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Original Paper

Regulation of the Voltage Gated K^+ Channel $K_{v1.3}$ by Recombinant Human Klotho Protein

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Key Words
Alpha Klotho protein • Recombinant human Klotho protein • $K_{v1.3}$ channel • *Xenopus* oocytes • Jcam cells

Abstract
Background/Aims: Klotho, a protein mainly produced in the kidney and released into circulating blood, contributes to the negative regulation of $1,25(OH)_2D_3$ formation and is thus a powerful regulator of mineral metabolism. As β-glucuronidase, alpha Klotho protein further regulates the stability of several carriers and channels in the plasma membrane and thus regulates channel and transporter activity. Accordingly, alpha Klotho protein participates in the regulation of diverse functions seemingly unrelated to mineral metabolism including lymphocyte function. The present study explored the impact of alpha Klotho protein on the voltage gated K^+ channel $K_{v1.3}$. ***Methods:*** cRNA encoding $K_{v1.3}$ (KCNA3) was injected into *Xenopus* oocytes and depolarization induced outward current in $K_{v1.3}$ expressing *Xenopus* oocytes determined utilizing dual electrode voltage clamp. Experiments were performed without or with prior treatment with recombinant human Klotho protein (50 ng/ml, 24 hours) in the absence or presence of a β-glucuronidase inhibitor D-saccharic acid-1,4-lactone (DSAL, 10 μM). Moreover, the voltage gated K^+ current was determined in Jcam lymphoma cells by whole cell patch clamp following 24 hours incubation without or with recombinant human Klotho protein (50 ng/ml, 24 hours). $K_{v1.3}$ protein abundance in Jcam cells was determined utilising fluorescent antibodies in flow cytometry. ***Results:*** In $K_{v1.3}$ expressing *Xenopus* oocytes the $K_{v1.3}$ currents and the protein abundance of $K_{v1.3}$ were both significantly enhanced after

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treatment with recombinant human Klotho protein (50 ng/ml, 24 hours), an effect reversed by presence of DSAL. Moreover, treatment with recombinant human Klotho protein increased K_v currents and $K_{v1.3}$ protein abundance in Jcam cells. ***Conclusion:*** Alpha Klotho protein enhances $K_{v1.3}$ channel abundance and $K_{v1.3}$ currents in the plasma membrane, an effect depending on its β-glucuronidase activity.

Introduction

Klotho, a protein expressed in diverse tissues including kidney, parathyroid glands, and choroid plexus [1, 2], is a major determinant of aging and life span [3, 4]. Klotho deficiency results in accelerated aging with early death [4] and Klotho overexpression enhances the life span [3, 4]. The extracellular domain of Klotho may function as β-glucuronidase and hormone [5-7]. Klotho is required for the inhibitory effect of FGF23 on 25-hydroxyvitamin D-1α-hydroxylase and thus formation of 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) [2, 3, 8-10], the active form of vitamin D [11], which is generated mainly in kidney [12], but may be produced in addition by B cells [13], dendritic cells and macrophages [14, 15]. $1,25(OH)_2D_3$ in turn stimulates the expression of FGF23 and Klotho [8, 16, 17]. $1,25(OH)_2D_3$ is further a powerful stimulator of intestinal and renal Ca^{2+} and phosphate transport [11, 18]. Klotho deficiency increases plasma $1,25(OH)_2D_3$, Ca^{2+} and phosphate [18, 19] concentration, resulting in vascular calcification [20], growth deficit [2] and rapid aging [2, 9, 10]. Klotho insufficiency further leads to a wide variety of disorders including hypoglycemia, hearing loss, cardiac arrhythmia, sudden cardiac death, and compromised immune response [1, 21, 22].

Klotho has been shown to modify the activity of several channels and transport proteins including Ca^{2+} channels [23], renal outer medullary K^+ channels [24], Na^+, phosphate cotransport [25, 26] and Na^+/K^+ ATPase [27]. It is at least in part effective as β-glucuronidase [5-7].

The present study explored whether Klotho modifies the function of voltage gated K^+ channel $K_{v1.3}$ (KCNA3), a channel expressed in many tissues and participating in the regulation of a wide variety of cellular functions, including excitability [28-31], cell proliferation [32], apoptosis [33], immune response [34, 35], insulin sensitivity [36], and platelet function [37]. Cells expressing $K_{v1.3}$ include Jurkat and Jcam lymphoma cells, where the channels participate in the regulation of apoptosis [33].

$K_{v1.3}$ has been expressed in *Xenopus* oocytes with or without subsequent treatment with human recombinant Klotho protein in the presence or absence of the β-glucuronidase inhibitor DSAL (D-saccharic acid-1,4-lactone). The $K_{v1.3}$ activity was determined by dual electrode voltage clamp and $K_{v1.3}$ protein abundance at the cell membrane quantified by chemiluminescence. Moreover, voltage gated $K_{v1.3}$ currents and $K_{v1.3}$ protein abundance were determined in Jcam cells without or with prior exposure to recombinant human alpha Klotho protein.

Materials and Methods

Ethics Statement

Xenopus oocytes were explanted from adult *Xenopus Laevis* (NASCO). *Xenopus Laevis* frogs were anaesthesized by a 0.1% Tricaine solution. After confirmation of anaesthesia and disinfection of the skin, a small abdominal incision was made and oocytes were removed, followed by closure of the skin by sutures. All animal experiments were conducted in accordance with the Helsinki Declaration of 1975 and according to the German law for the welfare of animals and the surgical procedures on the adult *Xenous laevis* were reviewed and approved by the respective government authority of the state Baden-Württemberg (Regierungspräsidium Tübingen) prior to the start of the study (Anzeige für Organentnahme nach §6).

Constructs

For generation of cRNA, a construct was used encoding wild-type mouse $K_{v1.3}$ (KCNA3) [38]. The construct was used for the generation of cRNA as described previously [39, 40].

Voltage clamp in Xenopus oocytes

Xenopus oocytes were prepared as previously described [41, 42]. cRNA encoding $K_{v1.3}$ (2.5 ng) was injected on the same or the second day of *Xenopus* oocytes preparation [43, 44]. All experiments were performed at room temperature 3 days after cRNA injection [45, 46]. The oocytes were maintained at 17°C in ND96 solution containing: 88.5.mM NaCl, 2mM KCl, 1mM $MgC1_2$, 1.8mM $CaC1_2$, 5mM HEPES. Tretracycline (50 mg/l), Ciprofloxacin (1.6 mg/l), Refobacin (100 mg/l) and Theophylline (90 mg/l) as well as sodium pyruvate (5mM) were added to the ND96, and pH was adjusted to 7.5 by addition of NaOH. The control superfusate (ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$,1 mM $MgCl_2$ and 5 mM HEPES; pH was adjusted to 7.4 by addition of NaOH. Where indicated, recombinant human Klotho protein (10, 30 or 50 ng/ml, 5334-KL-R&D Systems) was added for 6, 12 or 24 hours before voltage clamp measurements (66, 60 and 48 hours respectively after injection of cRNA encoding $K_{v1.3}$) and D-saccharic acid 1,4-lactone monohydrate (DSAL, 10μM, Sigma) was added for 24 hours before voltage clamp measurements. In dual electrode voltage-clamp experiments $K_{v1.3}$ channel currents were elicited every 15 second with 2 second depolarizing pulses up to +50 mV applied from a holding potential of -100 mV. Pulses were applied in 10 mV increments. The data were filtered at 2 kHz and recorded with a Digidata 1322A A/D-D/A converter and ClampexV .9.2 software for data acquisition (Axon Instruments) [47, 48]. The analysis of the data was performed with Clampfit 9.2 (Axon Instruments) software [49, 50].

Chemiluminescence

Chemiluminescence was employed for detection of $K_{v1.3}$ cell surface expression in oocytes, which are too large to be analysed by flow cytometry. Oocytes were first incubated with primary polyclonal rabbit anti-$K_{v1.3}$ antibody (extracellular) (1:200, Alomone Labs) and subsequently with secondary, HRP-conjugated anti-rabbit IgG antibody (1:2500, Cell Signaling). Individual oocytes were placed in 96 well plates with 20 μl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce) and chemiluminescence of single oocytes was quantified in a luminometer (Walter Wallac 2 plate reader, Perkin Elmer) by integrating the signal over a period of 1 sec [51]. Results display normalized relative light units. Integrity of the measured oocytes was assessed by visual control after the measurement to avoid unspecific light signals from the cytosol [52]. Where indicated, recombinant human Klotho protein (50 ng/ml, 5334-KL-R&D Systems) was added for 24 hours before chemiluminescence measurements (48 hours after injection of cRNA encoding $K_{v1.3}$) .

Culture of Jcam cells

Jcam cells were grown in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 m M L-glutamine (Gib-co-Invitrogen, Karlsruhe, Germany).

Patch clamp

Patch clamp experiments have been performed at room temperature in voltage-clamp, fast-whole-cell mode as described previously [53-55]. The cells were continuously superfused through a flow system inserted into the dish. Borosilicate glass pipettes (2-5 MΩ tip resistance; Harvard Apparatus, Kent, UK) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with a MS314 electrical micromanipulator (MW, Märzhäuser, Wetzlar, Germany) [56]. The currents were recorded by an EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, N.Y., USA). Whole-cell currents were elicited by 200 msec square wave voltage pulses from -100 to +100 mV in 20 mV steps delivered at 15 second intervals from a holding potential of -60 mV or by 200 millisecond square wave voltage pulses from -60 to +20 mV in 5 mV steps delivered at 15 second intervals from a holding potential of -60 mV. The currents were recorded with an acquisition frequency of 10 kHz and 3 kHz low-pass filtered. Since the time constant of activation decreased with the applied depolarizing voltage step and inactivation occurred especially during strong depolarization, the current was analyzed at its maximal amplitude value (peak current).

The Jcam cells were superfused with a bath solution containing (in mmol/l): 145 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 5 glucose and 10 HEPES, pH 7.4 (NaOH). The patch clamp pipettes were filled with an internal solution

containing (in mmol/l): 80 KCl, 60 K^+-gluconate, 1 $MgCl_2$, 1 Mg-ATP, 1 EGTA, 10 HEPES; pH was adjusted to 7.2 (KOH). Where indicated, recombinant human Klotho protein (50 ng/ml, 5334-KL-R&D Systems) was added 24 hours before patch clamp measurements.

The offset potentials between both electrodes were zeroed before sealing. The potentials were corrected for liquid junction potentials as estimated according to [57, 58]. The original whole-cell current traces are depicted and currents of the individual voltage square pulses are superimposed. 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 outward deflections of the original current traces.

Flow cytometry

For measurement of $K_{v1.3}$ protein abundance, 5x 10^5 Jcam cells were incubated without or with klotho protein [50 ng/ml], washed twice with PBS (Gibco, Invitrogen) and fixed for 5 minutes with 4% paraformaldehyde on ice. After additional two washes with PBS and permeablization with 0.1% Triton X-100 for 2mins cells were incubated for 1 h at 37°C with the primary $K_{v1.3}$ antibody (1:250, Alomone Labs, Israel) at 37°C. For detection the cells were washed twice with PBS-BSA (0,1%) and stained for 30 min at 37°C with CF™ 488A-labeled goat anti–rabbit secondary antibody (1: 250, Sigma-Aldrich, USA). Unbound secondary antibody was removed by repeated washing with PBS-BSA and the samples were analyzed by flow cytometry (BD FACS Calibur, BD Biosciences, USA) for median fluorescence intensity at excitation on 488 nm and emission on 530 nm.

Statistics

Data are provided as means ± SEM, *n* represents the number of independent experiments. All oocyte experiments were repeated with at least 3 batches of oocytes; in all repetitions qualitatively similar data were obtained. All data were tested for significance using ANOVA followed by posthoc analysis or unpaired t-test, where appropriate. Only results with $p < 0.05$ were considered statistically significant.

Results

The present study explored the effect of recombinant human Klotho protein on the voltage gated K^+ channel $K_{v1.3}$ (KCNA3). To this end, cRNA encoding $K_{v1.3}$ was injected in *Xenopus* oocytes and depolarization induced outward currents were recorded by dual electrode voltage clamp. The amplitude of the peak outward currents was taken as a measure of the voltage gated K^+ channel $K_{v1.3}$ conductance in *Xenopus* oocytes. As illustrated in Fig. 1-A,B, no appreciable outward $K_{v1.3}$-like currents were measured in *Xenopus* oocytes injected only with water. Thus, *Xenopus* oocytes do not express appreciable endogenous $K_{v1.3}$-like channels. In contrast, depolarization of *Xenopus* oocytes injected with cRNA encoding $K_{v1.3}$ was followed by a rapidly inactivating outward current typical for $K_{v1.3}$ channel. Further experiments explored whether the effect of recombinant human Klotho protein on $K_{v1.3}$ currents resulted from an effect of Klotho on $K_{v1.3}$ protein abundance in the cell membrane. The protein abundance was quantified by chemiluminescence. As shown in Fig. 1C, the cell surface expression of the $K_{v1.3}$ channel protein in *Xenopus* oocytes was indeed significantly increased by pretreatment with the recombinant human Klotho protein.

As illustrated in Fig. 2, the amplitude of the depolarization induced peak currents was significantly enhanced by a 24 hours pretreatment with recombinant human Klotho protein (50 ng/ml) prior to voltage clamp recording. The effect of recombinant human Klotho protein (50 ng/ml) on the depolarization induced outward current amplitude in $K_{v1.3}$ expressing *Xenopus* oocytes was time-dependent and reached statistical significance after 12 hours of incubation without reaching a plateau (Fig. 2). Fitting the mean peak current and the time course till 12 hours to Boltzman function yielded a half-life time $t_{1/2}$ of 3.8 hours.

In theory alpha Klotho protein could have been effective as beta-glucuronidase. In order to test whether recombinant human Klotho protein enhanced $K_{v1.3}$ currents by exerting beta-glucuronidase activity, the effect of recombinant human Klotho protein (50 ng/ml,

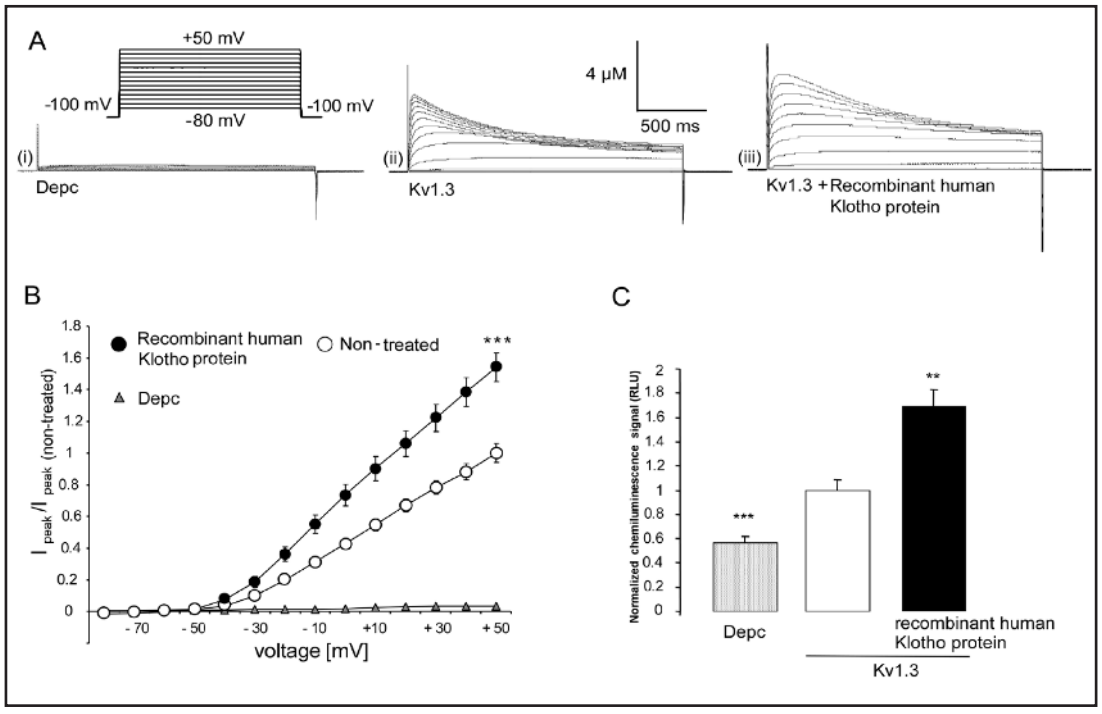


Fig. 1. Effect of recombinant human Klotho protein on $K_{v1.3}$ peak current amplitude in $K_{v1.3}$ expressing *Xenopus* oocytes and on $K_{v1.3}$-HA plasma membrane abundance in $K_{v1.3}$ –HA expressing *Xenopus* oocytes. A. Original tracings demonstrating outward $K_{v1.3}$ currents activated by depolarization from -80 to +50 mV in 20 second increments of 10 mV steps from a holding potential of -100 mV in *Xenopus* oocytes injected with water (i), or injected with cRNA encoding $K_{v1.3}$ without (ii) or with (iii) 24 hours treatment with recombinant human Klotho protein (50 ng/ml) prior to measurement. B. Arithmetic means ± SEM (n = 6-42) of the normalized depolarization-induced $K_{v1.3}$ peak current as a function of voltage in *Xenopus* oocytes injected with water (grey triangles), or with cRNA encoding $K_{v1.3}$ without (white circles) or with (black circles) 24 hours treatment with recombinant human Klotho protein (50 ng/ml) prior to measurement. Peak currents are normalized to the mean peak current at +50 mV in *Xenopus* oocytes injected with cRNA encoding $K_{v1.3}$ and not treated with recombinant human Klotho protein prior to measurement. *** ($p<0.001$) indicates statistically significant difference from *Xenopus* oocytes injected with cRNA encoding $K_{v1.3}$ and not treated with recombinant human Klotho protein prior to measurement. C. Arithmetic means ± SEM (n = 64-67) of the $K_{v1.3}$ protein abundance in the plasma membrane of *Xenopus* oocytes injected with water (dotted bar), or injected with cRNA encoding $K_{v1.3}$ without (white bar) or with (black bar) 24 hours treatment with recombinant human Klotho protein (50 ng/ml) prior to measurement. ** ($p<0.01$) indicates statistically significant difference from the protein abundance of $K_{v1.3}$ in untreated *Xenopus* oocytes injected with cRNA encoding $K_{v1.3}$.

24 hours) on the depolarization induced outward current in $K_{v1.3}$ expressing *Xenopus* oocytes was measured using dual electrode voltage clamp in the presence and absence of 10 µM of D-saccharic acid-1,4-lactone (DSAL), which is known to inhibit the beta glucuronidase activity of Klotho protein. As illustrated in Fig. 3, the effect of recombinant human Klotho protein (50 ng/ml, 24 hours) on the depolarization induced outward current in $K_{v1.3}$ expressing *Xenopus* oocytes was virtually abrogated in the presence of DSAL (10 µM).

In order to test, whether recombinant human Klotho protein is similarly effective in $K_{v1.3}$ expressing mammalian cells, a further series of experiments was performed in Jcam lymphoma cells. According to flow cytometry, Jcam cells expressed $K_{v1.3}$ (Fig. 4). The $K_{v1.3}$ protein abundance was significantly increased by treatment of Jcam lymphoma cells with recombinant human Klotho protein (50 ng/ml) for 24 hours (Fig. 4).

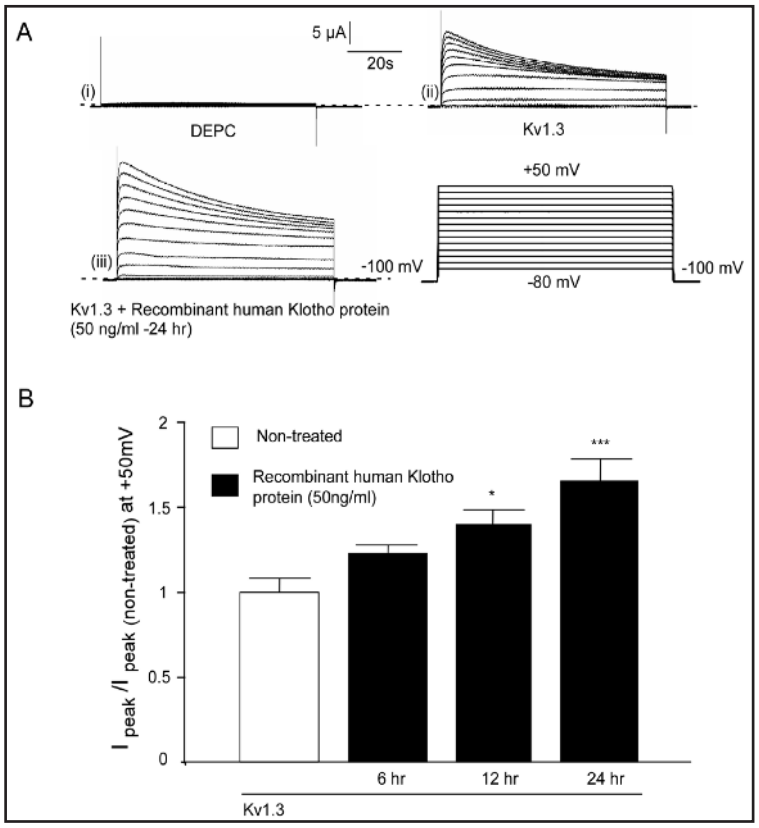


Fig. 2. Time course of the increase in $K_{v1.3}$ peak current amplitude following treatment with recombinant human Klotho protein in $K_{v1.3}$ expressing *Xenopus* oocytes. A. Original tracings demonstrating outward $K_{v1.3}$ currents activated by depolarization from -80 to +50 mV in 20 second increments of 10 mV steps from a holding potential of -100 mV in *Xenopus* oocytes injected with water (i), or injected with cRNA encoding $K_{v1.3}$ without (ii) or with (iii) 24 hours treatment with recombinant human Klotho protein (50 ng/ml) prior to measurement. B. Arithmetic means ± SEM (n = 8-17) of the normalized depolarization-induced $K_{v1.3}$ peak current at +50 mV in *Xenopus* oocytes injected with cRNA encoding $K_{v1.3}$ without (white bar) or with (black bars) 6, 12, or 24 hours treatment with recombinant human Klotho protein (50 ng/ml) prior to measurement. Peak currents are normalized to the mean peak current at +50 mV in *Xenopus* oocytes injected with cRNA encoding $K_{v1.3}$ and untreated with recombinant human Klotho protein prior to measurement. * ($p<0.05$), *** ($p<0.001$) indicates statistically significant difference from *Xenopus* oocytes injected with cRNA encoding $K_{v1.3}$ and not treated with recombinant human Klotho protein prior to measurement.

The voltage gated K^+ currents in Jcam lymphoma cells are illustrated in Fig. 5. Treatment of those cells with recombinant human Klotho protein (50 ng/ml) for 24 hours was followed by a significant increase of rapidly inactivating outward current typical for $K_{v1.3}$ channels [59]. The mean peak current density was increased from 51 ± 6 pA/pF (at +100 mV; n = 20) to 88 ± 13 pA/pF (at +100 mV; n = 21) following a 24 hours treatment of Jcam cells with recombinant human Klotho protein. Exposure to recombinant human Klotho protein increased the peak current density without appreciably affecting the other biophysical properties of the voltage gated K^+ currents in Jcam cells. Fitting the normalized conductance to Boltzman equation showed no difference in the half maximal activation voltage $V_{½}$ and the slope factor K before ($V_{½}$= -38.7±1.9 mV, $K_{\text{slope factor}}$ = 2.9 ±0.7, n=14) and after ($V_{½}$= -39.6±1.5 mV, $K_{\text{slope factor}}$ = 2.1±0.5, n=16) 24 hours treatment of Jcam cells with recombinant human Klotho protein (Fig.6). The activation time constants (τ_{act}) were calculated by fitting a single exponential function to the rising phase of currents at voltages from +20 to +100 mV. The mean activation time constant (τ_{act}) was not significantly different without (n = 11) or with (n = 12) a prior 24 hours exposure to recombinant human Klotho protein (Fig.6). The inactivation time constants (τ inactivation) were calculated by fitting single exponential function to the decaying phase of currents at voltages from +60 to +100 mV. The inactivation time constant (τ inactivation) was slightly different at +91 mV and at +71 mV between cells with or without prior Klotho treatment, a difference, however, not reaching

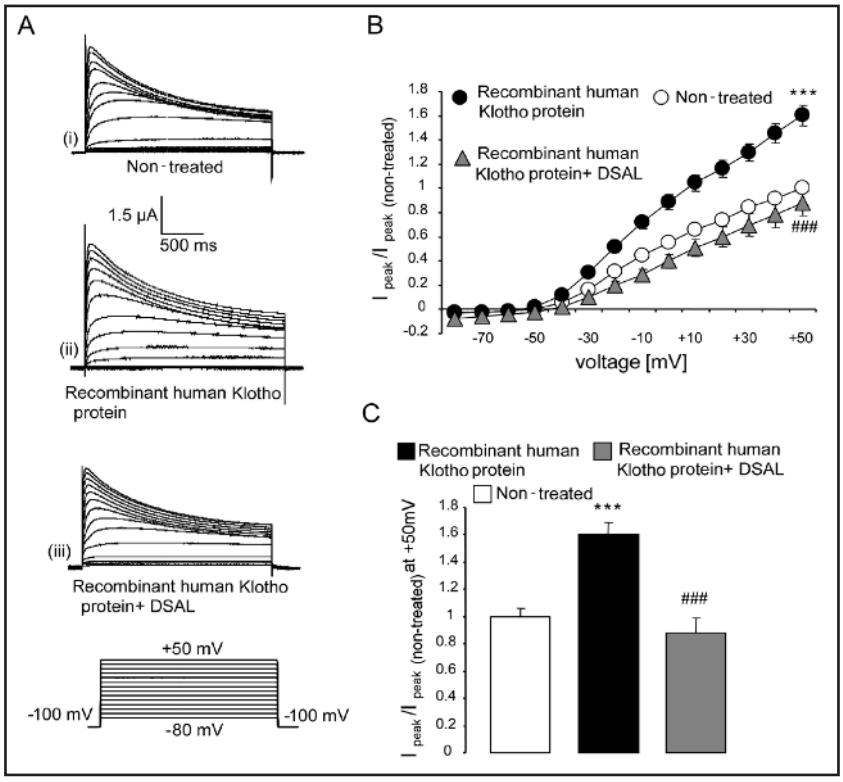
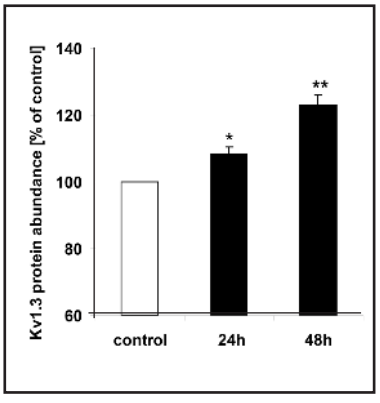


Fig. 3. Effect of recombinant human Klotho protein on $K_{v1.3}$ peak current amplitude in absence and presence of β-glucuronidase inhibitor (DSAL) in $K_{v1.3}$ expressing *Xenopus* oocytes. A. Original tracings demonstrating outward $K_{v1.3}$ currents activated by depolarization from -80 to +50 mV in 20 second increments of 10 mV steps from a holding potential of -100 mV in *Xenopus* oocytes injected with cRNA encoding $K_{v1.3}$ without (i) or following 24 hours pretreatment with recombinant human Klotho protein (50 ng/ml) in the absence (ii) and presence (iii) of D-saccharic acid-1,4-lactone, DSAL (10µM). B. Arithmetic means ± SEM (n = 39-53) of the normalized depolarization-induced $K_{v1.3}$ peak current as a function of voltage in *Xenopus* oocytes injected with cRNA encoding $K_{v1.3}$ without (white circles) or following 24 hours pretreatment with recombinant human Klotho protein (50 ng/ml) in the absence (black circles) and presence (grey triangles) of D-saccharic acid-1,4-lactone, DSAL (10µM). *** ($p<0.001$) indicates statistically significant difference from not treated $K_{v1.3}$expressing *Xenopus* oocytes. ### ($p<0.001$) indicates statistically significant difference from *Xenopus* oocytes injected with cRNA encoding $K_{v1.3}$ and treated with recombinant human Klotho protein for 24 hours in the absence of D-saccharic acid-1,4-lactone, DSAL (10µM). C. Arithmetic means ± SEM (n =39-53) of the normalized depolarization-induced $K_{v1.3}$ peak current at +50 mV in *Xenopus* oocytes injected with cRNA encoding $K_{v1.3}$ without (white bar) or following 24 hours pretreatment with recombinant human Klotho protein (50 ng/ml) in the absence (black bar) and presence (grey bar) of D-saccharic acid-1,4-lactone, DSAL (10 µM). *** ($p<0.001$) indicates statistically significant difference of from untreated $K_{v1.3}$ expressing *Xenopus* oocytes. ### ($p<0.001$) indicates statistically significant difference from *Xenopus* oocytes injected with cRNA encoding $K_{v1.3}$ and treated with recombinant human Klotho protein for 24 hours in the absence of D-saccharic acid-1,4-lactone, DSAL (10µM).

Fig. 4. $K_{v1.3}$ protein abundance in Jcam cells. Arithmetic means ± SEM (n = 4) of $K_{v1.3}$ protein abundance in Jcam cells without (white bar) and with (black bars) prior treatment with Klotho (50 ng/ml) protein for 24 or 48 hours.



statistical significance. The inactivation time constant at +50 mV was significantly ($p<0.01$) larger in Jcam cells treated with recombinant human Klotho protein (τ_{inact} = 145.9 ±17.55 msec, n= 21) than in not treated Jcam cells (τ_{inact} = 92.6 ±7.9 msec, n= 20) (Fig.6). The delayed inactivation after 24 hours pre-treatment with recombi-

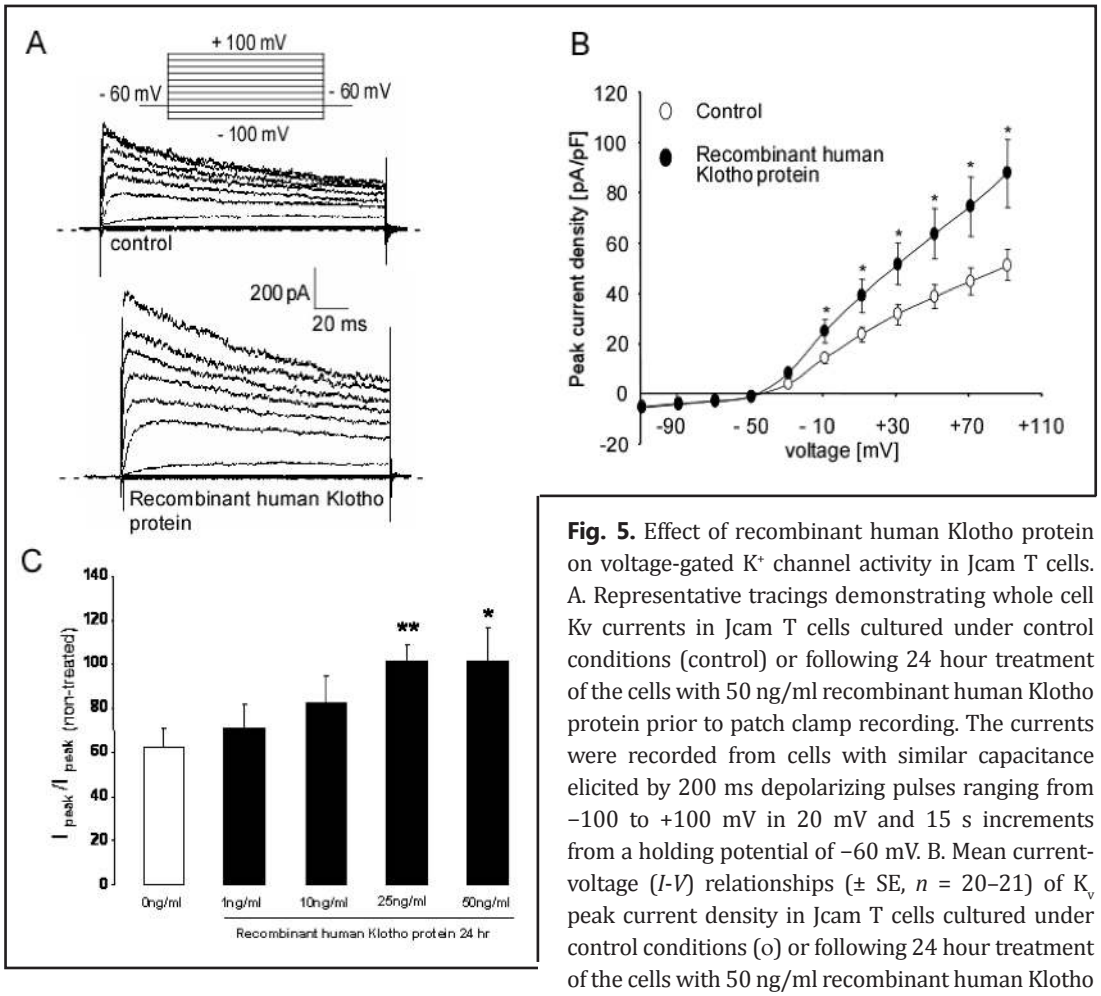


Fig. 5. Effect of recombinant human Klotho protein on voltage-gated K^+ channel activity in Jcam T cells. A. Representative tracings demonstrating whole cell Kv currents in Jcam T cells cultured under control conditions (control) or following 24 hour treatment of the cells with 50 ng/ml recombinant human Klotho protein prior to patch clamp recording. The currents were recorded from cells with similar capacitance elicited by 200 ms depolarizing pulses ranging from −100 to +100 mV in 20 mV and 15 s increments from a holding potential of −60 mV. B. Mean current-voltage (*I-V*) relationships (± SE, *n* = 20–21) of K_v peak current density in Jcam T cells cultured under control conditions (o) or following 24 hour treatment of the cells with 50 ng/ml recombinant human Klotho protein prior to patch clamp recording (●) . C. Arithmetic means ± SEM (n = 12-24) of whole cell K_v currents in Jcam T cells cultured under control conditions (white bar) and following 24 hour treatment of the cells with 1-50 ng/ml recombinant human Klotho protein (black bars) prior to patch clamp recording. The currents were recorded from the cells with similar capacitance elicited by 200-ms depolarizing pulses ranging from −100 to +100 mV in 20 mV and 15 s increments from a holding potential of −60 mV.

nant human Klotho protein could be explained by the increased protein abundance of $K_{v1.3}$ in the plasma membrane, which has previously been shown to slow down the channel inactivation kinetics [60].

Discussion

The present study reveals a novel function of Klotho, i.e. the up-regulation of the voltage gated K^+ channel $K_{v1.3}$ (KCNA3). Apparently Klotho increases the $K_{v1.3}$ channel protein abundance in the cell membrane without appreciably affecting other properties of the channel. The effect of Klotho is reversed by β-glucuronidase inhibitor DSAL and is thus most likely due to the enzyme activity of the Klotho protein. The present observations reveal that $K_{v1.3}$ channel activity is affected by both Klotho expression and exposure of the channels to soluble Klotho. The transmembrane Klotho protein serves as a co-receptor for FGF23 and is required for the inhibitory effect of FGF23 on the 25(OH) vitamin D 1α hydroxylase [61].

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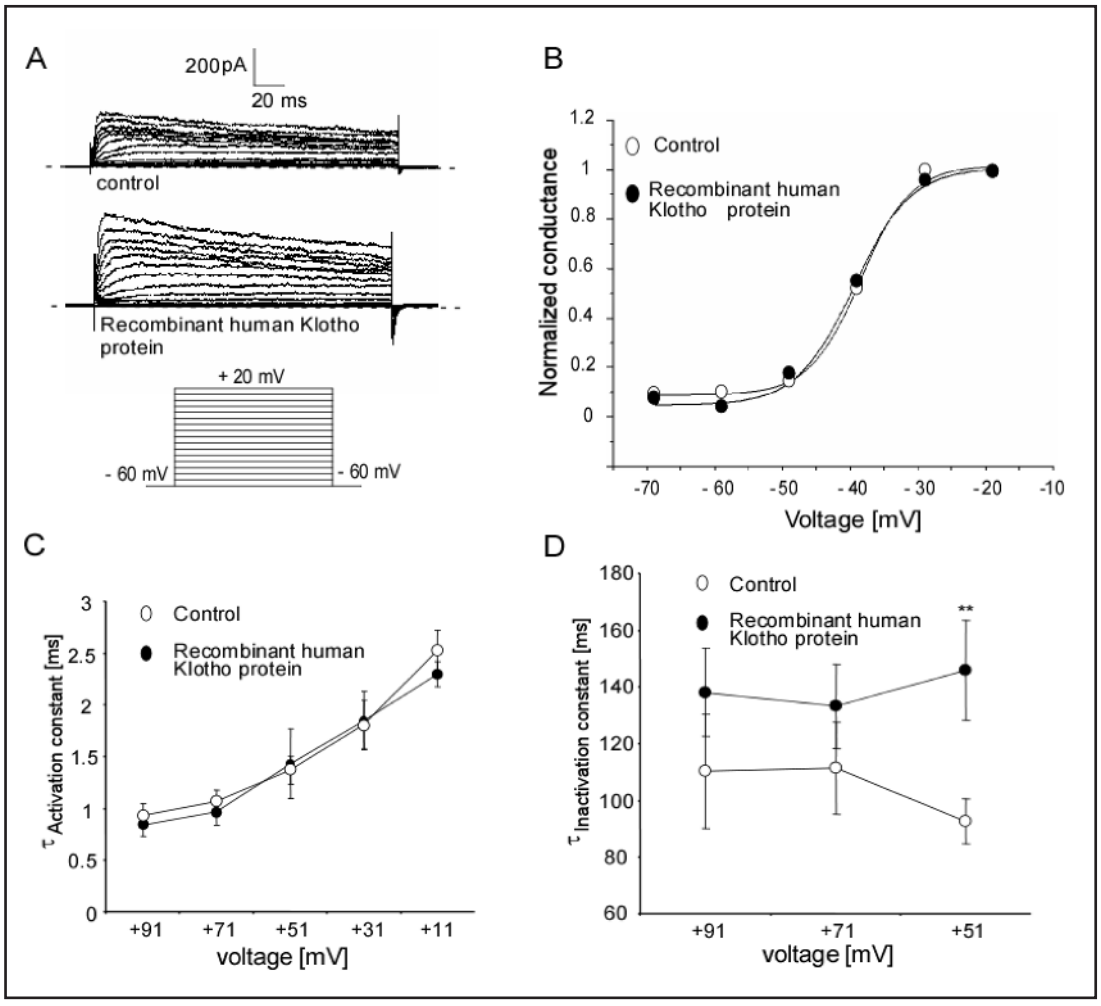

Fig. 6. Effect of recombinant human Klotho protein on biophysical properties of voltage-gated K^+ channels in Jcam T cells. A. Representative tracings demonstrating whole cell K_v currents in Jcam T cells cultured under control conditions (control) and following 24 hour treatment of the cells with 50 ng/ml recombinant human Klotho protein prior to patch clamp recording. The currents were recorded from the cells with similar capacitance elicited by 200-ms depolarizing pulses ranging from −60 to +20 mV in 5 mV and 15 s increments from a holding potential of −60 mV. B. Normalized conductance-voltage (G-V) relationship (n = 14–16) of K_v peak currents in Jcam T cells cultured under control conditions (o) or following 24 hour treatment of the cells with 50 ng/ml recombinant human Klotho protein prior to patch clamp recording (●). Conductance was normalized to the maximal conductance of K_v peak currents in each group, plotted to the voltage according to Boltzman equation, $V_{1/2}$ and slope factor K were deduced accordingly. The liquid junction potential ΔE between the pipette and the bath solutions was estimated and V corrected accordingly. C. Recombinant human Klotho protein does not affect onset of activation of K_v currents in Jcam T cells. Activation time constants (τ activation) were determined by fitting single exponential function to the rising phase of currents at voltages from +20 to +100 mV of control cells (n = 11) and cells pretreated with recombinant human Klotho protein (50 ng/ml, 24 hour, n = 12).
D. Recombinant human Klotho protein affects onset of inactivation of K_v currents in Jcam T cells. Inactivation time constants (τ inactivation) were determined by fitting single exponential function to the decaying phase of currents at voltages from +60 to +100 mV of control cells (n = 20) and cells pretreated with recombinant human Klotho protein (50 ng/ml, 24 hour, n = 21). The liquid junction potential ΔE between the pipette and the bath solutions was estimated and V corrected accordingly. *,** indicates statistically significant (P < 0.05, 0.01) difference from Jcam T cells cultured under control conditions (control) according to Student's t-test analysis.

The extracellular domain of Klotho may be cleaved off and function as β-glucuronidase and hormone [5-7]. Klotho is released into circulating blood, urine and cerebrospinal fluid and may influence a multitude of functions including transport, energy metabolism, oxidative stress, and hormone release [61].

Klotho has previously been shown to affect a variety of further channels, such as the epithelial Ca^{2+} channels TRPV5 [62-66] and TRPV6 [66], as well as the K^+ channels ROMK [24, 62], KCNQ1/KCNE1 [67] and hERG [68]. Klotho apparently does not appreciably affect the channels TRPV4 [66] or TRPM6 [66]. As shown for TRPV5, Klotho removes alpha2,6-linked sialic acids, which in turn fosters cell surface retention of the channels [63].

Klotho further participates in the regulation of carriers, such as the excitatory amino acid transporters EAAT 3 and EAAT4 [54] and the phosphate transporters NaPi-IIa and NaPi-IIb [25]. Moreover, Klotho up-regulates the Na^+/K^+ ATPase [27]. Klotho sensitive regulation of the excitatory amino acid transporters was also reversed by the β-glucuronidase inhibitor DSAL [54].

Klotho may, however, affect channels by other mechanisms. Klotho down-regulates the endothelial Ca^{2+} channel 1 (TRPC-1) by fostering VEGF-mediated internalization of the VEGFR-2/TRPC-1 complex [69]. Klotho may further affect channel and transporter activity by its involvement in the down-regulation of $1,25(OH)_2D_3$ formation. In *kl/kl* mice the unrestrained $1,25(OH)_2D_3$ formation leads to excessive plasma levels of $1,25(OH)_2D_3$ [8], which in turn directly or indirectly affects several channels, transporters and the Na^+/K^+ATPase [70-77]. The excessive formation of $1,25(OH)_2D_3$ in Klotho deficient animals leads, for instance, to decreased activity of the transcription factor NFκB with subsequent down-regulation of several NFκB sensitive genes including the Ca^{2+} release activated Ca^{2+} channel Orai1 [78]. Altered channel activity and transport in Klotho deficient animals thus does not only reflect direct effects of Klotho on the respective channel or transporter proteins, but as well the direct and indirect impact of excessive $1,25(OH)_2D_3$ formation.

In view of the Klotho sensitivity of $K_{v1.3}$ protein abundance in the lymphocyte cell membrane, Klotho is expected to modify the immune response. $K_{v1.3}$ channels participate in the regulation of the Ca^{2+} influx and short-term activation of lymphocytes [79, 80] and are thus decisive for the immune response [34, 35]. The Klotho sensitivity of lymphocyte $K_{v1.3}$ channels may thus well contribute to the known impact of Klotho on the immune system [22]. The concentration of recombinant Klotho employed was higher than the plasma concentrations of approximately 0.5 ng/ml in healthy individuals [81, 82]. On the other hand, the *in vivo* exposure time to plasma klotho is by far longer. Moreover, local concentrations of Klotho may be higher than the plasma concentration. Nevertheless, the quantitative impact of normal plasma klotho concentrations on lymphocyte $K_{v1.3}$ channel activity and function cannot be safely predicted from the present observations.

$K_{v1.3}$ channels are expressed in many further cell types and participate in the regulation of a wide variety of cellular functions, including excitability [28-31], cell proliferation [32], apoptosis [33], insulin sensitivity [36], and platelet function [37]. Future studies will be required to define the direct or indirect influence of the Klotho protein on those functions.

Conclusion

The present observations reveal that Klotho up-regulates surface protein abundance and activity of the voltage gated K^+ channel $K_{v1.3}$ (KCNA3) and may thus participate in the regulation of $K_{v1.3}$ dependent cellular functions.

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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