Original Paper

Up-Regulation of Excitatory Amino Acid Transporters EAAT1 and EAAT2 by β -Klotho

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Key Words

SLC1A3 • SLC1A2 • Neuronal excitation • Glutamate • Klotho • β -glucuronidase

Abstract

Background/Aims: Klotho, a transmembrane protein expressed in choroid plexus of the brain, kidney, and several other tissues, is required for inhibition of $1,25(\text{OH})_2\text{D}_3$ formation by FGF23. The extracellular domain of Klotho protein could be cleaved off, thus being released into blood or cerebrospinal fluid. At least in part by exerting β -glucuronidase activity, soluble klotho regulates several ion channels and carriers. Klotho protein deficiency accelerates the appearance of age related disorders including neurodegeneration and muscle wasting and eventually leads to premature death. The present study explored the effect of Klotho protein on the excitatory glutamate transporters EAAT1 (SLC1A3) and EAAT2 (SLC1A2), Na^+ coupled carriers clearing excitatory amino acids from the synaptic cleft and thus participating in the regulation of neuronal excitability. **Methods:** cRNA encoding EAAT1 or EAAT2 was injected into *Xenopus laevis* oocytes and glutamate (2 mM)-induced inward current (I_{Glu}) taken as measure of glutamate transport. Measurements were made without or with prior 24 h treatment with soluble β -Klotho protein (30 ng/ml) in the absence and presence of β -glucuronidase inhibitor D-saccharic acid 1,4-lactone monohydrate (DSAL, 10 μM). **Results:** I_{Glu} was observed in EAAT1 and in EAAT2 expressing oocytes but not in water injected oocytes. In both, EAAT1 and EAAT2 expressing oocytes I_{Glu} was significantly increased by treatment with soluble β -Klotho protein, an effect reversed by DSAL. Treatment with β -klotho protein increased significantly the maximal transport rate without significantly modifying the affinity of the carriers. **Conclusion:** β -Klotho up-regulates the excitatory glutamate transporters EAAT1 and EAAT2 and thus participates in the regulation of neuronal excitation.

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Introduction

Klotho is expressed mainly in kidney and choroid plexus [1, 2]. The extracellular domain of the Klotho protein may be cleaved off and function as enzyme with β -glucuronidase activity [3-7]. Klotho has a powerful impact on aging and life span [8, 9]. As shown in mice, klotho deficiency leads to severe growth retardation, accelerated appearance of several age related disorders, as well as dramatic shortening of the life span [9], whereas over-expression of klotho extends the life span [9, 10].

Klotho is effective in part by mediating the inhibitory effect of FGF23 on 1α -hydroxylase and thus $1,25(\text{OH})_2\text{D}_3$ (calcitriol) formation [2, 8, 11-14]. Calcitriol in turn stimulates Klotho expression [15] as well as intestinal and renal Ca^{2+} and phosphate transport [16, 17]. The excessive $1,25(\text{OH})_2\text{D}_3$ formation in klotho deficient mice [2, 13, 14] is followed by increase of plasma Ca^{2+} [18] and phosphate [17] concentrations as well as vascular calcification [19]. Klotho further more directly influences several transport proteins including Ca^{2+} channels [20], Na^+ ,phosphate cotransport [4, 21], Na^+/K^+ ATPase [22], renal outer medullary K^+ channels [23], KCNQ1/KCNE1 [24] and the excitatory amino acid transporters EAAT3 and EAAT4 [25].

Excitatory amino acid transporters (EAATs) influence neuroexcitation by clearance of the excitatory neurotransmitters glutamate and aspartate from synaptic clefts [26-30]. EAAT isoforms particularly important for the regulation of neuroexcitation are the excitatory amino acid transporter isoforms EAAT1 (SLC1A3) and EAAT2 (SLC1A2) [26, 31, 32]. EAAT1 [33-41] and EAAT2 [42] are both expressed in astrocytes. Moreover, EAAT1 is expressed in oligodendrocytes [43, 44], neurons [45-47], retina [48, 49], taste buds [50], cochlea [51, 52], vestibular organ [53], circumventricular organ [33], adrenal and pineal glands [54, 55] as well as bone cells [56-59]. EAAT2 may confer neuroprotection [60] and impaired expression or activity of EAAT2 is followed by neuroexcitotoxicity [61-64].

The present study explored, whether the function of EAAT1 and/or EAAT2 is sensitive to β -Klotho protein. To this end, EAAT1 or EAAT2 expressing *Xenopus laevis* oocytes were treated with β -Klotho protein and glutamate induced current determined by dual electrode voltage clamp.

Materials and Methods

Ethical Statement

All experiments conform with the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985) and were conducted according to the German law for the welfare of animals and the surgical procedures on the adult *Xenopus laevis* frogs were reviewed and approved by the respective government authority of the state Baden-Württemberg (Regierungspräsidium) prior to the start of the study (Anzeige für Organentnahme nach §36).

Constructs

Constructs encoding human wild-type EAAT1 [65] and human wild-type EAAT2 [66-68], were used for generation of cRNA as described previously [69, 70].

Voltage clamp in *Xenopus* oocytes

Xenopus oocytes were prepared as previously described [71, 72]. 10 ng cRNA encoding EAAT1 or EAAT2 were injected on the same day after preparation of the oocytes. The oocytes were maintained at 17°C in ND96-A, a solution containing (in mM): 88.5 NaCl, 2 KCl, 1 MgCl_2 , 1.8 CaCl_2 , 2.5 NaOH, 5 HEPES (pH 7.4), 5 sodium pyruvate ($\text{C}_3\text{H}_3\text{NaO}_3$), Gentamycin (100 mg/l), Tetracycline (50 mg/l), Ciprofloxacin (1.6 mg/l), and Theophiline (90 mg/l) [73, 74]. Where indicated, 30 ng/ml β -Klotho protein and/or 10 μM β -glucuronidase inhibitor DSAL were added to the respective solutions. The voltage clamp experiments were performed

at room temperature 3 days after the first injection [70, 75]. Glutamate induced currents were taken as a measure of glutamate transport [76, 77]. The holding potential was -70 mV. The data were filtered at 10 Hz and recorded with a Digidata A/D-D/A converter (1322A Axon Instruments) and Clampex 9.2 software for data acquisition and analysis (Axon Instruments) [78-80]. The control superfusate (ND96-B) contained (in mM): 93.5 NaCl, 2 KCl, 1.8 CaCl_2 , 1 MgCl_2 , 2.5 NaOH and 5 HEPES (pH 7.4). The flow rate of the superfusion was approx. 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s [81-83]. For kinetic analysis the glutamate induced-current (I_{Glu}) was plotted against the respective glutamate concentration (s) and maximal current (I_{max}) as well as concentration required for halfmaximal current (k_m) calculated using the equation $I_{\text{Glu}} = I_{\text{max}} \cdot s / (k_m + s)$.

Statistical analysis

Data are provided as means $\pm$ SEM, n represents the number of oocytes investigated. As different batches of oocytes may yield different results, comparisons were always made within a given oocyte batch. All voltage clamp experiments were repeated with at least 3 batches of oocytes; in all repetitions qualitatively similar data were obtained. Data were tested for significance using ANOVA or t-test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study explored the effect of the β -Klotho protein on electrogenic glutamate transport by the excitatory amino acid transporters EAAT1 and EAAT2. To this end, EAAT1 or EAAT2 expressing *Xenopus laevis* oocytes were left untreated or were treated with β -klotho protein (30 ng/ml) and glutamate-induced inward current (I_{Glu}) measured by dual electrode voltage clamp and taken as a measure of electrogenic glutamate transport.

Fig. 1. Effect of recombinant human β -Klotho protein on electrogenic glutamate transport in EAAT1 expressing *Xenopus laevis* oocytes. A: Representative original tracings of glutamate (2 mM) induced current in *Xenopus* oocytes injected with water (a,b), or with cRNA encoding EAAT1 (c,d) without (a,c) and with (b,d) prior β -Klotho protein (30 ng/ml, 24 h) treatment. B: Arithmetic means $\pm$ SEM (n = 10-14) of the normalized glutamate (2 mM) induced current in *Xenopus* oocytes injected with water (left bars) or expressing EAAT1 (right bars) without (white bars) or with (black bars) prior β -Klotho protein (30 ng/ml, 24 h) treatment. ** ($p < 0.01$) indicates statistically significant difference from EAAT1 expressing oocytes without β -Klotho protein treatment.

Fig. 2. Glutamate induced current in EAAT1 expressing *Xenopus laevis* oocytes as a function of glutamate concentration without and with prior β -Klotho protein treatment. Arithmetic means $\pm$ SEM (n = 3-5) of I_{glu} as a function of glutamate concentration in *Xenopus laevis* oocytes expressing EAAT1 without (black squares), or with (black circles) prior β -Klotho protein (30 ng/ml, 24 h) treatment. *** ($p < 0.001$) indicates statistically significant difference from untreated EAAT1 expressing oocytes.

Fig. 3. Effect of recombinant human β -Klotho protein in the absence and presence of β -glucuronidase inhibitor DSAL on electrogenic glutamate transport in EAAT1 expressing *Xenopus laevis* oocytes. A: Representative original tracings of glutamate (2 mM) induced current in *Xenopus* oocytes injected with water (a), or with cRNA encoding EAAT1 (b,c,d) without treatment (b) and with treatment with β -Klotho protein (30 ng/ml, 24 h) alone (c) or together with β -glucuronidase inhibitor D-saccharic acid 1,4-lactone monohydrate (DSAL, 10 μ M)(d). B: Arithmetic means $\pm$ SEM (n = 11-12) of the normalized glutamate (2 mM) induced current in *Xenopus* oocytes injected with water (dotted bar) or expressing EAAT1 without (white bars) or with prior β -Klotho protein (30 ng/ml, 24 h) treatment alone (black bar) or together with β -glucuronidase inhibitor D-saccharic acid 1,4-lactone monohydrate (DSAL, 10 μ M) (grey bar). *** ($p < 0.001$) indicates statistically significant difference from respective oocytes without β -Klotho protein treatment, ### ($p < 0.001$) indicates statistically significant difference from respective oocytes without presence of DSAL.

As illustrated in Fig. 1, I_{glu} was negligible in water-injected oocytes indicating that the oocytes did not express appreciable endogenous electrogenic glutamate transport. In contrast, glutamate (2 mM) triggered a sizable I_{glu} in EAAT1 expressing *Xenopus laevis* oocytes. The treatment of EAAT1 expressing oocytes with β -Klotho protein (30 ng/ml) was

Fig. 4. Effect of recombinant human β -Klotho protein on electrogenic glutamate transport in EAAT2 expressing *Xenopus laevis* oocytes. A: Representative original tracings of glutamate (2 mM) induced current in *Xenopus* oocytes injected with water (a), or with cRNA encoding EAAT2 without (b) or with (c) prior β -Klotho protein (30 ng/ml, 24 h) treatment. B: Arithmetic means $\pm$ SEM ($n = 7-10$) of the normalized glutamate (2 mM) induced current in *Xenopus* oocytes injected water (dotted bar) or expressing EAAT2 without (white bar) or with (black bars) prior β -Klotho protein (30 ng/ml, 24 h) treatment. * ($p < 0.05$) indicates statistically significant difference from EAAT2 expressing oocytes without β -Klotho protein treatment.

followed by a significant increase of I_{Glu} . Treatment of water injected oocytes with β -klotho protein (30 ng/ml) failed to increase I_{Glu} .

In order to test whether β -Klotho protein modifies the maximal I_{Glu} and/or the affinity of EAAT1, untreated or β -Klotho protein (30 ng/ml) treated EAAT1 expressing *Xenopus laevis* oocytes were exposed to L-glutamate concentrations ranging from 10 μM to 5000 μM . As illustrated in Fig. 2, I_{Glu} was a function of the extracellular glutamate concentration. Maximal I_{Glu} was significantly ($p < 0.05$) higher in β -Klotho protein treated (146.3 ± 12.4 nA, $n = 5$) than in untreated (102.9 ± 11.3 nA, $n = 3$) EAAT1 expressing *Xenopus laevis* oocytes. The concentration required for half maximal I_{Glu} (apparent K_m) tended to be lower in β -Klotho protein treated (208 ± 85 μM , $n = 5$) than in untreated (553 ± 227 μM , $n = 3$) EAAT1 expressing *Xenopus laevis* oocytes, a difference, however, not reaching statistical significance.

A further series of experiments explored whether the effect of β -Klotho protein is related to its β -glucuronidase activity. To this end EAAT1 expressing *Xenopus laevis* oocytes were treated with β -Klotho protein in the absence and presence of β -glucuronidase inhibitor D-saccharic acid 1,4-lactone monohydrate (DSAL). As illustrated in Fig. 3, the effect of β -Klotho protein on electrogenic glutamate transport in EAAT1 expressing *Xenopus laevis* oocytes was virtually abolished by DSAL (10 μM). This observation is highly suggestive that klotho is effective as enzyme.

Similar observations were made in EAAT2 expressing *Xenopus laevis* oocytes. As illustrated in Fig. 4, glutamate (2 mM) triggered a sizable I_{Glu} in EAAT2 expressing *Xenopus laevis* oocytes. The treatment of EAAT2 expressing oocytes with β -klotho protein (30 ng/ml) was followed by a significant increase of I_{Glu} .

Fig. 5. Glutamate induced current in EAAT2 expressing *Xenopus laevis* oocytes as a function of glutamate concentration without and with prior β -Klotho protein treatment. Arithmetic means $\pm$ SEM (n = 6-7) of I_{glu} as a function of glutamate concentration in *Xenopus laevis* oocytes expressing EAAT2 without (black squares), or with (black circles) prior β -Klotho protein (30 ng/ml, 24 h) treatment. *** ($p < 0.001$) indicates statistically significant difference from untreated EAAT2 expressing oocytes.

Exposure of EAAT2 expressing oocytes to glutamate concentrations ranging from 10 μM to 5000 μM revealed the dependence of I_{glu} on extracellular L-glutamate concentration (Fig. 5). Maximal I_{glu} was again significantly ($p < 0.05$) higher in β -Klotho protein treated (310.0 ± 14.6 nA, $n = 7$) than in untreated (242.6 ± 21.6 nA, $n = 6$) EAAT2 expressing *Xenopus laevis* oocytes. The concentration required for half maximal I_{glu} (apparent K_m) tended again to be lower in β -Klotho protein treated (354 ± 38 μM , $n = 7$) than in untreated (550 ± 71 μM , $n = 6$) EAAT2 expressing *Xenopus laevis* oocytes, a difference again not reaching statistical significance.

A further series of experiments again revealed that the effect of β -Klotho protein was related to its β -glucuronidase activity. As illustrated in Fig. 6, the effect of β -Klotho protein on electrogenic glutamate transport in EAAT2 expressing *Xenopus laevis* oocytes was virtually abolished by DSAL (10 μM).

Discussion

The present study reveals a novel function of β -Klotho protein, i.e. the up-regulation of the excitatory amino acid transporters EAAT1 and EAAT2. Treatment of either, EAAT1 and EAAT2 expressing oocytes with human recombinant β -Klotho significantly increased the glutamate-induced inward current (I_{glu}). Kinetic analysis reveals that β -Klotho is in large part effective by increasing maximal transport rate of the carriers. The large scatter of the calculated concentrations required for half maximal current precludes safe conclusions about the effect of klotho on carrier affinity. The effect of β -Klotho on I_{glu} is virtually abolished by β -glucuronidase inhibitor D-saccharic acid 1,4-lactone monohydrate DSAL, an observation suggesting that β -klotho is effective as enzyme. The beta-glucuronidase Klotho belongs to the β -glycosidase family [84]. As first shown for the Ca^{2+} channel TRPV5, the enzyme hydrolyzes extracellular sugar residues of target membrane proteins and by this means stabilizes the proteins in the cell membrane [84]. Thus, klotho may increase EAAT activity by stabilizing the carrier protein in the cell membrane. However, the current was significantly lower in klotho and DSAL treated oocytes than in untreated oocytes. We thus cannot exclude that DSAL modifies EAAT1 and EAAT2 by mechanisms other than inhibition of klotho enzyme activity. Along those lines, we cannot rule out that klotho is effective by mechanisms other than beta-glucuronidase activity.

Up-regulation of EAAT1 and EAAT2 by β -Klotho were expected to stimulate the clearance of glutamate from the synaptic cleft and thus to accelerate termination of excitation. Up-regulation of excitatory glutamate transporters would thus decrease neuroexcitability.

Fig. 6. Effect of recombinant human β -Klotho protein in the absence and presence of β -glucuronidase inhibitor DSAL on electrogenic glutamate transport in EAAT2 expressing *Xenopus laevis* oocytes. A: Representative original tracings of glutamate (2 mM) induced current in *Xenopus* oocytes injected with water (a), or with cRNA encoding EAAT2 (b,c,d) without treatment (b) and with treatment with β -Klotho protein (30 ng/ml, 24 h) alone (c) or together with β -glucuronidase inhibitor D-saccharic acid 1,4-lactone monohydrate (DSAL, 10 μ M) (d). B: Arithmetic means $\pm$ SEM (n = 14-16) of the normalized glutamate (2 mM) induced current in *Xenopus* oocytes injected water (dotted bar) or expressing EAAT2 without (white bars) or with prior β -Klotho protein (30 ng/ml, 24 h) treatment alone (black bar) or together with β -glucuronidase inhibitor D-saccharic acid 1,4-lactone monohydrate (DSAL, 10 μ M) (grey bar). *** ($p < 0.001$) indicates statistically significant difference from respective oocytes without β -Klotho protein treatment, ### ($p < 0.001$) indicates statistically significant difference from respective oocytes without presence of DSAL.

Ample evidence points to a role of EAAT1 and EAAT2 in the regulation of neuroexcitation. EAAT1 deficiency in mice results in locomotor hyperactivity, abnormal behavior with reduced preference for a novel social stimulus, reduced acoustic startle response, and impaired memory consolidation in mice [85, 86]. EAAT2 deficiency in humans may be involved in the pathophysiology of several neurological disorders including Alzheimer disease [87, 88], schizophrenia [89], HIV associated dementia [90], multiple sclerosis [91, 92], leukomalacia [93], epilepsy [94, 95], brain trauma [96], hypoxia and stroke [30, 97-99], reward dependence [100], as well as amyotrophic lateral sclerosis (ALS) [61, 101, 102]. Additional experimental evidence is needed to test whether altered expression and/or function of β -Klotho contributes to the clinical course of those diseases.

Beyond its influence on EAAT1 and EAAT2, Klotho modifies a variety of channels, carriers, and the Na^+/K^+ ATPase, which are partially expected to impact on neuronal function. Moreover, the beta-glucuronidase activity could modify and stabilize further cell membrane proteins, such as receptors. Clearly, additional experimentation is required to dissect the various actions of Klotho on neuronal function.

In conclusion, β -Klotho up-regulates the activity of the glutamate transporters EAAT1 and EAAT2. The effect could contribute to the complex regulation of neuronal excitability.

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Disclosure Statement

The authors declare that they have nothing to disclose.

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