nitric oxide production in cultured endothelial cells. Cell Physiol Biochem 2011;27:661-668.
61 Guessous I, Bochud M, Bonny O, Burnier M: Calcium, vitamin D and cardiovascular disease. Kidney Blood Press Res 2011;34:404-417.
62 Vedralova M, Kotrbova-Kozak A, Zelezniakova V, Zoubkova H, Rychlik I, Vendl L, Cerna M: Polymorphisms in the Vitamin D Receptor Gene and Parathyroid Hormone Gene in the Development and Progression of Diabetes Mellitus and its Chronic Complications, Diabetic Nephropathy and Non-Diabetic Renal Disease. Kidney Blood Press Res 2012;36:1-9.
63 Ikushima M, Rakugi H, Ishikawa K, Maekawa Y, Yamamoto K, Ohta J, Chihara Y, Kida I, Ogihara T: Anti-apoptotic and anti-senescence effects of Klotho on vascular endothelial cells. Biochem Biophys Res Commun 2006;339:827-832.
64 Kusaba T, Okigaki M, Matui A, Murakami M, Ishikawa K, Kimura T, Sonomura K, Adachi Y, Shibuya M, Shirayama T, Tanda S, Hatta T, Sasaki S, Mori Y, Matsubara H: Klotho is associated with VEGF receptor-2 and the transient receptor potential canonical-1 Ca2+ channel to maintain endothelial integrity. Proc Natl Acad Sci U S A 2010;107:19308-19313.
65 Satoh M, Nagasu H, Morita Y, Yamaguchi TP, Kanwar YS, Kashihara N: Klotho protects against mouse renal fibrosis by inhibiting Wnt signalling. Am J Physiol Renal Physiol 2012;303:F1641-F1651.
66 Calvier L, Miana M, Rebold R, Cacheleto A, Martinez-Martinez E, de Boer RA, Poirier F, Lacolley P, Zannad F, Rossignol P, Lopez-Andres N: Galectin-3 Mediates Aldosterone-Induced Vascular Fibrosis. Arterioscler Thromb Vasc Biol 2013;33:67-75.
67 Barrera-Chimal J, Perez-Villalva R, Rodriguez-Romo R, Reyna J, Uribe N, Gamba G, Bobadilla NA: Spironolactone prevents chronic kidney disease caused by ischemic acute kidney injury. Kidney Int 2013;83:93-103.
68 Quaschning T, Rutschitzka F, Shaw S, Luscher TF: Aldosterone receptor antagonism normalizes vascular function in liquorice-induced hypertension. Hypertension 2001;37:801-805.
69 Trachtman H, Weiser AC, Valderrama E, Morgado M, Palmer LS: Prevention of renal fibrosis by spironolactone in mice with complete unilateral ureteral obstruction. J Urol 2004;172:1590-1594.
70 Ritz E, Koleganova N: Aldosterone in uremia - beyond blood pressure. Blood Purif 2010;29:111-113.
71 Bianchi S, Bigazzi R, Campese VM: Antagonists of aldosterone and proteinuria in patients with CKD: an uncontrolled pilot study. Am J Kidney Dis 2005;46:45-51.
72 Edwards NC, Steeds RP, Chue CD, Stewart PM, Ferro CJ, Townend JN: The safety and tolerability of spironolactone in patients with mild to moderate chronic kidney disease. Br J Clin Pharmacol 2012;73:447-454.
73 Heshka J, Ruzicka M, Hiremath S, McCormick BB: Spironolactone for difficult to control hypertension in chronic kidney disease: an analysis of safety and efficacy. J Am Soc Hypertens 2010;4:295-301.