Original Paper

Down-Regulation of Renal Klotho Expression by Shiga Toxin 2

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Ke Words

25-hydroxyvitamin D₃ 1- α -hydroxylase • 1,25(OH)₂D₃ • klotho • FGF23 • phosphate • p38 MAPK

Abstract

Background/Aims: Shiga toxin 2 may trigger classical hemolytic uremic syndrome (HUS) eventually leading to renal failure. Klotho, a transmembrane protein, protease and hormone mainly expressed in kidney is involved in the regulation of renal phosphate excretion and also retains renal protective effects. Renal failure is associated with renal depletion of klotho. The present study explored the influence of Shiga toxin 2 on renal klotho expression. **Methods:** Mice were injected with either solvent or Shiga toxin 2 and urinary flow rate and phosphate excretion were determined in metabolic cages. Renal transcript levels were measured by quantitative RT-PCR and renal protein abundance by Western blotting. Plasma concentrations of 1,25(OH)₂D₃ and FGF23 were determined by ELISA and plasma phosphate and urea concentrations by photometry. **Results:** Shiga toxin 2 treatment was followed by increase of plasma urea concentration, urinary flow rate and renal phosphate excretion but not of plasma phosphate concentration. Shiga toxin 2 treatment strongly decreased *klotho* mRNA expression and klotho protein abundance in renal tissue. Shiga toxin 2 treatment further increased tumor necrosis factor (*Tnf α*) mRNA levels, as well as protein abundance of phosphorylated p38 MAPK in renal tissue. The treatment significantly increased renal *Cyp27b1* and decreased renal *Cyp24a1* mRNA levels without significantly altering plasma 1,25(OH)₂D₃ levels. Shiga toxin 2 treatment was further followed by increase of plasma FGF23 concentrations. **Conclusion:** Shiga toxin 2 treatment stimulated *Tnf α* transcription, down-regulated renal klotho expression and increased FGF23 formation, effects presumably contributing to renal tissue injury.

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Introduction

Infections with Shiga toxin producing *Escherichia coli* may lead to haemolytic uremic syndrome (HUS) [1-4], a disorder characterized by haemolytic anaemia with fragmented erythrocytes, thrombocytopenia and acute renal failure [2, 5-10]. *Escherichia coli* may produce Shiga toxins 1 and/or 2 (Vero toxins or Vero cytotoxins) [2, 11]. Mechanisms involved in triggering of acute renal injury following Shiga toxin exposure include damaging vascular endothelial cells, triggering cytokine secretion and/or complement activation [1]. Shiga toxin induced renal damage involves acute tubular damage [12].

Acute renal injury may be paralleled and fostered by decreased formation of klotho [13], a transmembrane protein mainly expressed in the kidney [14-17]. Klotho is secreted into blood, urine and spinal fluid [18, 19]. Klotho serves a wide variety of functions including regulation of phosphate homeostasis [17, 20]. Reductions of klotho are discussed as a sensitive biomarker of renal disease [13].

Klotho deficiency results in hyperphosphatemia with severe calcifications and dramatically reduced lifespan in mice [21, 22]. Klotho is required for the inhibitory effect of FGF23 on 25-hydroxyvitamin D₃ 1- α -hydroxylase (encoded by the *Cyp27b1* gene). Accordingly, klotho deficiency results in excessive formation of 1,25(OH)₂D₃, which stimulates intestinal phosphate and Ca²⁺ absorption. The resulting hyperphosphatemia is followed by calcium phosphate precipitations and vascular calcification [22-24]. 1,25(OH)₂D₃ stimulates klotho expression thereby closing a negative feedback loop [16]. Klotho expression is down-regulated by several inflammatory mediators and by aldosterone [25-27]. It is up-regulated by angiotensin II blockade [28]. Klotho is down-regulated by mineralocorticoid receptor activation and ADH [26] and is up-regulated by the moderately selective mineralocorticoid receptor antagonist spironolactone [29].

Besides its role in phosphate homeostasis, klotho further regulates several ion channels, influences epithelial-to-mesenchymal transition and counteracts aging [17, 22, 30, 31]. Klotho further inhibits apoptosis [32]. Klotho confers some protection of the kidney [27, 33, 34] and klotho deficiency may aggravate acute kidney damage [13]. Beyond that, klotho counteracts vascular calcification [22, 35, 36] as well as pathological cardiac remodelling [37]. In chronic kidney disease, klotho deficiency accelerates the progression of renal disease and the appearance of extra-renal complications [13]. The decrease of klotho expression in renal injury leads to FGF23 resistance [19]. In chronic kidney disease the decrease of klotho expression contributes to the development of hyperphosphatemia despite reduced 1,25(OH)₂D₃ levels [17].

Due to the diverse functions of klotho, the present study explored whether Shiga toxin 2 influences klotho expression in renal tissue.

Materials and Methods

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. Where indicated, mice were treated with intraperitoneal injection of Shiga toxin 2 (1ng/20g body weight) at days 0, 3, and 6 [38]. After 8 days of treatment, mice were sacrificed and kidney tissues were rapidly removed and immediately snap frozen.

To determine urinary flow rate, the mice were placed individually in metabolic cages (Tecniplast, Hohenpeissenberg, Germany) as described previously [39, 40]. The urinary phosphate concentration was determined colorimetrically utilizing a commercial diagnostic kit (Roche Diagnostics, Mannheim, Germany). Plasma urea and phosphate concentrations were determined by a photometric method (FUJI FDC 3500i, Sysmex, Norsted, Germany). ELISA was utilized to determine the plasma concentration of 1,25(OH)₂D₃ (IDS, Frankfurt/Main, Germany) and of FGF23 (Immutopics, San Clemente, USA).

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 [41]. 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). 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):

Cyp24a1 fw: GTGAAGCGTGCGCCAAAAG;
Cyp24a1 rev: CTCACCGTCGGTCATCAGC;
Cyp27b1 fw: CAGTTTACGTTGCCGACCCTA;
Cyp27b1 rev: GGACAGTGACTTCTTGTCGC;
Gapdh fw: AGGTCGGTGTGAACGGATTTG;
Gapdh rev: TGTAGACCATGTAGTTGAGGTCA;
Klotho fw: CCCTGTGACTTTGCTTGGG;
Klotho rev: CCCACAGATAGACATTCGGGT;
Tnfr fw: CTGAACCTTCGGGGTGATCGG;
Tnfr rev: GGCTTGCTCACTCGAATTTGAGA.

The specificity of the PCR products was confirmed by analysis of the melting curves. All PCRs were performed in duplicate, and mRNA fold changes were calculated by the $2^{-\Delta\Delta C_t}$ method using *Gapdh* as internal reference.

Western blot analysis

Renal tissue was lysed with ice-cold lysis buffer (Thermo Fisher Scientific, Rockford, IL, 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 5 min, separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated overnight at 4°C with rabbit anti-phospho p38 MAPK (Thr180/Tyr182), rabbit anti-p38 (dilution 1:1000, Cell Signaling, Danvers, MA, USA), rat anti-α-klotho antibody (dilution 1:1000, kindly provided by Kyowa Hakko Kirin Co. Ltd, Japan) or rabbit anti-GAPDH antibody (dilution 1:1000; Cell Signaling, Danvers, MA, USA) and then with secondary anti-rabbit HRP-conjugated antibody (dilution 1:1000; Cell Signaling, Danvers, MA) or secondary anti-rat HRP-conjugated antibody (dilution 1:1000; Cell Signaling, Danvers, MA) for 1 hour at RT. For loading controls, the membranes were stripped in stripping buffer (Carl Roth, Karlsruhe, Germany) at 60°C for 5 min. Antibody binding was detected with the ECL Western Blotting Substrate (Pierce, Rockford, IL, USA). 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 for significance using unpaired Student *t*-test (normally distributed data) or Mann-Whitney test (non-normally distributed data) according to Shapiro-Wilk test. Only results with *p* < 0.05 were considered statistically significant.

Results

The present study explored the renal effects of Shiga toxin 2, which is involved in the development of haemolytic uremic syndrome, leading to renal injury. Shiga toxin 2 was injected (1ng/20g body weight) at days 0, 3, and 6. After 8 days of treatment, Shiga toxin 2

Fig. 1. Effect of Shiga toxin 2 injection on plasma urea levels, urinary flow rate, urinary phosphate excretion and plasma phosphate concentration. Arithmetic means $\pm$ SEM (n=4-5 mice/group) of blood urea nitrogen (A, BUN, mg/dl), urinary flow rate (B, μ l/24h/g BW), urinary phosphate excretion (C, μ mol/24h/g BW) and plasma phosphate concentration (D, mg/dl) in mice after treatment with vehicle (Ctr, white bars) or with Shiga toxin 2 (HUS, black bars). *(p<0.05), ** (p<0.01) indicate statistically significant differences from control treated mice.

Fig. 2. Effect of Shiga toxin 2 injection on renal klotho transcript and protein levels. A. Arithmetic means $\pm$ SEM (n=5, arbitrary units) of relative *klotho* mRNA expression in renal tissues from mice treated with vehicle (Ctr, white bar) or with Shiga toxin 2 (HUS, black bar). B. Representative original western blots and arithmetic means $\pm$ SEM (n=5, arbitrary units) of klotho to Gapdh protein ratio in renal tissue from mice treated with vehicle (Ctr, white bar) or with Shiga toxin 2 (HUS, black bar). *(p<0.05), *** (p<0.001) indicate statistically significant differences from control treated mice.

induced a substantial increase of blood urea nitrogen (BUN, Fig. 1A). Furthermore, Shiga toxin 2 treatment caused a significant increase of urinary flow rate (Fig. 1B). This was associated with an increased renal phosphate excretion (Fig. 1C). The plasma phosphate levels were not significantly modified by the treatment with Shiga toxin 2 (Fig. 1D).

After sacrificing the mice, kidneys were removed and transcript levels were quantified by RT-PCR, as well as protein abundance determined by Western blotting. As illustrated in Fig. 2A, Shiga toxin 2 injection significantly decreased the renal *klotho* mRNA levels. In accordance, the renal protein abundance of klotho was strongly reduced by Shiga toxin 2 treatments (Fig. 2B).

Fig. 3. Effect of Shiga toxin 2 injection on renal *Tnfa* expression and phospho-p38 MAPK and total p38 MAPK protein abundance. A. Arithmetic means $\pm$ SEM (n=5, arbitrary units) of relative *Tnfa* mRNA expression in renal tissues from mice treated with vehicle (Ctr; white bar) or with Shiga toxin 2 (HUS; black bar). B. Representative original western blots showing phosphorylated p38 MAPK, total p38 MAPK and Gapdh protein abundance in renal tissues from mice treated with vehicle (Ctr) or with Shiga toxin 2 (HUS). Arithmetic means $\pm$ SEM (n=5, arbitrary units) of normalized phosphorylated p38 MAPK to Gapdh protein ratio (C) and p38 MAPK to Gapdh protein ratio (D) in renal tissues from mice treated with vehicle (Ctr; white bars) or with Shiga toxin 2 (HUS; black bars). * (p<0.05), ** (p<0.01) indicate statistically significant differences from control treated mice.

To investigate a possible underlying cause for the reduction in klotho expression, the renal expression of *Tnfa* was determined. As a result, Shiga toxin 2 injection strongly increased the transcript levels of *Tnfa* in kidney tissues (Fig. 3A). Shiga toxin 2 injection was further followed by an increase of p38 MAPK phosphorylation in renal tissue. The total p38 MAPK protein abundance was not significantly modified by Shiga toxin 2 in the murine kidneys (Fig. 3B-D).

To investigate, whether the effects of Shiga toxin 2 on klotho expression affected the vitamin D₃ metabolism, the transcript levels of 25-hydroxyvitamin D₃ 1- α -hydroxylase (*Cyp27b1*) and of 24-hydroxyvitamin D₃ 1- α -hydroxylase (*Cyp24a1*) were determined. As a result, the effect of Shiga toxin 2 on klotho expression was paralleled by a significant increase of *Cyp27b1* mRNA expression and a significant decrease of *Cyp24a1* mRNA expression (Fig. 4A,B). In addition, Shiga toxin 2 treatment led to an increase of plasma FGF23 concentrations (Fig. 4C). The plasma 1,25(OH)₂D₃ concentration tended to increase slightly, an effect, however, not reaching statistical significance (Fig. 4D).

Discussion

The present study reveals novel effects of Shiga toxin 2 from *Escherichia coli*, a causative agent of haemolytic uremic syndrome (HUS) [1-4]. Shiga toxin 2 treatment caused a marked reduction in renal klotho expression and increased plasma FGF23 levels. Injection of Shiga toxin 2 was followed by increase of plasma urea concentration and polyuria. Shiga toxin has previously been shown to interfere with urinary concentration [42].

The shiga toxin 2 treatment was paralleled by increase of renal *Tnfa* mRNA expression. Increased *Tnfa* signaling following Shiga toxin 2 treatment presumably contributes to the observed decrease of klotho expression, which was shown to be down-regulated by *Tnfa* [25]. *Tnfa* further downregulates klotho in endothelial cells [43]. Klotho deficiency is associated with increased p38 MAPK activity, which is in turn associated with down-regulation of klotho [44, 45]. The p38 MAPK kinase is causally involved in the effects of Shiga toxin 2 and promotes *Tnfa* expression [46-49]. Along those lines the Shiga toxin-induced increase of *Tnfa* in renal tubular epithelial cells can be attenuated by a p38 inhibitor [50].

Fig. 4. Effects of Shiga toxin 2 injection on renal *Cyp27b1* and *Cyp24a1* expression and plasma $1,25(\text{OH})_2\text{D}_3$ and FGF23 concentrations. Arithmetic means $\pm$ SEM (n=5, arbitrary units) of relative mRNA expression of *Cyp27b1* (A) and *Cyp24a1* (B) in renal tissues from mice treated for 8 days with vehicle (Ctr, white bars) or with Shiga toxin 2 (HUS, black bars). Arithmetic means $\pm$ SEM (n=5 mice/group) of plasma FGF23 (C, pg/ml) and $1,25(\text{OH})_2\text{D}_3$ (D, pmol/l) concentrations in mice after treatment with vehicle (Ctr, white bars) or with Shiga toxin 2 (HUS, black bars). *(p<0.05), **(p<0.01) indicate statistically significant differences from control treated mice.

The effect of Shiga toxin 2 treatment on *klotho* mRNA levels and *klotho* protein abundance presumably accounts for the increase of renal *Cyp27b1* and decrease of renal *Cyp24a1* transcript levels. The decline of *klotho* expression compromises FGF23 signaling down-regulating *Cyp27b1* and up-regulating *Cyp24a1* expression [16, 51, 52]. The alterations of *Cyp27b1* and *Cyp24a1* transcript levels are thus presumably, at least in part, secondary to decreased *klotho* expression. Stimulation of *Cyp27b1* enhances the formation of $1,25(\text{OH})_2\text{D}_3$ and inhibition of *Cyp24a1* disrupts $1,25(\text{OH})_2\text{D}_3$ degradation. Nonetheless, no significant effects on plasma $1,25(\text{OH})_2\text{D}_3$ were observed. FGF23 release could be a compensatory mechanism for the polyuria [53]. The increased urinary phosphate excretion could, at least partially, result from increased FGF23 release. The present observations do, however, not rule out more direct effects of Shiga toxin 2 on *klotho*, *Cyp27b1* and *Cyp24a1* expression and/or FGF23 release.

The *klotho* depleting effect of Shiga toxin 2 could contribute to the pathophysiology of HUS. *Klotho* is a biomarker of renal function, and could also serve to detect and quantify the renal effects of haemolytic uremic syndrome [19]. Besides its role as a biomarker, *klotho* depletion may be causally linked to renal damage. The down-regulation of *klotho* may sensitize the kidneys to injury [13]. *Klotho* deficiency has previously been shown to accelerate the progression of renal disease and the appearance of extra-renal complications, especially in the vasculature [13]. Moreover, *klotho* counteracts vascular dysfunction, renal fibrosis and aging [31, 35, 54-56].

Conclusion

Shiga toxin 2 treatment stimulates renal *Tnfa* expression, up-regulates plasma FGF23 concentrations and down-regulates renal *klotho* expression.

Disclosure Statement

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

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