

Original Paper

β -Klotho as a Negative Regulator of the Peptide Transporters PEPT1 and PEPT2

Abeer Abousaab^a Jamshed Warsi^{a,b} Madhuri S. Salker^a Florian Lang^{a,c}^aDepartment of Cardiology, Vascular Medicine and Physiology, University of Tuebingen, Tuebingen, Germany; ^bDepartment of Physiology, University of Sindh, Jamshoro, Pakistan; ^cDepartment of Molecular Medicine II, Heinrich Heine University Duesseldorf, Duesseldorf Germany**Key Words**PEPT1 • PEPT2 • DSAL • Glycine-glycine • β -glucuronidase • Ageing • Intestine**Abstract**

Background/Aims: β -Klotho, a transmembrane protein expressed in several tissues including the brain and the kidney, is critically important 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. Soluble klotho is a β -glucuronidase participating in the regulation of several ion channels and carriers. The present study explored the effect of β -Klotho protein on the peptide transporters PEPT1 and PEPT2. **Methods:** cRNA encoding PEPT1 or PEPT2 was injected into *Xenopus laevis* oocytes and glycine-glycine (2 mM)-induced inward current (I_{Gly}) taken as measure of glycine-glycine 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). Using chamber experiments were employed to determine electrogenic peptide transport across intestinal epithelia of klotho deficient ($kl^{-/-}$) and corresponding wild type ($kl^{+/+}$) mice. **Results:** I_{Gly} was observed in PEPT1 and in PEPT2 expressing oocytes but not in water injected oocytes. In both, PEPT1 and PEPT2 expressing oocytes I_{Gly} was significantly decreased by treatment with soluble β -Klotho protein. As shown for PEPT1, β -klotho protein decreased significantly the maximal transport rate without significantly modifying the affinity of the carrier. The effect of β -Klotho on PEPT1 was reversed by DSAL. Intestinal I_{Gly} was significantly larger in $kl^{-/-}$ than in $kl^{+/+}$ mice. **Conclusion:** β -Klotho participates in the regulation of the peptide transporters PEPT1 and PEPT2.

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Published by S. Karger AG, Basel**Introduction**

Klotho is a transmembrane protein expressed in several tissues with particular strong expression in kidney and choroid plexus [1, 2]. The extracellular domain of the Klotho protein with β -glucuronidase activity may be cleaved off and released into blood and cerebrospinal

A. Abousaab and J. Warsi contributed equally thus shared the first authorship.

Prof. Dr. Florian Lang

Department of Physiology, University of Tuebingen,
Gmelinstr. 5, D-72076 Tuebingen, (Germany)
Tel. +49 7071/2972194, Fax +49 7071/295618, E-Mail florian.lang@uni-tuebingen.de

fluid [3-7]. Klotho has a powerful impact on ageing [8, 9]. Klotho deficiency results in severe growth retardation, accelerated appearance of several age related disorders, and dramatic shortening of the life span [9]. Conversely, klotho over-expression extends the life span [9, 10].

Klotho is required for the inhibitory effect of FGF23 on 1α -hydroxylase and thus $1,25(\text{OH})_2\text{D}_3$ formation [2, 8, 11-14]. Effects of $1,25(\text{OH})_2\text{D}_3$ include stimulation of klotho expression [15] as well as intestinal and renal Ca^{2+} and phosphate absorption [16, 17]. Klotho deficiency is followed by excessive $1,25(\text{OH})_2\text{D}_3$ formation [2, 13, 14] leading to increase of plasma Ca^{2+} [18] and phosphate [17] concentrations as well as vascular calcification [19]. Besides its effect on $1,25(\text{OH})_2\text{D}_3$ formation, klotho modifies the function of several transport proteins including Ca^{2+} channels [20], Na^+ , phosphate cotransport [4, 21], Na^+/K^+ ATPase [22], renal outer medullary K^+ channels [23], Kv1.3 K^+ channels [24], KCNQ1/KCNE1 [25], the creatine transporter CreaT [26], and the excitatory amino acid transporters EAAT1, EAAT2, EAAT3 and EAAT4 [27, 28].

Carriers impacting on ageing include the peptide transporter PEPT1 [32]. Intestinal peptide transport is a major source of nutritional amino acids and is thus decisive for growth and development as well as signaling regulating metabolism and ageing [32]. The possibility was thus considered that klotho may influence peptide transport which in turn may affect ageing.

The present study thus explored, whether Klotho impacts on the function of the peptide transporters 1 (PEPT1) and/or 2 (PEPT2), which accomplish electrogenic cellular uptake of di- and tripeptides [29-31] including peptide-like drugs [30, 31].

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. 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 rabbit wild-type PEPT1 and PEPT2 [33, 34], were used for generation of cRNA as described previously [35, 36].

Voltage clamp in *Xenopus* oocytes

Xenopus oocytes were prepared as previously described [37, 38]. Ten ng cRNA encoding PEPT1 or Twenty ng cRNA encoding PEPT2 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) [39, 40]. 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 of cRNA encoding PEPT1 and 4 days after the injection of PEPT2 [41, 42]. Glycine-glycine induced currents were taken as a measure of glycine-glycine transport [43, 44]. The holding potential was -70mV . 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) [33, 45]. 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 [46, 47]. For kinetic analysis the glycine-glycine induced-current (I_{Glu}) was plotted against the respective glycine-glycine concentration (s) and maximal current (I_{max}) as well as concentration required for half maximal current (k_m) calculated using the equation $I_{\text{Glu}} = I_{\text{max}} \cdot s / (k_m + s)$.

Ussing chamber experiments

All animal experiments were conducted according to the German law for the welfare of animals and according to the guidelines of the American Physiological Society and were approved by local authorities (Regierungspräsidium Tübingen). Experiments were performed using jejunal segments from 6-week old female klotho-deficient mice (*kl^{-/-}*) and wild-type mice. The origin of the mice, breeding and genotyping were described previously [9]. Prior to the experiments mice had access to food (1314, Altromin, Heidenau, Germany) and water *ad libitum*, and were kept under constant humidity ($55 \pm 10\%$), temperature ($22 \pm 2^\circ\text{C}$) and 12h light-dark cycle conditions. Mice were fasted for 6 hours prior to experiments. Peptide transport rate was estimated from glycine-glycine or from glycine-Sar induced current across the intestinal epithelium. After removing the outer serosal and the muscular layer of jejunum under a microscope, tissues were mounted onto a custom-made mini-Ussing chamber with an opening diameter of 0.99 mm and an opening area of 0.00769 cm². Transepithelial potential difference (V_{te}) was determined continuously and transepithelial resistance (R_{te}) estimated from the voltage deflections (ΔV_{te}) elicited by imposing rectangular test currents of 1 μA and 1.2 s duration at a rate of 8/min. R_{te} was calculated according to Ohm's law [48]. The serosal and luminal perfusate contained (in mM): 145 NaCl, 1 MgCl₂, 2.6 Ca-gluconate, 0.4 KH₂PO₄, 1.6 K₂HPO₄, 5 glucose. To assess peptide transport, glycine-glycine (10 mM) or glycine-Sar (10 mM) was added to the luminal perfusate.

Statistical analysis

Data are provided as means \pm SEM, n represents the number of replicates. 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 Student's t-test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study explored whether soluble recombinant β -Klotho protein modifies the electrogenic glycine-glycine transport by the peptide transporters PEPT1 and PEPT2. To this end, PEPT1 or PEPT2 expressing *Xenopus laevis* oocytes were left untreated or were treated with β -Klotho protein (30 ng/ml) and glycine-glycine-induced inward current (I_{Gly}) measured by dual electrode voltage clamp taken as a measure of electrogenic glycine-glycine transport.

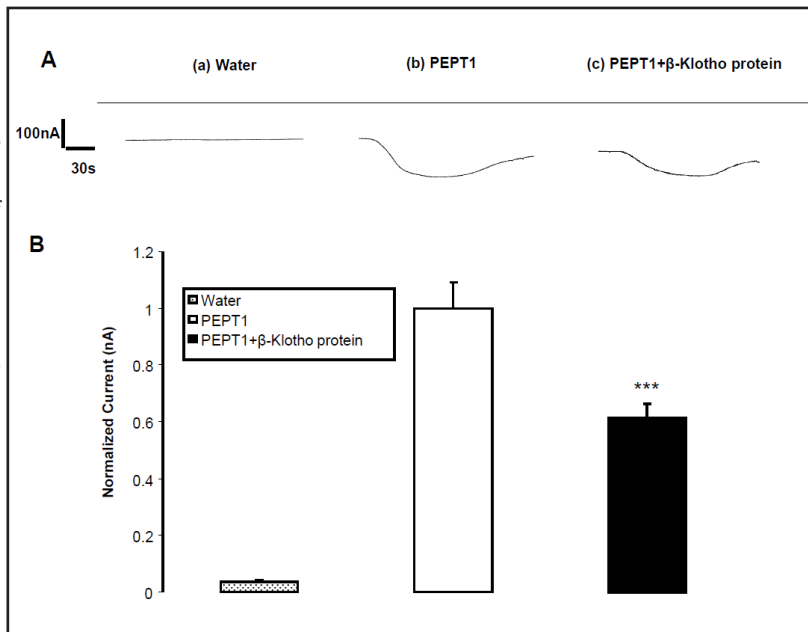
Effect of β -Klotho on PEPT1 in *Xenopus laevis* oocytes

As illustrated in Fig. 1, I_{Gly} was negligible in water-injected oocytes indicating that the oocytes did not express significant endogenous electrogenic glycine-glycine transport. In contrast, glycine-glycine (2 mM) triggered a sizable I_{Gly} in PEPT1 expressing *Xenopus laevis* oocytes. The treatment of PEPT1 expressing oocytes with β -Klotho protein (30 ng/ml) was followed by a significant decrease of I_{Gly} .

In order to test whether β -Klotho protein modifies the maximal I_{Gly} and/or the affinity of PEPT1, untreated or β -Klotho protein (30 ng/ml) treated PEPT1 expressing *Xenopus laevis* oocytes were exposed to glycine-glycine concentrations ranging from 10 μM to 5000 μM . As illustrated in Fig. 2, I_{Gly} was a function of the extracellular glycine-glycine concentration. Maximal I_{Gly} was significantly ($p < 0.05$) lower in β -Klotho protein treated (44.3 ± 3.8 nA, $n = 9$) than in untreated (62.4 ± 6.8 nA, $n = 11$) PEPT1 expressing *Xenopus laevis* oocytes. The concentration required for half maximal I_{Gly} (apparent K_m) tended to be lower in β -Klotho protein treated (367.9 ± 40.1 μM , $n = 9$) than in untreated (271.9 ± 79.9 μM , $n = 11$) PEPT1 expressing *Xenopus laevis* oocytes, a difference, however, not reaching statistical significance.

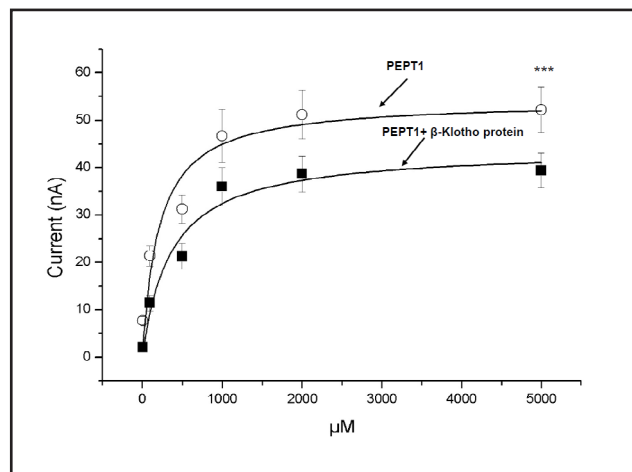
A further series of experiments explored whether the effect of β -Klotho protein is related to its β -glucuronidase activity. To this end PEPT1 expressing *Xenopus laevis* oocytes were treated with β -Klotho protein in the absence and presence of β -glucuronidase inhibitor D-saccharic acid 1,4-lactone monohydrate (DSAL). As illustrated in Fig. 3, the effect of β -Klotho protein on electrogenic glycine-glycine transport in PEPT1 expressing *Xenopus laevis* oocytes was virtually abolished by DSAL (10 μM).

Fig. 1. Effect of recombinant human β -Klotho protein on electrogenic peptide transport in PEPT1 expressing *Xenopus laevis* oocytes. A: Representative original tracings of glycine-glycine (2 mM) - induced current (I_{gly}) in *Xenopus laevis* oocytes injected with water (a) or with cRNA encoding PEPT1 without (b) and with (c) prior β -Klotho protein (30 ng/ml, 24 h) treatment. B: Arithmetic means \pm SEM (n = 15-17) of the normalized glycine-glycine (2 mM) - induced current in *Xenopus* oocytes



injected with water (dotted bar) or expressing PEPT1 without (white bar) or with (black bar) prior β -Klotho protein (30 ng/ml, 24 h) treatment. Control I_{gly} amounted to 79.8 ± 6.9 nA (n = 17). *** ($p < 0.001$) indicates statistically significant difference from PEPT1 expressing oocytes without β -Klotho protein treatment.

Fig. 2. Glycine-glycine induced current in PEPT1 expressing *Xenopus laevis* oocytes as a function of glycine-glycine concentration without and with prior β -Klotho protein treatment. Arithmetic means \pm SEM (n = 9-11) of I_{gly} as a function of glycine-glycine concentration in *Xenopus laevis* oocytes expressing PEPT1 without (white circles), or with (black squares) prior β -Klotho protein (30 ng/ml, 24 h) treatment. *** ($p < 0.001$) indicates statistically significant difference from PEPT1 expressing oocytes without β -Klotho protein treatment.



Effect of β -Klotho on PEPT2 in *Xenopus laevis* oocytes

An additional series of experiments explored whether klotho was similarly effective in PEPT2 expressing *Xenopus laevis* oocytes. As illustrated in Fig. 4, glycine-glycine (2 mM) triggered a sizable I_{gly} in PEPT2 expressing *Xenopus laevis* oocytes. Treatment with β -Klotho protein (30 ng/ml) of PEPT2 expressing oocytes was followed by a significant decrease of I_{gly} .

In vivo β -Klotho sensitivity of intestinal peptide transport

In order to test whether klotho sensitivity plays a role *in vivo*, glycine-glycine (10 mM)-induced current was measured in intestinal epithelia utilizing Ussing chambers. As illustrated in Fig. 5, the intestinal glycine-glycine induced current (I_{gly}) was significantly larger in klotho deficient ($kl^{-/-}$) mice than in wild-type mice ($kl^{+/+}$). Similar observations were made with the peptide Gly-Sar (Fig. 6)

Fig. 3. Effect of recombinant human β -Klotho protein in the absence and presence of β -glucuronidase inhibitor DSAL on electrogenic glycine-glycine transport in PEPT1 expressing *Xenopus laevis* oocytes. A: Representative original tracings of glycine-glycine (2 mM) induced current in *Xenopus* oocytes injected with cRNA encoding PEPT1 without treatment (a) and with treatment with β -Klotho protein (30 ng/ml, 24 h) alone (b) or together with β -glucuronidase inhibitor D-saccharic acid 1,4-lactone monohydrate (DSAL, 10 μ M) (c). B: Arithmetic means \pm SEM (n = 15-20) of the normalized glycine-glycine (2 mM) induced current in *Xenopus* oocytes expressing PEPT1 without treatment (white bar) 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). Control I_{gly} amounted to 53.4 ± 4.3 nA (n = 17). *** ($p < 0.001$) indicates statistically significant difference from respective oocytes without β -Klotho protein treatment, ## ($p < 0.01$) indicates statistically significant difference from respective oocytes without presence of DSAL.

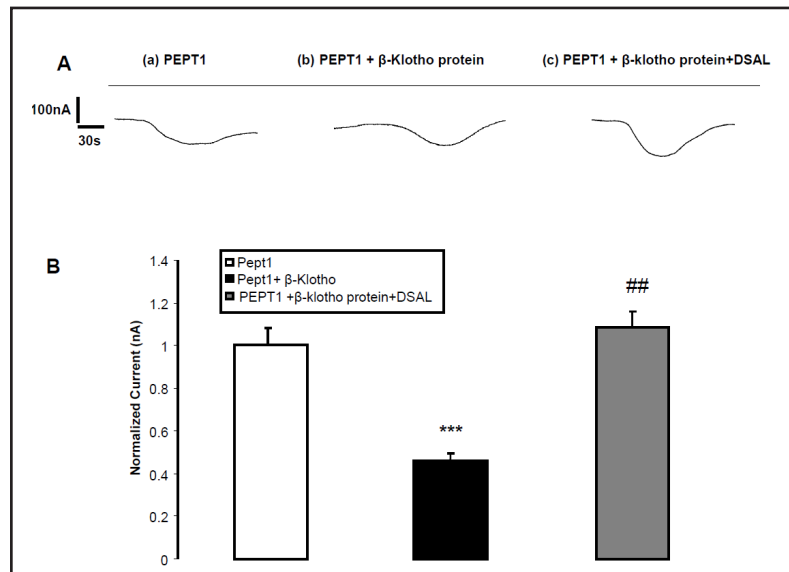
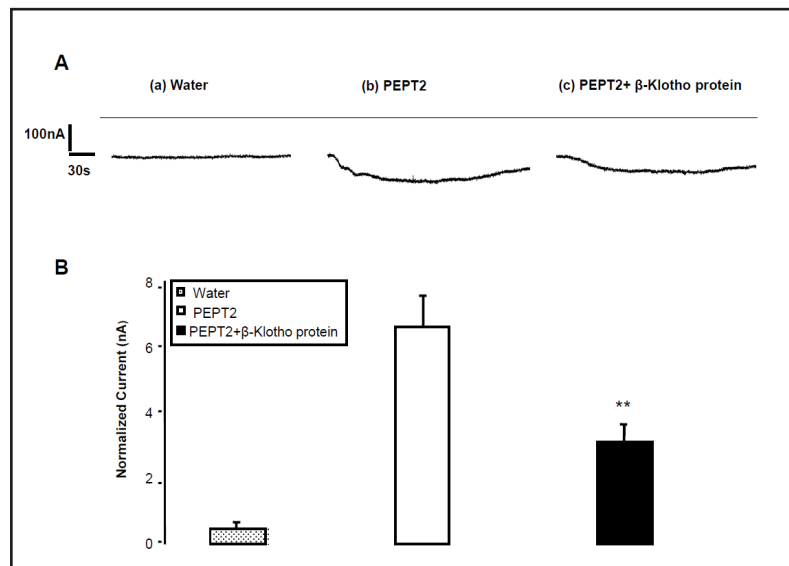


Fig. 4. Effect of recombinant human β -Klotho protein on electrogenic glycine-glycine transport in PEPT2 expressing *Xenopus laevis* oocytes. A: Representative original tracings of glycine-glycine (2 mM) induced current in *Xenopus* oocytes injected with water (a), or with cRNA encoding PEPT2 without (b) or with (c) prior β -Klotho protein (30 ng/ml, 24 h) treatment. B: Arithmetic means \pm SEM (n = 12-13) of the normalized glycine-glycine (2 mM) induced current in *Xenopus* oocytes injected water (dotted bar) or expressing PEPT2 without (white bar) or with (black bars) prior β -Klotho protein (30 ng/ml, 24 h) treatment. ** ($p < 0.01$) indicates statistically significant difference from PEPT2 expressing oocytes without β -Klotho protein treatment. Control I_{gly} amounted to 70.2 ± 5.1 nA (n = 18).



Discussion

The present study uncovers a novel function of β -Klotho protein, i.e. the down-regulation of the peptide transporters PEPT1 and PEPT2. Treatment of either, PEPT1 and PEPT2 expressing oocytes with human recombinant β -Klotho significantly decreased the glycine-

Fig. 5. Glycine-glycine induced transepithelial current in intestinal epithelia from klotho-deficient mice ($kl^{-/-}$) and wild-type ($kl^{+/+}$) mice. A. Representative original tracings showing the effect of glycine-glycine (gly-gly) (10 mM) on the transepithelial intestinal potential difference in wild-type mice ($kl^{+/+}$) and klotho-deficient mice (right). Arrows highlight the addition of 10 mM glycine-glycine. B. Arithmetic means \pm SEM (n = 4-5) of the glycine-glycine (10 mM) induced equivalent short-circuit current across intestinal epithelium from wild-type mice (white bar) and klotho-deficient mice (black bar). * ($p < 0.05$) indicates statistically significant difference from $kl^{-/-}$ mice (unpaired t-test).

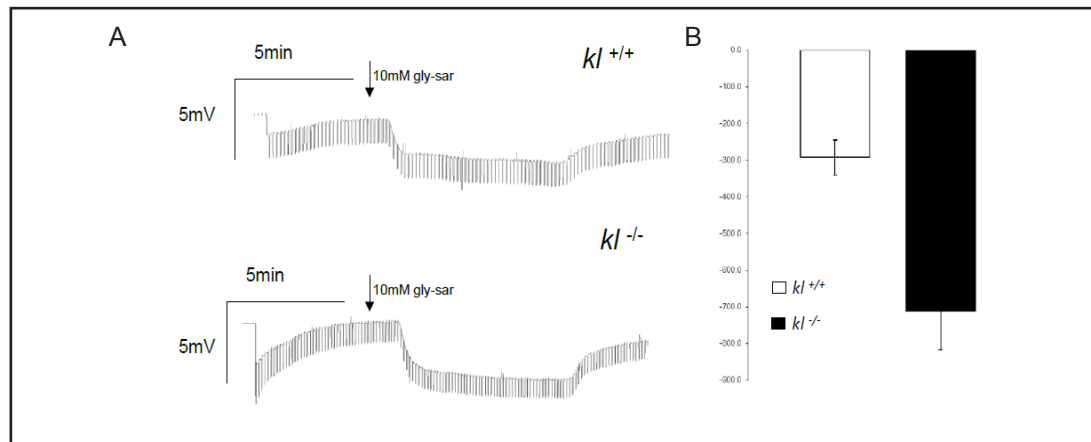
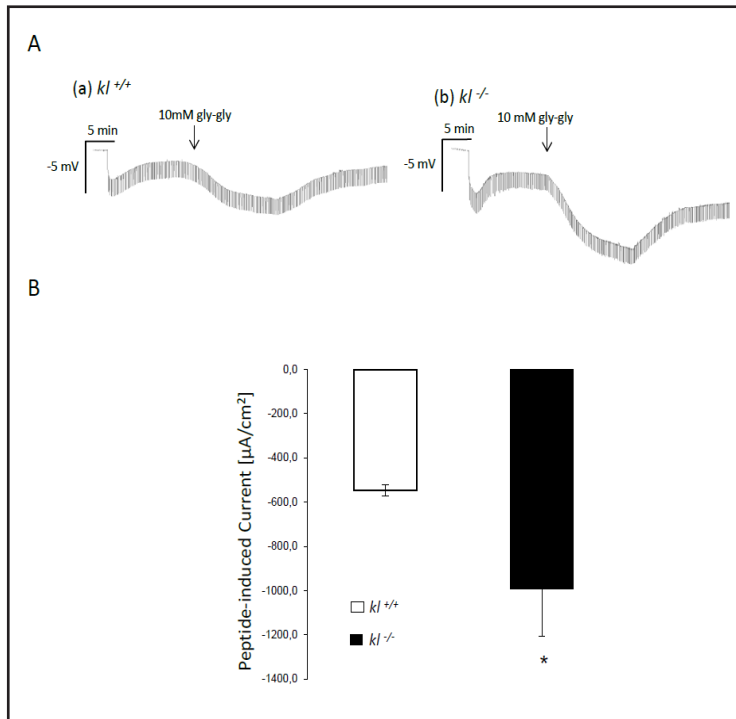


Fig. 6. Glycine-Sar induced transepithelial current in intestinal epithelia from klotho-deficient mice ($kl^{-/-}$) and wild-type ($kl^{+/+}$) mice. A. Representative original tracings showing the effect of glycine-sar (gly-sar) (10 mM) on the transepithelial intestinal potential difference in wild-type mice (Upper) and klotho-deficient mice (Lower). Arrows highlight the addition of 10 mM gly-Sar. B. Arithmetic means \pm SEM (n = 3-4) of the gly-sar (10 mM) induced equivalent short-circuit current across intestinal epithelium from wild-type mice (white bar) and klotho-deficient mice (black bar). * ($p < 0.05$) indicates statistically significant difference from $kl^{-/-}$ mice (unpaired t-test).

glycine-induced inward current (I_{Gly}). Kinetic analysis revealed that β -Klotho was in large part effective by decreasing the maximal transport rate of the carriers. The large scatter of the calculated concentrations required for half maximal current precludes safe conclusions about effects of klotho on carrier affinity. The effect of β -Klotho on I_{Gly} was reversed in the presence of the β -glucuronidase inhibitor D-saccharic acid 1,4-lactone monohydrate DSAL. Thus, β -klotho was apparently effective as β -glucuronidase. Klotho belongs to the β -glycosidase family [49]. Klotho has previously been shown to hydrolyze extracellular sugar residues of target membrane proteins [49]. As shown for the epithelial Ca^{2+} channel TRPV5 [49] and the excitatory amino acid transporters [28], klotho may stabilize the transport

proteins in the cell membrane. In contrast to its effect on TRPV5 and excitatory amino acid transporters, klotho down-regulates the peptide carriers. The present observations do not rule out that klotho modifies peptide transport in PEPT1 or PEPT2 expressing oocytes by mechanisms other than β -glucuronidase activity and other than destabilization of the carrier proteins in the cell membrane. Whatever mechanism involved, the effect of klotho obviously affects intestinal peptide transport. Presumably due to lacking down-regulation by klotho of peptide transporters, the peptide transport was markedly enhanced in klotho deficient mice. We can, however, not rule out the involvement of more indirect mechanisms modifying peptide transport in klotho deficient mice.

In view of the impact of peptide transporters on ageing [32], it is tempting to speculate that the down-regulation of intestinal peptide transport contributes to the impact of klotho on ageing. Even though up-regulation of peptide transporters is expected to accelerate ageing [32], the dramatic acceleration of ageing in klotho deficient mice is, however, obviously in large part the result of deranged mineral metabolism and tissue calcification [19].

In conclusion, β -Klotho down-regulates the activity of the peptide transporters PEPT1 and PEPT2. The effect apparently modifies intestinal peptide transport and may contribute a small part to the impact of klotho on ageing.

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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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