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Downregulation of Klotho expression by dehydration

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Tang C, Pathare G, Michael D, Fajol A, Eichenmüller M, Lang F. Downregulation of Klotho expression by dehydration. *Am J Physiol Renal Physiol* 301: F745–F750, 2011. First published July 6, 2011; doi:10.1152/ajprenal.00037.2011.—Klotho, a transmembrane protein, protease, and hormone mainly expressed in renal tissue counteracts aging. Overexpression of Klotho substantially prolongs the life span. Klotho deficiency leads to excessive formation of $1,25(OH)_2D_3$, growth deficit, accelerated aging, and early death. Aging is frequently paralleled by dehydration, which is considered to accelerate the development of age-related disorders. The present study explored the possibility that dehydration influences Klotho expression. Klotho transcript levels were determined by RT-PCR, and Klotho protein abundance was detected by Western blotting in renal tissue from hydrated and 36-h-dehydrated mice as well as in human embryonic kidney (HEK293) cells. Dehydration was followed by a significant decline of renal Klotho transcript levels and protein abundance, accompanied by an increase in plasma osmolarity as well as plasma ADH, aldosterone, and $1,25(OH)_2D_3$ levels. Antidiuretic hormone (ADH; 50 nM) and aldosterone (1 μM) significantly decreased Klotho transcription and protein expression in HEK293 cells. In conclusion, the present observations disclose a powerful effect of dehydration on Klotho expression, an effect at least partially mediated by enhanced release of ADH and aldosterone.

ADH; aldosterone; osmolarity; $1,25(OH)_2D_3$; aging

KLOTHO IS A TRANSMEMBRANE protein mainly expressed in the kidney, parathyroid glands, and choroid plexus (5, 19, 38, 40). The extracellular domain may be cleaved off and released into cerebrospinal fluid and blood (15). Klotho counteracts aging and is thus a powerful regulator of life span (19, 20). In mice, Klotho deficiency leads to severe growth retardation and accelerated aging and death within <5 mo (20). Conversely, overexpression of Klotho leads to substantial extension of life span (19, 21). Klotho deficiency results in accelerated appearance of several age-related disorders such as hypogonadotropic hypogonadism, skin atrophy, muscle dystrophy, vascular calcification, osteopenia, pulmonary emphysema, cognition impairment, hearing loss, and motor neuron degeneration (14, 19).

The impact of Klotho on life span is considered to result at least partially from its stimulating effect on $1,25(OH)_2D_3$ formation, and the life span of Klotho-deficient mice can be substantially extended by a low-vitamin D diet (7, 19, 29, 40, 41). Excessive $1,25(OH)_2D_3$ contributes to hyperphosphatemia of the Klotho-deficient mice, which in turn fosters vascular calcification (13, 19, 25).

Early frailty, incident disability, and mortality in the elderly are observed in individuals with enhanced plasma osmolarity (37) and may thus be fostered by dehydration. In the elderly, dehydration is common, resulting from several reasons, including deficit of thirst perception, renal concentrating ability, and vasopressin effectiveness (22). Dehydration is encountered particularly in elderly diabetic patients and enhances their morbidity and mortality (9). Dehydration has further been shown to increase the risk of renal injury in the elderly (3).

The present paper pursued the hypothesis that dehydration may downregulate the expression of Klotho, which could thus contribute to the premature development of frailty and early death of the affected individuals. To this end, mice were exposed to a 36-h period of water deprivation and Klotho expression was determined by RT-PCR and Western blotting. Dehydration indeed decreased renal Klotho transcript levels and protein abundance, an effect paralleled by an increase in plasma antidiuretic hormone (ADH), aldosterone, and $1,25(OH)_2D_3$ levels. To test for a possible role of ADH and aldosterone in the regulation of Klotho expression, human embryonic kidney (HEK293) cells were treated with the hormones and Klotho expression was determined.

MATERIALS AND METHODS

Mice. All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities.

The mice (8 females, 8 males, aged 10 wk) had access to control food (Altromin 1310) and either access to water ad libitum or were deprived of fluid for 36 h. Female and male mice were studied to rule out gender-specific effects. To obtain blood, mice were anesthetized with diethylether (Roth, Karlsruhe, Germany), and blood specimens (50–200 μl) were withdrawn into capillaries containing EDTA by puncturing the retro-orbital plexus. To obtain renal tissue, the animals were anesthetized with diethylether and euthanized by cervical dislocation. The kidneys were then rapidly removed and snap frozen.

Cells. Human embryonic kidney (HEK293) cells were cultured in DMEM, supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin at 5% CO_2 and 37°C. For serum starvation, DMEM with 0.5% FBS was employed for 6 h before treatment. HEK293 cells were treated with ADH (50 nM), aldosterone (1 μM), or $1,25(OH)_2D_3$ (0.1 μM) for indicated time periods, and thereafter were harvested for RT-PCR or Western blotting.

Plasma osmolarity, ADH, aldosterone, and vitamin D measurements. Plasma was separated by centrifugation of blood at 4,000 rpm for 10 min. Plasma osmolarity was measured by the vapor pressure method. Commercial EIA kits were utilized to determine ADH (AVP EIA kit, Phoenix Europe, Karlsruhe, Germany) and $1,25(OH)_2D_3$ concentrations (IDS, Boldon, UK). The plasma aldosterone concentrations were determined using a commercial Radioimmunoassay kit (Demeditec, Kiel, Germany). All kits were used according to the manufacturer's instructions.

RNA extraction and real-time RT-PCR. Total RNA was isolated from human embryonic kidney (HEK293) cells or snap-frozen kidneys (5 kidneys for each condition) by using a QIA shredder and RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA were generated from 2 μg RNA by using random hexamer primers (Roche Diagnostics, Penzberg, Germany) and SuperScriptII reverse transcriptase (Invitrogen). To determine Klotho transcript levels, quantitative real-time PCR with the CFX96 real-time sys-

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Table 1. *Primer sequences used for RT-PCR*

Origin	Gene	Sequence
Human	*TBP*	5′-GCCCGAAACGCCGAATAT-3′
		5′-CCGTGGTTCGTGGCTCTCT-3′
	KLOTHO	5′-GTTTGTCTCAGGGACCACCAA-3′
		5′-CCACCCCATCCAGCTTGAT-3′
	AVPR1A	5′-GATGTGGTCTGTCTGGGATCC-3′
		5′-GATTACAGCAGCTATTCAAGGAACC-3′
	AVPR1B	5′-CTTCAGTGTCCAGATGTGGTCC-3′
		5′-TGCCCAAAAGCATAGAGATGG-3′
	AVPR2	5′-CCCTTTGTGCTACTCATGTTGC-3′
		5′-GCTCTGAGGACACGCTGCT-3′
	NR3C2	5′-ACTGACCAAGCTGCTGGACTC-3′
		5′-CTTCAGCGCATGGGACTCT-3′
Mouse	*Tbp*	5′-CACTCCTGCCACACCAGCTT-3′
		5′-TGGTCTTTAGGTCAAGTTTACAGCC-3′
	Klotho	5′-CCCTGTGACTTTGCTTGGG-3′
		5′-CCCACAGATAGACATTCGGGT-3′

tem (Bio-Rad Laboratories, München, Germany) was applied. The primers used for quantification of mRNA transcripts are listed in Table 1. PCR reactions were performed in a final mixture volume of 20 μl, containing 40 ng cDNA, 500 nM forward and reverse primers, and 10 μl Taq SYBG Green Supermix (Bio-Rad). The target genes were amplified with either three-step or two-step PCR. Three-step PCR involved 40 cycles, denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 70°C for 20 s. Two-step PCR involved 40 cycles, denaturation at 95°C for 10 s, and 20-s annealing and extension at 58°C. The relative gene expression was quantified to the amount of TATA-Box-binding-Protein (TBP) of each sample and calculated using the $\Delta\Delta C_t$ method as described earlier (26).

Western blotting. To determine Klotho protein abundance from hydrated and dehydrated mice, the kidneys were removed and immediately shock-frozen in liquid nitrogen. The renal tissues were then homogenized in RIPA lysis buffer (Cell Signaling Technology, Danvers, MA), containing 20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM sodium orthovanadate, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1% sodium deoxycholate, 1% NP-40, 1 μg/ml leupeptin, 1 mM PMSF (freshly added), and a protease inhibitor cocktail tablet (Complete mini, Roche, Mannheim, Germany) for each 10 ml lysis buffer (freshly added). The homogenates were incubated on ice for 20 min and then centrifuged at 11.000 *g*, 4°C for 20 min. The supernatant was removed and used for Western blotting. The protein lysate from HEK293 cells was achieved by using RIPA lysis buffer and followed by centrifugation as well. Total protein (90 μg) was separated by SDS-PAGE and thereafter transferred to nitrocellulose membranes (Whatman) and blocked in 5% nonfat dry milk/Tris-buffered saline Tween 20 (TBST) at room temperature for 1 h. Membranes were probed overnight at 4°C with polyclonal antibodies against Klotho (Abcam, Cambridge, MA or kindly provided by Akiko Saito, Kyowa Hakko Kirin) or an antibody against tubulin (Cell Signaling), diluted 1:1,000 in 5% milk/TSBT, followed by the incubation with horseradish peroxidase-labeled anti-rabbit secondary antibodies (1:5,000, Cell Signaling) or anti-rat secondary antibodies (1:2,000, Cell Signaling) for 1 h at room temperature. The bands were visualized with enhanced chemiluminescence (Amersham, Piscataway, NJ), according to the manufacturer's instructions. Densitometric analysis of Klotho and tubulin was performed using Quantity One software (Bio-Rad).

Statistics. Data are provided as means ± SE, and *n* represents the number of independent experiments. All data were tested for significance using ANOVA or a paired or unpaired Student *t*-test. The results with $P < 0.05$ were considered statistically significant.

RESULTS

Mice were either allowed access to tap water ad libitum or dehydrated for 36 h. Subsequently, the animals were lightly anesthetized with diethylether and ~50–200 μl of blood was withdrawn into heparinized capillaries for determina-

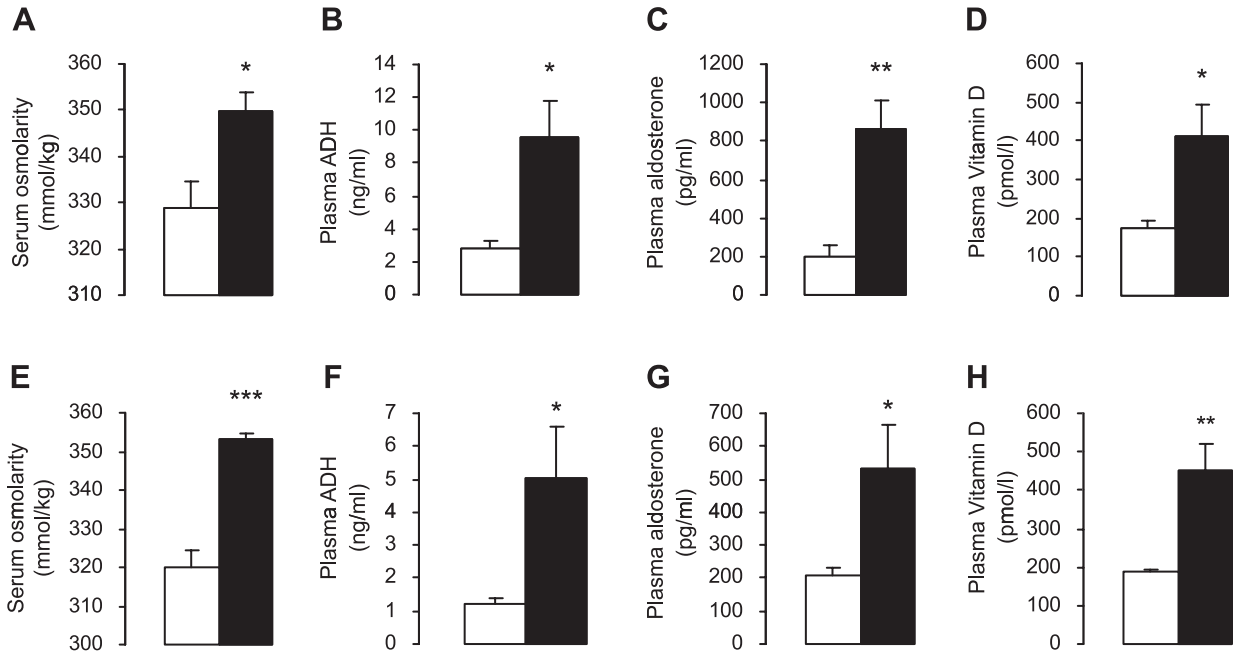

Fig. 1. Serum osmolarity, antidiuretic hormone (ADH), aldosterone, and $1,25(OH)_2D_3$ levels in hydrated and dehydrated wild-type mice. Values are means ± SE of serum osmolarity (*A* and *E*), plasma ADH concentration (*B* and *F*), plasma aldosterone concentration (*C* and *G*), and plasma $1,25(OH)_2D_3$ concentration (*D* and *H*) in hydrated (open bars) and 36-h-dehydrated (filled bars) female (*A–D*, $n = 5$ hydrated and 5 dehydrated mice) and male mice (*E–H*, $n = 5$ hydrated and 5 dehydrated mice). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant differences from the respective hydrated animals (ANOVA).

tion of plasma osmolarity and plasma hormone concentrations. As shown in Fig. 1, dehydration was followed by a significant increase in plasma osmolarity (Fig. 1, *A* and *E*), as well as in the plasma concentrations of ADH (Fig. 1, *B* and *F*) and aldosterone (Fig. 1, *C* and *G*). Moreover, dehydration led to a significant increase in 1,25 $(OH)_2D_3$ plasma concentration (Fig. 1, *D* and *H*). Similar alterations in plasma osmolarity and plasma hormone concentrations were observed in female (Fig. 1, *A–D*) and in male (Fig. 1, *E–H*) animals.

The animals were then euthanized by cervical dislocation in continued diethylether anesthesia, the abdomen was opened, and both kidneys were removed. Renal tissue was analyzed for Klotho transcript levels and protein abundance. To this end, total RNA was subsequently isolated from one kidney as well as protein from the other kidney. According to quantitative real-time PCR, dehydration was followed by a significant decrease in Klotho transcript levels (Fig. 2, *B* and *D*). According to Western blotting, dehydration further led to a significant reduction of Klotho protein expression (Fig. 2, *C* and *E*). Again, a similar decline of Klotho transcript levels and protein was observed in female (Fig. 2, *B* and *D*) and male (Fig. 2, *C* and *E*) animals.

To explore whether the observed increases in hormone levels could contribute to the observed alterations of Klotho expression, serum-deprived human embryonic kidney (HEK293) cells were treated with ADH or aldosterone. As illustrated in Fig. 3*B*, exposure of serum-deprived HEK293 cells to 50 nM ADH was followed by a gradual decline of Klotho transcription levels, an effect reaching statistical significance after a 4 h period of ADH exposure. The decline of Klotho transcript levels was paralleled by a decline of Klotho protein abundance, an effect reaching statistical significance after a 12-h period of ADH exposure (Fig. 4, *A* and *B*). As shown in Fig. 3*C*, treatment of serum-deprived HEK293 cells with aldosterone (1 μM) was again followed by a gradual decline of Klotho transcription levels, an effect reaching statistical significance after a 2-h period of aldosterone exposure. The decline of Klotho transcript levels was again paralleled by a decline of Klotho protein abundance, an effect reaching statistical significance after an 8-h period of aldosterone exposure (Fig. 3, *B* and *C*).

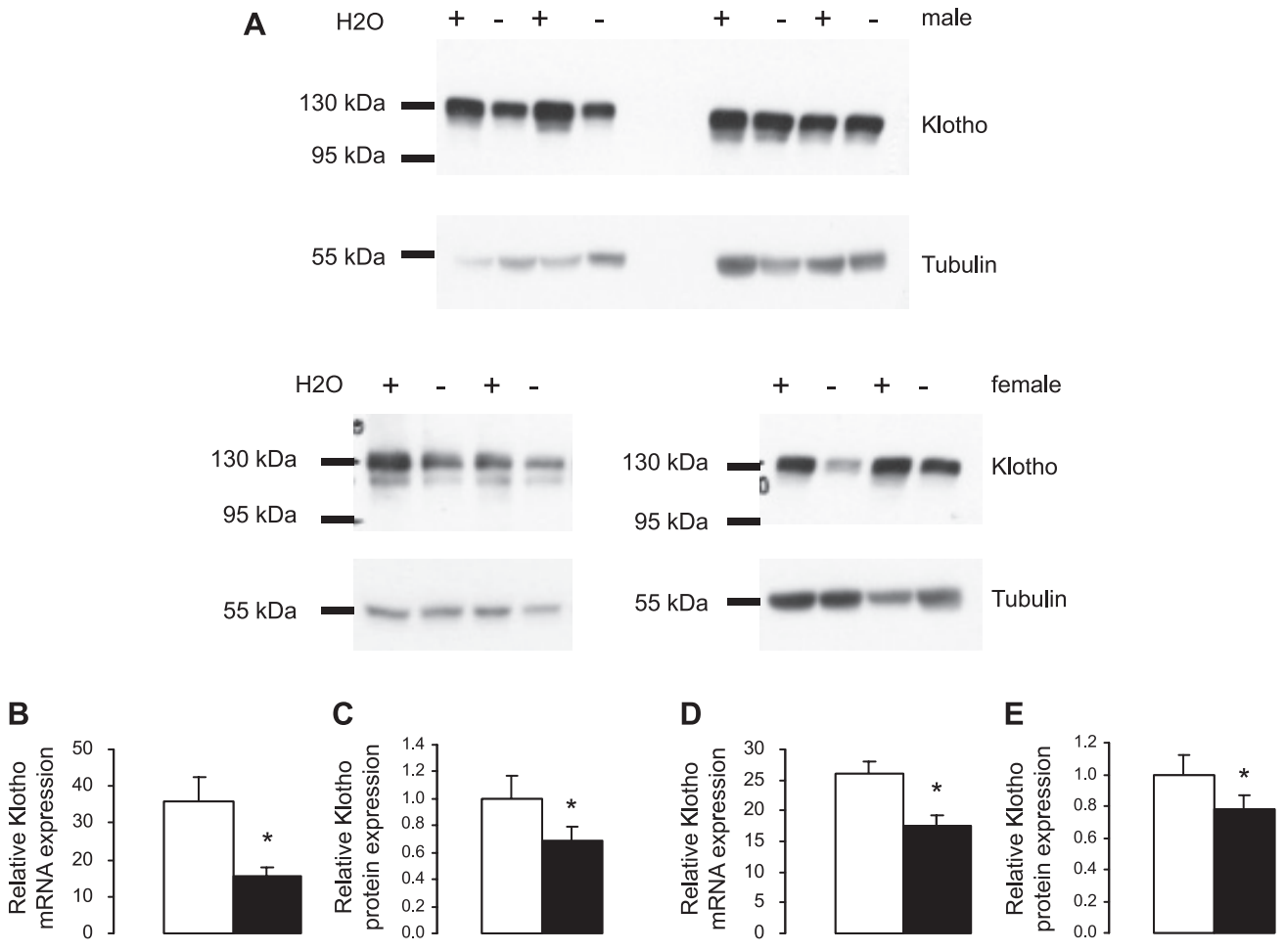

Fig. 2. Klotho expression in kidneys from hydrated and dehydrated wild-type mice. *A*: original Western blots demonstrating Klotho protein expression in kidneys from dehydrated female and male wild-type mice ($-H_2O$) and in kidneys from hydrated female and male wild-type mice ($+H_2O$). Tubulin was used as a loading control for Klotho protein expression. *B–E*: means ± SE of Klotho transcript levels as determined by quantitative real-time PCR and normalized to TATA box binding protein (TBP; *B* and *D*) and of Klotho protein abundance (*C* and *E*) in hydrated (open bars) and dehydrated (filled bars) female mice (*B* and *C*, $n = 4$ hydrated and 4 dehydrated mice) and male mice (*D* and *E*, $n = 4$ hydrated and 4 dehydrated mice). *$P < 0.05$, significant differences from the respective hydrated animals (unpaired Student's *t*-test).

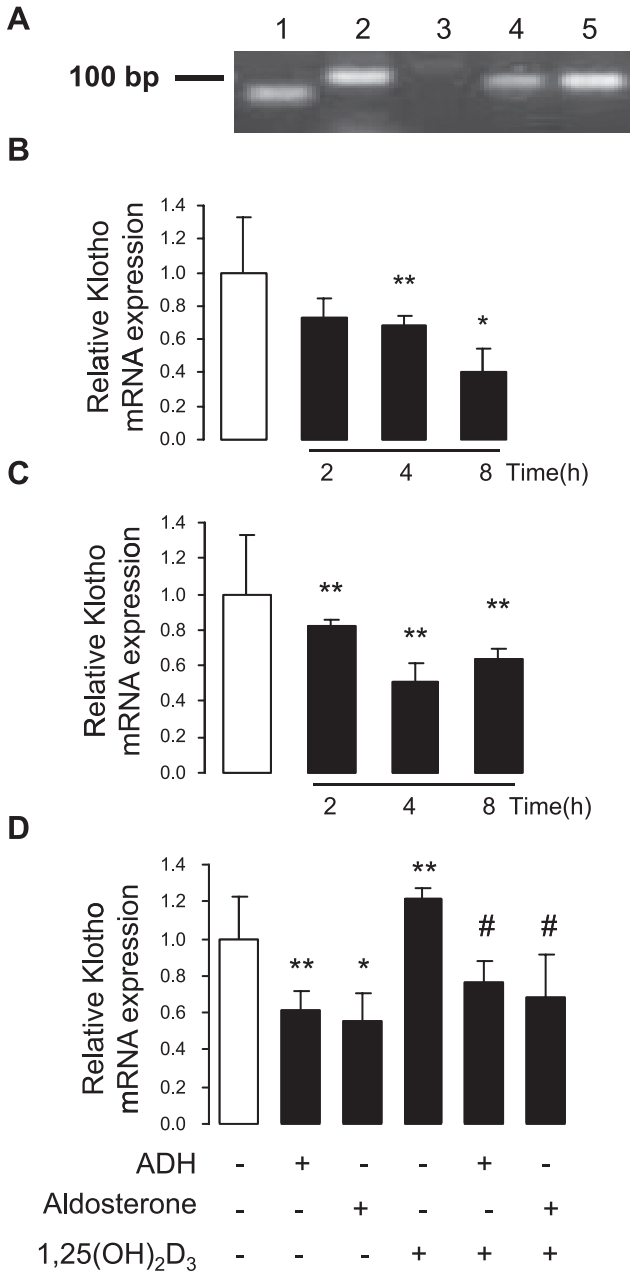

Fig. 3. Effect of ADH, aldosterone, and $1,25(OH)_2D_3$ on Klotho transcript levels in HEK293 cells. *A*: PCR showing transcript abundance of TBP (*lane 1*), ADH receptors AVPR1A (*lane 2*), AVPR1B (*lane 3*), and AVPR2 (*lane 4*) as well as mineralocorticoid receptor NR3C2 (*lane 5*) in HEK293 cells. *B–D*: means ± SE ($n = 3$ each group) of Klotho transcript levels, determined by quantitative real-time PCR and normalized to TBP in untreated HEK293 cells (open bars) or in HEK293 cells treated with 50 nM ADH (*B*), 1 μM aldosterone (*C*), or ADH or aldosterone (*D*) with or without additional treatment with $1,25(OH)_2D_3$ (0.1 μM) for the indicated time periods (filled bars). $*P < 0.05$, $**P < 0.01$, significant differences from the respective value without hormones (ANOVA). $\#P < 0.05$, significant differences from the respective value in the presence of $1,25(OH)_2D_3$ only (ANOVA).

Additional experiments were performed to explore whether the effects of ADH and aldosterone were influenced by the additional administration of $1,25(OH)_2D_3$ (0.1 μM). As illustrated in Fig. 3*D*, the administration of $1,25(OH)_2D_3$ tended to increase Klotho transcript levels within 24 h (Fig. 3*D*). However, the presence of $1,25(OH)_2D_3$ did not significantly blunt the significant decline of Klotho transcript levels following treatment of the cells with either ADH (50 nM) or aldosterone (1 μM) for 8 h.

Further experiments have been performed to define the time course of declining Klotho protein abundance following treatment of HEK293 cells with either 50 nM ADH (Fig. 4, *A* and *B*) or 1 μM aldosterone (Fig. 4, *A* and *C*). Disruption of formation of new Klotho by treatment of HEK293 cells with actinomycin (1 μg/ml) resulted in a significant decline of Klotho protein by 44 ± 16% ($n = 4$) within 24 h (data not shown).

DISCUSSION

The present observations reveal that dehydration leads to marked downregulation of Klotho transcription and protein expression. Dehydration was followed by the expected increase in plasma osmolarity and stimulation of ADH and aldosterone release. More importantly, the present observations disclose that the two hormones are potent negative regulators of Klotho expression. The downregulation of Klotho transcript levels and protein abundance by ADH and aldosterone contributes to the decline of Klotho transcript levels and protein abundance during dehydration. In a recent study, angiotensin II has been shown to downregulate Klotho expression (24). As angiotensin II is increased during dehydration (4), enhanced release of angiotensin II could well contribute to the decline of Klotho expression following dehydration.

Klotho has previously been shown to be downregulated by reactive oxygen species and NF-κB (31). As formation of reactive oxygen species and the activation of NF-κB may both result from mineralocorticoid receptor activation (6, 8, 27), they could well contribute to or even account for the observed downregulation of Klotho.

Klotho expression is further downregulated by the soluble amyloid precursor protein (23) sirolimus (10), hyperparathyroidism (1), TNF, and INF-γ (39). TNF and IFN-γ are effective by stimulating the expression of inducible nitric oxide synthase (iNOS) with subsequent NO production (39). Klotho expression is upregulated by vitamin D (11), calcitonin gene-related peptide (44), peroxisome proliferator-activated receptor-γ (43), and epidermal growth factor (2). Uremia has been reported to increase (12) or decrease (17, 42) Klotho expression.

Dehydration further leads to an increase in $1,25(OH)_2D_3$ plasma concentrations, an effect which may be secondary to downregulation of Klotho. Klotho is known to inhibit 1α-hydroxylase and thus to decrease $1,25(OH)_2D_3$ production (29, 40, 41). Accordingly, Klotho deficiency is followed by excessive $1,25(OH)_2D_3$ formation (29, 40, 41). $1,25(OH)_2D_3$ in turn stimulates Klotho expression (11). Thus enhanced $1,25(OH)_2D_3$ plasma levels in dehydrated animals does not contribute to but rather attenuates the downregulation of Klotho expression.

The hormone $1,25(OH)_2D_3$ stimulates intestinal and renal Ca^{2+} and phosphate transport (28, 30). Thus excess $1,25(OH)_2D_3$ contributes to the increase in plasma Ca^{2+} (18) and phosphate (30) concentration in Klotho-deficient mice (29, 40, 41). Hyperphosphatemia is considered to be a decisive determinant of life span (19). For instance, $CaHPO_4$ precipi-

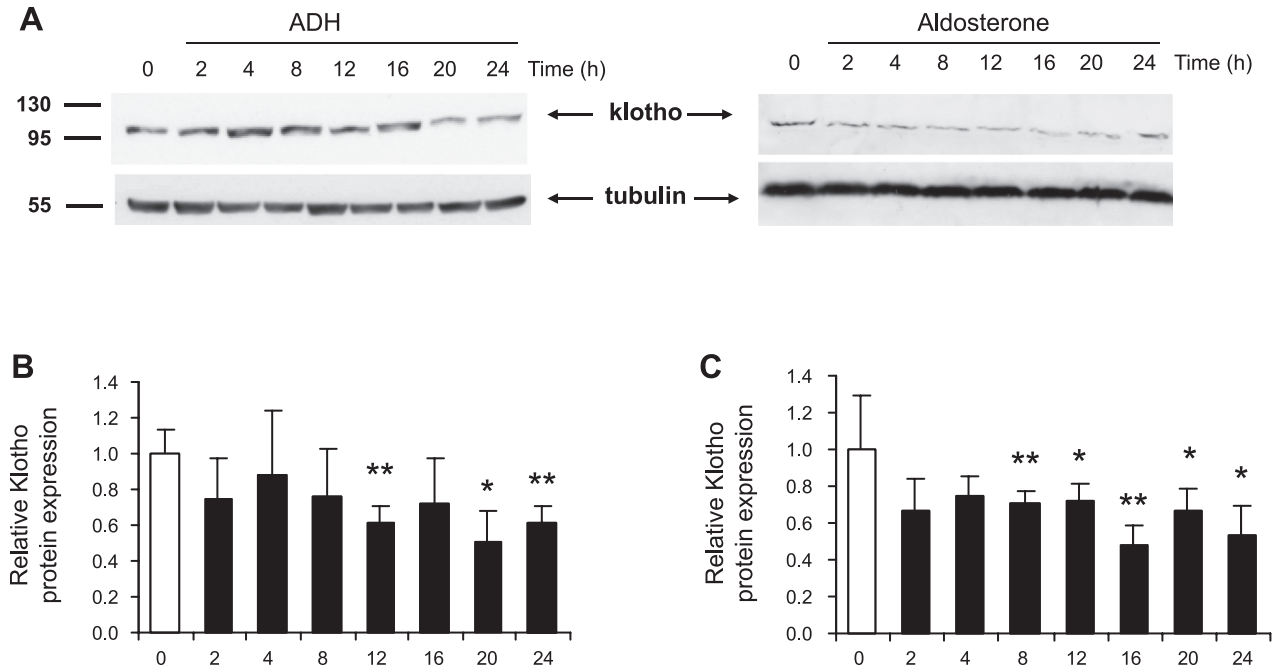

Fig. 4. Effect of ADH and aldosterone on Klotho protein expression in HEK293 cells. *A*: original Western blot of Klotho and tubulin protein expression in HEK293 cells treated with ADH (*left*) or aldosterone (*right*). *B* and *C*: means ± SE ($n = 4$ each group) of Klotho protein abundance according to Western blotting following incubation of HEK293 cells without (open bars) or with (filled bars) presence of 50 nM ADH (*B*) or 1 μM aldosterone (*C*). *$P < 0.05$, **$P < 0.01$, significant differences from the respective protein abundance without hormones (ANOVA).

tations during hyperphosphatemia could contribute to vascular calcification. Accordingly, vitamin D restriction reverses the untoward effect of Klotho deficiency on life span (40), growth deficit (40), and erythrocyte survival (16).

The present observations may shed new light on the impact of volume depletion and hyperaldosteronism in Klotho hypomorphic mice (7). The life span of these mice may be substantially extended by saline (7). In theory, hyperaldosteronism of these mice could have led to downregulation of residual Klotho expression, and the decline of plasma aldosterone levels following treatment with saline could have augmented residual Klotho expression in these mice. Alternatively, saline may have been effective more directly by counteracting extracellular volume depletion of the mice.

Dehydration is relatively common in the elderly (32, 36). Decreased water intake or plasma hyperosmolarity impacts body weight (33, 34), development of diabetes (35), and early frailty, incident disability, and mortality (37). The present observations may provide a mechanistic link between dehydration and development of frailty and reduced life span.

In conclusion, dehydration leads to downregulation of Klotho. The effect may accelerate the development of age-related disorders.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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