Original Paper

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

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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.

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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. s14777, 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 $1,25(\text{OH})_2\text{D}_3$ 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: GTGAAGCGTGCGCCAAAAG; *Cyp24a1* rev: CTCACCGTCGGTCATCAGC;
Cyp27b1 fw: CAGTTTACGTTGCCGACCCTA; *Cyp27b1* rev: GGACAGTGACTTTCTTGTCTCGC;
Gapdh fw: AGGTCGGTGTGAACGGATTTG; *Gapdh* rev: TGTAGACCATGTAGTTGAGTGCA;
Klotho fw: CCCTGTGACTTTGCTTGGG; *Klotho* rev: CCCACAGATAGACATTCGGGT;
Vdr fw: GATGCCACCAACAAGACCTAC; *Vdr* rev: GTCTGCACGAATTGGAGGC.

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

CYP27B1 fw: GGAACCTGAACAACGTAGTC; *CYP27B1* rev: AGTCCGAACCTGTAAAATTCCCC;
GAPDH fw: GAGTCAACGGATTTGGTCGT; *GAPDH* rev: GACAAGCTTCCCGTTCTCAG;
KLOTHO fw: GGTGTCCATTGCCCTAAGCTC; *KLOTHO* rev: TCGGTCATTCTTCGAGGATTGA;
NR3C2 fw: AGCAGAACCAACAAGGAAGCA; *NR3C2* rev: GTGTTACACAACCTTAGAGTGGA;
VDR fw: TCTCCAATCTGGATCTGAGTGAA; *VDR* rev: ACAGCTCTAGGGTCACAGAAG.

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 $2^{-\Delta\Delta\text{Ct}}$ 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 $\pm$ 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 μ M). 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

Fig. 1. Effects of aldosterone and spironolactone on *KLOTHO* and *CYP27B1* gene expression in HEK293 cells. Arithmetic means $\pm$ SEM (n=5-6, arbitrary units) of *KLOTHO* (A) and *CYP27B1* (B) relative mRNA levels in HEK293 cells after a 24 hours treatment with vehicle alone (white bar; Ctr), with 100 nM aldosterone alone (black bar; Aldo), with 100 nM aldosterone and 10 μ M spironolactone (dark grey bar; Aldo + Spiro) or with 10 μ M spironolactone alone (light grey bar; Spiro). *(p<0.05), **(p<0.01) indicate statistically significant differences from HEK293 cells treated with vehicle alone; ###(p<0.001) indicate statistically significant differences from HEK293 cells treated with aldosterone.

Fig. 2. Effects of DOCA and spironolactone treatment on *Klotho* and *Cyp27b1* gene expression in the kidney. Arithmetic means $\pm$ SEM (n=8, arbitrary units) of *Klotho* (A) and *Cyp27b1* (B) relative mRNA levels in kidney tissue from mice treated for 8 hours with vehicle (white bar; Ctr), with DOCA alone (black bar; DOCA), with DOCA and spironolactone (dark grey bar; DOCA + Spiro) or with spironolactone alone (light grey bar; Spiro). *(p<0.05), **(p<0.01) indicate statistically significant differences from control treated mice. # (p<0.05), ###(p<0.001) indicate statistically significant differences from DOCA treated mice.

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 μ M spironolactone (Spiro). (B) Arithmetic means $\pm$ 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 μ M 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 μ M spironolactone (Spiro). (D) Arithmetic means $\pm$ 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 μ M 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 $\pm$ 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 $\pm$ 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 μ M spironolactone (Spiro, right bars). ***(p<0.001), ***(p<0.001) indicate statistically significant differences from HEK293 cells silenced with negative control siRNA and treated with vehicle alone.

Arithmetic means $\pm$ 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 μ M spironolactone (Spiro, right bars). ***(p<0.001), ***(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 D₃ 1- α -hydroxylase or vitamin D₃ receptor in HEK293 cells. (A) Representative original bands of 25-hydroxyvitamin D₃ 1- α -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 $\pm$ 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 $\pm$ 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 μ M spironolactone (Spiro, right bars). (D) Representative original bands of vitamin D₃ 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 $\pm$ 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 $\pm$ 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 μ M 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 μ M spironolactone.

levels in HEK293 cells was mediated by the 25-hydroxyvitamin D₃ 1- α -hydroxylase and/or the vitamin D₃ 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 D₃ receptor (VDR). Silencing of VDR did not significantly modify *KLOTHO* transcript levels. However, treatment

Fig. 6. Regulation of renal *Klotho* and *Cyp27b1* gene expression following 5 days treatment with spironolactone. Arithmetic means $\pm$ SEM (n=8-9, arbitrary units) of *Klotho* (A) and *Cyp27b1* (B) relative mRNA levels in kidney tissue from mice following treatment for 5 days with control solution (Ctr; white bar) or with spironolactone (Spiro, black bar) in drinking water. *(p<0.05) indicates statistically significant differences from control treated mice.

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)₂D₃ levels (145.3 $\pm$ 6.2 and 150.4 $\pm$ 6.2 pmol/l; n=6; in control treated and spironolactone treated mice, respectively), renal *Cyp24a1* mRNA expression (1.10 $\pm$ 0.18 and 1.41 $\pm$ 0.29 a.u.; n=8-9; in control treated and spironolactone treated mice, respectively) and renal *Vdr* mRNA expression (1.02 $\pm$ 0.07 and 1.09 $\pm$ 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 FBS 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- α -hydroxylase or the vitamin D₃ receptor. Thus, spironolactone stimulates the 25-hydroxyvitamin D₃ 1- α -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 heterogenous 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- α -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 NF κ B, spironolactone additionally inhibits NF κ B in a MR-independent manner [51, 52]. NF κ B represses 25-hydroxyvitamin D₃ 1- α -hydroxylase expression and treatment of HEK293 cells with an NF κ B 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 NF κ B 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.

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