Original Paper

Upregulation of the Creatine Transporter Slc6A8 by Klotho

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Key Words

Creatine • CreaT • β -glucuronidase • DSAL • Brain

Abstract

Background/Aims: The transmembrane Klotho protein contributes to inhibition of $1,25(\text{OH})_2\text{D}_3$ formation. The extracellular domain of Klotho protein could function as an enzyme with e.g. β -glucuronidase activity, be cleaved off and be released into blood and cerebrospinal fluid. Klotho regulates several cellular transporters. Klotho protein deficiency accelerates the appearance of age related disorders including neurodegeneration and muscle wasting and eventually leads to premature death. The main site of Klotho protein expression is the kidney. Klotho protein is also appreciably expressed in other tissues including chorioid plexus. The present study explored the effect of Klotho protein on the creatine transporter CreaT (Slc6A8), which participates in the maintenance of neuronal function and survival. **Methods:** To this end cRNA encoding Slc6A8 was injected into *Xenopus* oocytes with and without additional injection of cRNA encoding Klotho protein. Creatine transporter CreaT (Slc6A8) activity was estimated from creatine induced current determined by two-electrode voltage-clamp. **Results:** Coexpression of Klotho protein significantly increased creatine-induced current in Slc6A8 expressing *Xenopus* oocytes. Coexpression of Klotho protein delayed the decline of creatine induced current following inhibition of carrier insertion into the cell membrane by brefeldin A (5 μM). The increase of creatine induced current by coexpression of Klotho protein in Slc6A8 expressing *Xenopus* oocytes was reversed by β -glucuronidase inhibitor (DSAL). Similarly, treatment of Slc6A8 expressing *Xenopus* oocytes with recombinant human alpha Klotho protein significantly increased creatine induced current. **Conclusion:** Klotho protein up-regulates

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the activity of creatine transporter CreaT (Slc6A8) by stabilizing the carrier protein in the cell membrane, an effect requiring β -glucuronidase activity of Klotho protein.

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Introduction

Klotho is expressed mainly in kidney and choroid plexus [1, 2]. The extracellular domain of the Klotho protein may function as enzyme with β -glucuronidase activity and, after being cleaved off, as hormone [3-7]. Klotho expression is a decisive determinant of ageing and life span [8, 9]. As shown in mice, Klotho deficiency leads to severe growth retardation and accelerates the appearance of several age related disorders resulting in shortening the life span to less than 5 months [8]. Conversely, Klotho overexpression extends substantially the life span [8, 9].

Klotho is required for the inhibitory effect of FGF23 on 1α -hydroxylase and thus $1,25(\text{OH})_2\text{D}_3$ formation [2, 9-11]. $1,25(\text{OH})_2\text{D}_3$ stimulates Klotho expression [12] as well as intestinal and renal Ca^{2+} and phosphate transport [13, 14]. Lack of Klotho in Klotho-deficient mice [2, 10, 11] leads to excessive $1,25(\text{OH})_2\text{D}_3$ formation with increase of plasma Ca^{2+} [15] and phosphate [14] concentration, vascular calcification [16] and growth deficit [2]. In addition to its influence on $1,25(\text{OH})_2\text{D}_3$ formation [2, 9-11, 17, 18] and thus $1,25(\text{OH})_2\text{D}_3$ dependent regulation of Ca^{2+} and phosphate transport Klotho influences more directly Ca^{2+} channels [19] and Na^+ ,phosphate cotransport [4, 20]. Moreover, Klotho up-regulates the Na^+/K^+ ATPase [21], renal outer medullary K^+ channels ROMK [22] voltage gated channels, KCNQ1/KCNE1 [23] and excitatory amino acid transporters EAAT 3 and EAAT4 [24]. Thus, Klotho exerts some of its effects by regulating transport mechanisms in the cell membrane.

Carriers expressed in both, brain and kidney include the widely expressed creatine transporter CreaT (Slc6A8) [25-28], a member of the Na^+,Cl^- coupled transporter superfamily for neurotransmitters (e.g. dopamine, GABA, serotonin and norepinephrine), amino acids (e.g. glycine) [29-31] as well as the organic osmolytes betaine [32] and taurine [33]. Slc6A8 activity is regulated by AMP activated kinase [34], cyclosporine A [35], mTOR [36], serum and glucocorticoid inducible kinase isoforms [37], PIKfyve [38], as well as Src [39], and its expression is increased by growth hormone [40]. Slc6A8 is further regulated by extracellular and cytosolic creatine levels [41, 42].

The present study explored whether Klotho participates in the regulation of creatine transporter CreaT (Slc6A8). To this end, cRNA encoding Slc6A8 was injected into *Xenopus* oocytes either without or with additional injection of cRNA encoding Klotho. Moreover, Slc6A8 and Klotho expressing *Xenopus* oocytes were treated with β -glucuronidase inhibitor DSAL (10 μM). To estimate creatine transporter CreaT (Slc6A8) activity, the creatine induced current was determined utilizing dual electrode voltage clamp.

Materials and Methods

Constructs

Murine full-length Klotho was subcloned from pCR-XL-TOPO vector (Imagines, Berlin, Germany) into pSGEM, a *Xenopus* oocyte expression vector using XhoI - SpeI restriction sites. For generation of cRNA [43, 44] constructs were used encoding Klotho [20] and CreaT (Slc6A8) [30, 45]. The constructs were used for the generation of cRNA as described previously [46-48].

Voltage clamp in *Xenopus* oocytes

Xenopus oocytes were prepared as previously described [49, 50]. *Xenopus* oocytes were injected with DEPC-water and 15 ng cRNA encoding Slc6A8 with or without additional co-injection of 10 ng cRNA encoding Klotho protein on the same day of preparation of the *Xenopus* oocytes. The oocytes were maintained at 17 °C in ND96 solution containing: 88.5 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , 1.8 mM CaCl_2 , 5 mM HEPES. Tetracycline

(50 mg/l), Ciprofloxacin (1.6 mg/l), Refobacin (100 mg/l) and Theophyllin (90 mg/l) as well as sodium pyruvate (5mM) were added to the ND96, and the pH was adjusted to 7.5 by addition of NaOH. The control superfusate (ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES. The pH was adjusted to 7.4 by addition of NaOH [51, 52]. Where indicated, Brefeldin A (5μM, Sigma), recombinant human alpha Klotho protein (30ng/ml, 5334-KL-R&D Systems) and D-saccharic acid 1,4-lactone monohydrate (DSAL, 10μM, Sigma) were added. All experiments were performed at room temperature (about 22°C) 3-5 days after injection. Pipettes were filled with 3 M KCl and had resistances of 0.3-3.0 MΩ. The substrate (creatine) was added to the solutions at a concentration of 2 mM unless otherwise stated. The flow rate of the superfusion was approximately 20 ml/min, and a complete exchange of the solution in the recording bath was reached within about 10 sec. Where indicated, brefeldin A at the concentration of 5μM was added to the solutions in order to test for alterations of CreaT protein stability in the plasma membrane. Brefeldin A is involved in plasma membrane trafficking and the treatment of *Xenopus* oocytes with brefeldin A prevents further insertion of new carrier protein into the plasma membrane through the vesicular pathway. Therefore, the consequent decay of carrier activity could be taken as a measure of carrier protein clearance from the plasma membrane. Two-electrode voltage-clamp recordings were performed as described previously [53, 54] at a holding potential of -60 mV. The data were filtered at 10 Hz and recorded with a GeneClamp 500 amplifier, a DigiData 1300 A/D-D/A converter and the pClamp 9.2 software packages for data acquisition and analysis (Axon Instruments, Foster City, CA, USA) [53, 55]. The offset potentials between both electrodes were zeroed before sealing. The applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. The outward currents, defined as flow of positive charge from the cytoplasmic to the extracellular membrane face, are positive currents and depicted as upward deflections of the original current traces.

Statistical analysis

Data are provided as means ± SEM, n represents the number of experiments. All oocyte experiments were repeated with at least 2 batches of oocytes; in all repetitions qualitatively similar data were obtained. Data were tested for significance using ANOVA, as appropriate, and results with $p < 0.05$ were considered statistically significant.

Results

The present study explored whether Klotho protein participates in the regulation of the Na⁺-Cl⁻ coupled creatine transporter CreaT (Slc6A8). To this end, cRNA encoding Slc6A8 was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding Klotho protein. The Slc6A8 activity was estimated from the creatine induced current generated by electrogenic creatine transport. As shown in Fig. 1, creatine did not generate an appreciable current in water-injected *Xenopus* oocytes or in oocytes expressing Klotho protein alone, indicating that *Xenopus* oocytes do not express appreciable endogenous electrogenic creatine transporters. In contrast, creatine induced a large current in *Xenopus* oocytes expressing Slc6A8. The additional coexpression of Klotho was followed by a significant increase of the creatine induced current (Fig. 1).

In order to elucidate whether Klotho protein coexpression modifies the maximal creatine induced current or the affinity of the carrier, *Xenopus* oocytes expressing Slc6A8 without or with additional expression of Klotho protein were exposed to different concentrations of creatine for kinetic analysis. As shown in Fig. 2, the creatine induced current was a function of the extracellular creatine concentration. The maximal creatine induced current was higher in *Xenopus* oocytes expressing Slc6A8 together with Klotho (29.5 ± 3.5 nA, $n = 8$) than in *Xenopus* oocytes expressing Slc6A8 alone (20.2 ± 3.7 nA, $n = 10$). The concentration required for halfmaximal creatine induced current was not significantly different between *Xenopus* oocytes expressing Slc6A8 together with Klotho (37.4 ± 9.0 μM, $n = 10$) and *Xenopus* oocytes expressing Slc6A8 alone (34.4 ± 8.3 μM, $n = 8$). The increased maximal transport rate pointed to an increase of creatine transporter CreaT abundance in the plasma membrane of Slc6A8 expressing *Xenopus* oocytes following coexpression of Klotho.

Fig. 1. Effect of Klotho coexpression on electrogenic creatine transport in Slc6A8-expressing *Xenopus* oocytes. A: Representative original tracings of creatine (2 mM) induced current in *Xenopus* oocytes injected with water (a), or with cRNA encoding Klotho alone (b),

CreaT (Slc6A8) alone (c) or CreaT (Slc6A8) and Klotho (d). B: Arithmetic means $\pm$ SEM ($n = 19-30$) of creatine (2 mM) -induced current in *Xenopus* oocytes without (left bars, - CreaT) or with (right bars, + CreaT) injection of cRNA encoding CreaT (Slc6A8) without (white bars) or with (black bars) additional injection of cRNA encoding Klotho. ** ($p < 0.01$) indicates statistically significant difference from the absence of Klotho coexpression.

Fig. 2. Creatine induced currents as a function of creatine concentration in Slc6A8 expressing oocytes. Arithmetic means $\pm$ SEM ($n = 8-10$) of creatine induced current as a function of creatine concentration in *Xenopus* oocytes expressing CreaT (Slc6A8) without (black triangles) and with (black circles) additional coexpression of Klotho.

In theory, Klotho could modify carrier protein abundance in the cell membrane by either, accelerating insertion of carrier protein into the cell membrane or delaying retrieval of carrier protein from the cell membrane. In order to discriminate between those two possibilities, *Xenopus* oocytes expressing either both, Slc6A8 and Klotho, or Slc6A8 alone, were treated with brefeldin A (5 μ M), an inhibitor of protein insertion into the cell membrane. As shown in Fig. 3, brefeldin A treatment was followed by a decay of the current in both groups, an effect, which was faster in *Xenopus* oocytes expressing Slc6A8 alone than in *Xenopus* oocytes expressing both Slc6A8 and Klotho. The ratio of the decay of creatine induced current in Slc6A8 and Klotho expressing *Xenopus* oocytes over the current in *Xenopus* oocytes expressing Slc6A8 alone, increased from 0.96 ± 0.40 ($n = 11$) in the absence of brefeldin A to 2.01 ± 0.57 ($n = 11$) following a 12 hours treatment with brefeldin A. The observations disclose an effect of klotho in the absence of carrier insertion and thus strongly suggest that the effect of klotho is secondary to stabilization of carrier protein within the cell membrane. A further series of experiments elucidated whether the β -glucuronidase activity of Klotho is required for its effect on Slc6A8 activity. To this end, *Xenopus* oocytes expressing both, Slc6A8 and Klotho were treated with β -glucuronidase inhibitor DSAL (10 μ M) for 48 h

Fig. 3. Effect of brefeldin A in Slc6A8 expressing *Xenopus* oocytes with or without coexpression of Klotho. Arithmetic means $\pm$ SEM ($n = 10-18$) of the normalized creatine (2 mM) induced current in *Xenopus* oocytes injected with cRNA encoding CreaT, without (white bars) or with additional expression of Klotho (black bars) in the absence (left bars) and presence of 5 μ M Brefeldin A for 12 hours (middle bars) or 24 hours (right bars) prior to the measurement. * ($p < 0.05$), ** ($p < 0.005$), *** ($p < 0.001$) indicates statistically significant difference from the absence of Klotho coexpression.

Fig. 4. Effect of β -glucuronidase inhibitor (DSAL) and recombinant human alpha Klotho protein on electrogenic creatine transporter CreaT (Slc6A8). A: Arithmetic means $\pm$ SEM ($n = 11-18$) of the normalized creatine (2 mM) induced current in *Xenopus* oocytes expressing Slc6A8 alone (white bar) or Slc6A8 together with Klotho without (1st black bar) and with (2nd black bar) treatment with β -glucuronidase inhibitor DSAL (10 μ M) for 48 hours. * ($p < 0.05$) indicates statistically significant difference from respective oocytes expressing Slc6A8 alone, # ($p < 0.05$), indicates statistically significant difference from Slc6A8 and Klotho expressing oocytes without DSAL treatment. B: Arithmetic means $\pm$ SEM ($n = 15-18$) of the normalized creatine (2 mM) induced current in *Xenopus* oocytes expressing Slc6A8 alone (white bar) or in *Xenopus* oocytes injected with cRNA encoding Slc6A8 alone with pretreatment for 24 hours (1st black bar) or 48 hours (2nd black bar), respectively, with recombinant human alpha Klotho protein (30 ng/ml). * ($p < 0.05$), *** ($p < 0.001$) indicates statistically significant difference from respective oocytes expressing Slc6A8 alone.

in order to suppress β -glucuronidase activity of expressed klotho. As shown in Fig. 4A, the treatment of Slc6A8 and Klotho-expressing *Xenopus* oocytes with DSAL reversed the effect of Klotho coexpression on the creatine induced inward current.

Additional experiments were performed to test, whether the effect of Klotho coexpression on creatine induced current was mimicked by treatment of Slc6A8-expressing *Xenopus* oocytes with recombinant human alpha Klotho protein (30 ng/ml). To possibly disclose a stabilizing effect of klotho on the carrier protein in the cell membrane, the oocytes were exposed for 24 h or 48 h to klotho protein. As shown in Fig. 4B, treatment of Slc6A8 expressing *Xenopus* oocytes with recombinant human alpha Klotho protein resulted in a significant increase of creatine induced inward current.

Discussion

The present observations disclose a novel function of Klotho, i.e. the up-regulation of the creatine transporter CreaT (Slc6A8). Klotho increases the maximal transport rate of the carrier presumably by stabilizing the carrier protein in the cell membrane. The Klotho concentration required was within the range of concentrations encountered *in vivo* [56]. The effect of Klotho was reversed in the presence of the β -glucuronidase inhibitor DSAL added from the beginning of Klotho expression in order to continuously suppress β -glucuronidase activity of expressed Klotho. The reversal of the effect of Klotho indicates that the effect of Klotho on Slc6A8 indeed requires β -glucuronidase activity of Klotho. Previous studies suggested that Klotho may regulate ion channels by removing terminal sialic acids [5, 6].

Klotho further up-regulates the Na^+/K^+ ATPase [21], which maintains the electrochemical gradient for Na^+ coupled transport [57]. Thus, Klotho could modify Na^+ coupled creatine transport in part by up-regulation of Na^+/K^+ ATPase activity. The Na^+/K^+ ATPase further maintains the intracellular K^+ concentration, which is in turn decisive for cell survival [58-62].

Slc6A8 is critically important for proper function of the brain, as genetic defects of Slc6A8 lead to mental retardation with seizures [63-80]. Slc6A8 deficient mice suffer from learning and memory deficits resembling human Slc6A8 deficiency [63]. Consequences of Klotho deficiency include degeneration of mesencephalic dopaminergic neurons [81], an effect, however, reversed by vitamin D restriction [81] and thus presumably not due to direct influence of Klotho on Slc6A8. Klotho deficiency further leads to cognitive deficits [82]. Klotho is required for proper maturation of rat primary oligodendrocytic progenitor cells [82] and Klotho deficiency is paralleled by lack of oligodendrocytes with decreased abundance of major myelin protein [82]. Downregulation of Klotho in the aged brain may contribute to a decrease of white matter and myelin abnormalities during cerebral ageing [83]. To which extent compromised up-regulation of Slc6A8 by Klotho contributes to the deterioration of cerebral function in Klotho deficiency or in the aging brain, remains to be shown. Clearly, additional studies will be required to define the *in vivo* relevance of Klotho sensitive creatine transport.

Klotho confers cardioprotection [84], an effect in large part due to down-regulation of TRPC6 channels. It is noteworthy, however, that Slc6A8 deficiency may impair cardiac function [85] and that decreased Slc6A8 abundance is observed in the failing heart [86]. Thus, at least in theory, up-regulation of Slc6A8 could contribute to cardioprotection.

Conclusion

The present study demonstrates that Klotho is a powerful stimulator of the creatine transporter CreaT (Slc6A8). Klotho dependent regulation of Slc6A8 may contribute to neurodegeneration and cardiac failure in Klotho deficiency.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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