NH₄Cl Treatment Prevents Tissue Calcification in Klotho Deficiency

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ABSTRACT

Klotho, a cofactor in suppressing 1,25(OH)₂D₃ formation, is a powerful regulator of mineral metabolism. Klotho-hypomorphic mice (kl/kl) exhibit excessive plasma 1,25(OH)₂D₃, Ca²⁺, and phosphate concentrations, severe tissue calcification, volume depletion with hyperaldosteronism, and early death. Calcification is paralleled by overexpression of osteoinductive transcription factor Runx2/Cbfa1, Alpl, and senescence-associated molecules Tgfb1, Pai-1, p21, and Glb1. Here, we show that NH₄Cl treatment in drinking water (0.28 M) prevented soft tissue and vascular calcification and increased the life span of kl/kl mice. 12-fold in males and 4-fold in females without significantly affecting extracellular pH or plasma concentrations of 1,25(OH)₂D₃, Ca²⁺, and phosphate. NH₄Cl treatment significantly decreased plasma aldosterone and antidiuretic hormone concentrations and reversed the increase of Runx2/Cbfa1, Alpl, Tgfb1, Pai-1, p21, and Glb1 expression in aorta of kl/kl mice. Similarly, in primary human aortic smooth muscle cells (HAoSMCs), NH₄Cl treatment reduced phosphate-induced mRNA expression of RUNX2/CBFA1, ALPL, and senescence-associated molecules. In both kl/kl mice and phosphate-treated HAoSMCs, levels of osmosensitive transcription factor NFAT5 and NFAT5-downstream mediator SOX9 were higher than in controls and decreased after NH₄Cl treatment. Overexpression of NFAT5 in HAoSMCs mimicked the effect of phosphate and abrogated the effect of NH₄Cl on SOX9, RUNX2/CBFA1, and ALPL mRNA expression. TGFβ1 treatment of HAoSMCs upregulated NFAT5 expression and prevented the decrease of phosphate-induced NFAT5 expression after NH₄Cl treatment. In conclusion, NH₄Cl treatment prevents tissue calcification, reduces vascular senescence, and extends survival of klotho-hypomorphic mice. The effects of NH₄Cl on vascular osteoinduction involve decrease of TGFβ1 and inhibition of NFAT5-dependent osteochondrogenic signaling.


Klotho is expressed mainly in the kidney, parathyroid glands, and choroid plexus.¹ The extracellular domain of the transmembrane protein may be cleaved off and released into blood or cerebrospinal fluid.¹ Klotho participates in the inhibition of 25-hydroxyvitamin D₃ 1-α-hydroxylase and thus limits the production of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃).¹ Moreover, klotho influences the mineral metabolism more directly by upregulating the renal epithelial Ca²⁺ channel² and downregulating renal tubular phosphate transport.³ Klotho further stimulates Na⁺/K⁺-ATPase activity¹ and lack of klotho leads to extracellular volume depletion with secondary increase of antidiuretic hormone (ADH) and aldosterone release.⁴ Klotho-hypomorphic mice (kl/kl) carrying a disruption in the promoter sequence

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of the klotho gene suffer from severe tissue calcification, as well as a wide variety of age-related disorders. Accordingly, the life span is severely reduced in klotho-hypomorphic mice, and it is substantially increased in mice overexpressing klotho. Klotho has similarly been suggested as a factor influencing the life span of humans.

Vascular calcification is a major pathophysiologic mechanism limiting the life span of patients with CKD. Mineral bone disorder is closely associated with survival of patients with CKD, which is determined by vascular calcification. In patients with CKD, the impaired phosphate excretion is compounded by decreased klotho levels. Along those lines, a klotho gene variant is associated with survival in dialysis patients.

Vascular calcification is a complex active process involving transition of vascular smooth muscle cells into an osteogenic and chondrogenic phenotype. Reprogramming of vascular cells into osteogenic phenotypes is triggered by increased extracellular phosphate concentrations. The excessive tissue calcification of kl/kl mice and patients with CKD is associated with osteogenic differentiation of vascular smooth muscle cells as well as increased expression of the osteogenic transcription factor Core binding factor α-1 (Cbfa1), which triggers transformation of mesenchymal cells into osteoblast- and chondroblast-like cells. Cbfa1 stimulates the expression of alkaline phosphatase, which fosters calcification by degradation of the inhibitor pyrophosphate. Osteoinductive reprogramming of vascular smooth muscle cells is stimulated by aldosterone and fostered by hyperaldosteronism of kl/kl mice. Phosphate-induced osteoinductive reprogramming is closely intertwined with cellular senescence.

Acidosis is a common complication in klotho-hypomorphic mice and patients with CKD. The pH is a major determinant of the solubility of hydroxyapatite and low pH prevents the precipitation of calcium and phosphate by increasing their solubility. Moreover, acidosis decreases calcification by downregulation of the type III sodium-dependent phosphate transporter Pit1. Renal tubular phosphate transport is decreased by acidosis and, at least in theory, acidosis could foster renal phosphate elimination, thus countering hyperphosphatemia and calcification. However, acidosis has previously been shown to increase plasma phosphate concentration. Conflicting observations have been reported on the effect of acidosis on chronic renal disease. On the one hand, metabolic acidosis has been reported to slow the progression of renal disease in rats. On the other hand, acidosis has been shown to worsen experimental CKD, especially cystic kidney disease. Moreover, administration of alkali retards the progression of CKD in rodents. In humans with CKD, acidosis is associated with worse kidney function, whereas the administration of bicarbonate, as shown in several studies, is associated with the slowing of progressive kidney disease. Acidosis can be induced by dietary NH4Cl. NH4+ may further dissociate to H+ and NH3, which easily crosses membranes, thus entering cells and cellular compartments. In acidic compartments, NH3 binds H+ and is thus trapped as NH4+. As a result, NH4Cl alkalizes acidic cellular compartments and swells cells. Cell swelling downregulates the cell volume–sensitive transcription factor Tonicity-Responsive Enhancer Binding Protein or nuclear factor of activated T cells (NFAT5), which has been implicated in the regulation of Cbfa1 expression, a function involving the transcription factor Sox9. Beyond that, lysosomal alkalinization prevents maturation of several proteins including TGFβ1, which in turn is known to upregulate NFAT5 and participate in osteogenic signaling.

This study explored the effect of NH4Cl treatment on the phenotype of klotho-hypomorphic mice.

RESULTS

Effect of NH4Cl Treatment on Phenotype, Body Weight, and Survival of kl/kl Mice

As illustrated in Figure 1A, klotho-hypomorphic mice (kl/kl) suffer from a severe growth deficit compared with corresponding wild-type mice. The body weight of kl/kl mice was significantly lower than the body weight of wild-type mice (Figure 1B). After NH4Cl treatment (0.28 M in tap water), the body weight of kl/kl mice was significantly increased, approaching the body weight of wild-type mice. Applying an equimolar dose of NaCl (0.28 M in tap water) did not significantly modify the body weight of kl/kl mice. NH4Cl treatment further influenced the survival of kl/kl mice (Figure 1C). Whereas none of the untreated male kl/kl mice survived >95 days, all treated male kl/kl mice survived >729 days. Similarly, NH4Cl treatment increased the average life span of female kl/kl mice significantly from 84±4 days (n=9) to 355±46 days (n=7). Male kl/kl mice even regained fertility after NH4Cl treatment (Table 1). Treatment with NaCl (0.28 M in tap water) instead of NH4Cl had only a modest effect on the lifespan of kl/kl mice. After 87 days of NaCl treatment, four of nine animals survived.

Effect of NH4Cl Treatment on Plasma NH3, CaHPO4, and Hormone Concentrations

Plasma ammonia concentration tended to be slightly higher in untreated kl/kl mice than in untreated wild-type mice; however, the difference did not reach statistical significance (Figure 2A). Treatment with NH4Cl increased plasma ammonia concentration in both wild-type and kl/kl mice. Plasma phosphate (Figure 2B) and Ca2+ (Figure 2C) concentrations were significantly higher in kl/kl mice than in wild-type mice, whereby the differences were not significantly affected by treatment with NH4Cl. Similarly, plasma 1,25(OH)2D3 (Figure 2D) concentration was significantly higher in kl/kl mice than in wild-type mice, a difference that again was not significantly
affected by NH₄Cl treatment. C-terminal fibroblast growth factor 23 (FGF23) (Figure 2E) and intact FGF23 (Figure 2F) were significantly higher in kl/kl mice than in wild-type mice. NH₄Cl treatment significantly decreased only plasma levels of intact FGF23 (Figure 2F). Plasma parathyroid hormone (PTH) concentrations were significantly higher in kl/kl mice than in wild-type mice. NH₄Cl treatment did not significantly modify blood ionized Ca²⁺ in kl/kl mice or wild-type mice (Figure 3F). Blood Cl⁻ levels signiﬁcantly increased in kl/kl mice but not in wild-type mice (Figure 3G). Blood pH levels were signiﬁcantly lower in untreated kl/kl mice than in untreated wild-type mice. When treated with NH₄Cl, K⁺ levels signiﬁcantly increased in kl/kl mice but not in wild-type mice (Figure 3H). There were no differences in blood Na⁺ levels among the different groups (Figure 3E), but plasma K⁺ levels were signiﬁcantly lower in untreated kl/kl mice than in untreated wild-type mice. When treated with NH₄Cl, K⁺ levels signiﬁcantly increased in kl/kl mice but not in wild-type mice (Figure 3F). Blood Cl⁻ levels also increased in both genotypes after NH₄Cl treatment; however, the effect reached statistical signiﬁcance only in kl/kl mice (Figure 3G). Blood ionized Ca²⁺ was signiﬁcantly higher in untreated kl/kl mice than in untreated wild-type mice. NH₄Cl treatment did not signiﬁcantly modify blood ionized Ca²⁺ in kl/kl mice or wild-type mice (Figure 3H).

Effect of NH₄Cl Treatment on Tissue Calcification of kl/kl Mice
To further define the mechanisms contributing to or accounting for the effects of NH₄Cl on survival of kl/kl mice, tissues from untreated and NH₄Cl-treated kl/kl mice were subjected to histologic analysis. As illustrated in Figure 4, extensive calciﬁcations were observed in trachea, lung, kidney, stomach, intestine, and vascular tissue of kl/kl mice. NH₄Cl treatment strongly reduced the tissue calcification of kl/kl mice.

Effect of NH₄Cl Treatment on Procalcification Reprogramming in Aortic Tissues of kl/kl Mice
NH₄Cl treatment could have reduced vascular calcification in kl/kl mice by inhibition of the active procalcification reprogramming in vascular tissue. As illustrated in Figure 5, the transcript levels of osteogenic transcription factor Cbfα1 and of
alkaline phosphatase (Alpl) were significantly higher in aortic tissue of kl/kl mice than in aortic tissue of wild-type mice, differences that were significantly blunted by NH4Cl treatment (Figure 5, C and D). The alterations of Cbfa1 and Alpl transcript levels were paralleled by similar alterations of Nfat5 and of Sox9 transcript levels, which were significantly higher in aortic tissue from kl/kl mice than in aortic tissue from wild-type mice. NH4Cl treatment again decreased Nfat5 and Sox9 transcript levels in aortic tissue of kl/kl mice (Figure 5, A and B).

**Effect of NH4Cl Treatment on Vascular Senescence in kl/kl Mice and in HAoSMCs**

Because vascular calcification is fostered by cellular senescence of vascular smooth muscle cells, the senescence-associated molecules Tgfβ1, Pai-1, p21, and SA-β-galactosidase (Glb1) were measured in aortic tissue of kl/kl mice and in HAoSMCs. As illustrated in Figure 7, A–D, the transcript levels of Tgfβ1, Pai-1, p21, and Glb1 were significantly higher in aortic tissue of kl/kl mice than in aortic tissue of wild-type mice. NH4Cl treatment significantly suppressed the expression of Tgfβ1, Pai-1, p21, and Glb1 in the aortic tissue of kl/kl mice and virtually cotreatment with 500 μM NH4Cl. Treatment of HAoSMCs with β-glycerophosphate significantly increased Nfat5, Sox9, Cbfa1, and Alpl mRNA expression (Figure 6), effects that were significantly suppressed by cotreatment with NH4Cl.

**Figure 2.** Plasma NH4+, phosphate, Ca2+, 1,25(OH)2D3, FGF23, intact FGF23 (jFGF23), PTH, ADH, and aldosterone concentrations of wild-type mice and kl/kl mice with or without NH4Cl treatment. Arithmetic means±SEM (n=5–8) of plasma ammonia (A), phosphate (B), Ca2+ (C), 1,25(OH)2D3 (D), FGF23 (C-Term) (E), intact FGF23 (F), PTH (G), ADH (H), and aldosterone (I) concentrations of wild-type mice (WT, white bars) and klotho-hypomorphic mice (kl/kl, black bars) without (control, left bars) and with (NH4Cl, right bars) NH4Cl treatment (0.28 M in tap water). *P<0.05 and **P<0.01, statistically significant differences from respective wild-type mice; †P<0.05 and ††P<0.01, statistically significant differences from respective untreated mice.
abrogated the differences in aortic senescence-associated molecules transcript levels between kl/kl mice and wild-type mice. In HAoSMCs, phosphate treatment induced the mRNA expression of TGFB1, PAI-1, P21, and GLB1 (Figure 7, E–H), effects again reversed by cotreatment with NH4Cl.

Effect of NFAT5 Overexpression on Osteoinductive Signaling and Effect of TGFB1 on NFAT5 Expression in HAoSMCs

Additional experiments were performed to test whether the regulation of SOX9, CBFA1, and ALPL mRNA levels in empty vector–transfected SOX9, CBFA1, and ALPL mRNA levels was followed by marked and significant increase of SOX9, CBFA1, and ALPL mRNA levels with NFAT5 overexpression in HAoSMCs. To this end, HAoSMCs were transfected for 48 hours with or without subsequent encoding NFAT5 with or without control (left bars) and with NH4Cl (right bars). NH4Cl treatment caused an effect completely reversed by additional cotreatment with NH4Cl.
NH4Cl interferes with aortic osteoinductive signaling in kl/kl mice. Arithmetic means±SEM (n=10; arbitrary units [a.u.]) of NFAT5 (A), Sox9 (B), Cbfa1 (C), and ALPL (D) relative mRNA expression (exp.) in aortic tissue of wild-type mice (WT, white bars) and klotho-hypomorphic mice (kl/kl, black bars), without (control, left bars) and with (NH4Cl, right bars) NH4Cl treatment (0.28 M in tap water). *P<0.05, **P<0.01, and ***P<0.001, statistically significant differences from respective wild-type mice; #P<0.05 and ###P<0.001 indicate statistically significant differences from respective untreated mice.

Renal Function
Average kidney weight was 5.8±0.2 mg/g body wt in untreated wild-type mice and 5.7±0.2 mg/g body wt in untreated kl/kl mice. NH4Cl treatment increased the kidney mass significantly (P<0.001) to 7.1±0.2 mg/g body w in wild-type mice and to 7.0±0.1 mg/g body wt in kl/kl mice. Before and after NH4Cl treatment the ratio of kidney mass-to-body weight was similar in wild-type mice and kl/kl mice. Measurements in metabolic cages were performed in wild-type mice, NH4Cl-treated wild-type mice, and NH4Cl-treated kl/kl mice. Because untreated kl/kl mice do not tolerate this procedure, the treatment was stopped at the age of 7 weeks in a fourth group of NH4Cl-treated kl/kl mice. After a 3-week washout phase, the animals were housed in the metabolic cages. According to the measurements (Supplemental Figures 1–3), body weight, food and fluid intake, urinary volume and pH, fecal mass, plasma creatinine concentration, creatinine excretion, creatinine clearance, plasma protein, plasma albumin, urinary albumin excretion, plasma cystatin C, urinary cystatin C excretion, urinary urea excretion, and urinary ammonia excretion were similar in kl/kl mice and wild-type mice. Plasma urea concentration was, however, significantly higher and plasma ammonia concentration tended to be higher in the previously NH4Cl-treated kl/kl mice than in wild-type mice. In both kl/kl mice and wild-type mice, NH4Cl treatment significantly decreased urinary pH, and significantly increased plasma ammonia concentration, urinary urea excretion, urinary ammonia excretion, and, in wild-type mice, plasma urea concentration. All other measured parameters were not significantly modified by the NH4Cl treatment.

DISCUSSION
Our observations reveal a profound protective effect of NH4Cl treatment on the phenotype of the kl/kl mouse. The NH4Cl treatment abolished the growth deficit and extended the life...
NH₄Cl interferes with vascular senescence in kl/kl mice and in phosphate-treated HAoSMCs. (A–D) Arithmetic means±SEM (n=10; arbitrary units [a.u.]) of Tgfb1 (A), Pai-1 (B), p21 (C), and Glb1 (D) relative mRNA expression (exp.) in aortic tissue of wild-type mice (WT, white bars) and klotho-hypomorphic mice (kl/kl, black bars) without (control [CTR], left bars) or with (NH₄Cl, right bars) NH₄Cl treatment (0.28 M in tap water). *P<0.05, **P<0.01, and ***P<0.001, statistically significant difference from respective untreated mice; #P<0.05, ##P<0.01, and ###P<0.001, statistically significant difference from respective wild-type mice. (E–H) Arithmetic means±SEM (n=6; arbitrary units) of TGFβ1 (E), PAI-1 (F), p21 (G), and GLB1 (H) relative mRNA expression in untreated HAoSMCs (control, white bars) and in HAoSMCs after 24 hours of treatment with 2 mM β-glycerophosphate alone (Pi, black bars) or with 2 mM β-glycerophosphate and 500 μM NH₄Cl (Pi+NH₄Cl, gray bars). *P<0.05, **P<0.01, and ***P<0.001, statistically significant differences from untreated HAoSMCs; #P<0.05, ##P<0.01, and ###P<0.001, statistically significant differences from HAoSMCs treated with 2 mM β-glycerophosphate alone.

span >12-fold in male mice and >4-fold in female mice. Thus, NH₄Cl dramatically slowed the aging of kl/kl mice. Male kl/kl mice even became fertile (Table 1). The effect was paralleled by and at least in part due to a dramatic decrease in tissue calcification. Vascular calcification is a hallmark of aging,57-59 It is triggered by increased extracellular phosphate concentration,14 a predictor of mortality.60 NH₄Cl treatment inhibits tissue calcification in kl/kl mice without appreciably affecting plasma 1,25(OH)₂D₃, Ca²⁺, and phosphate concentrations. In theory, the inhibition of calcification could have resulted from extracellular fluid acidification, which is known to inhibit calcification in uremic rats.28,32,33 However, according to Figure 3A, NH₄Cl treatment did not significantly aggravate the existing calcification of the kl/kl mice. As revealed by this study, the untreated kl/kl mice suffer from respiratory acidosis most likely resulting from the severe lung emphysema in untreated kl/kl mice. NH₄Cl imposes a metabolic acidosis but by the same token prevents the development of lung emphysema (Figure 4). As a result, the respiratory acidosis is replaced by metabolic acidosis with little change of extracellular pH. Thus, tissue acidosis may contribute to but hardly accounts for the effect of NH₄Cl on tissue calcification and survival of kl/kl mice. The preexisting acidosis would have been expected to counteract calcification. However, the respiratory acidosis is expected to develop only after birth in parallel to the development of lung emphysema. In later stages, the excessive osteogenic signaling obviously overrides the inhibiting effect of acidosis, which cannot prevent premature aging and dramatic extraskeletal calcification. According to pH measurements in the media (Supplemental Methods), the in vitro effect of NH₄Cl on osteogenic signaling was similarly not explained by acidosis. In view of the pK of NH₃/NH₄⁺ of 8.9,61 the addition of 500 μM NH₄Cl is not expected to modify the pH in a well buffered solution.

NH₄Cl treatment further reverses the increased ADH release and hyperaldosteronism, which have previously been observed in kl/kl mice.4 The decrease of plasma aldosterone concentration after NH₄Cl treatment could have contributed to the decrease of vascular calcification, because aldosterone stimulates vascular osteoinduction, an effect at least in part due to upregulation of Pit1 expression.16 However, the survival benefit of aldosterone receptor blockade by spironolactone is only modest in kl/kl mice.16 Thus, reversal of hyperaldosteronism presumably contributes to, but does not fully account for, the spectacular survival benefit and phenotypic recovery of kl/kl mice by NH₄Cl treatment. In wild-type mice, the plasma aldosterone and ADH concentrations were low and not significantly modified by NH₄Cl treatment. Thus, NH₄Cl intake reverses the severe dehydration of kl/kl mice, but does not significantly modify volume regulation of euvolemic wild-type mice. To test whether NH₄Cl exerts its effect on the lifespan of kl/kl mice only by extracellular fluid expansion, we added controls.
receiving an equimolar aqueous solution of NaCl instead of NH4Cl. The effect of the NaCl treatment on the life span of the mice was only modest.

NH4Cl treatment did not significantly modify the plasma concentration of the C-terminal FGF23 fragment but significantly decreased the plasma concentrations of intact FGF23. In theory, the effect of NH4Cl treatment on the intact FGF23 levels could have contributed to the extended life span of kl/kl mice. However, according to earlier observations,62 the growth deficit and shortened life span of klotho-deficient mice is not significantly modified by additional knockout of FGF23.

NH4Cl counteracts osteoinductive signaling under high-phosphate conditions. Both in vivo and in vitro, NH4Cl reduces expression of Cbfal, a transcription factor decisive for the stimulation of osteoblastic differentiation.18 Cbfal stimulates the expression of alkaline phosphatase Alp,63 which in turn degrades pyrophosphate and thus fosters precipitation of calcium phosphate. The vascular osteoinduction is closely associated with vascular smooth muscle cell senescence,21 a key factor in vascular aging and injury.64 NH4Cl treatment blunted the expression of senescence indicators Pai-1, p21, and Gli1.65

Excessive extracellular phosphate concentration promotes Tgfβ1 production.56 Tgfβ1 stimulates cellular senescence, thus contributing to aging and vascular osteoinduction.66 Disruption of Tgfβ1–Pai-1 signaling ameliorates vascular calcification.67 We observed enhanced Tgfβ1 mRNA expression in untreated kl/kl mice and in HAOsMCs after phosphate treatment. The increased Tgfβ1 transcript levels were significantly decreased by NH4Cl treatment. NH4Cl has previously been shown to hinder the maturation of Tgfβ1.51

According to our observations, expression of Nfat5 and Sox9 is upregulated in kl/kl mice and decreased by NH4Cl treatment. Nfat5 in turn upregulates the transcript levels of Cbfal and Alpl. Nfat5 further upregulates the transcript levels of Sox9, a downstream mediator of Nfat5-induced Cbfal expression.50 Sox9 is involved in the chondrogenic gene expression in vascular smooth muscle cells.68 Sox9 is upregulated in vascular tissue of uremic rats.69 The effects of β-glycerophosphate are mimicked and those of NH4Cl are abolished by Nfat5 overexpression. Nfat5 thus contributes to the detrimental vascular effects of excessive phosphate and their inhibition by NH4Cl. Further experiments in HAOsMCs

Figure 8. The effect of phosphate-induced osteoinductive signaling is mimicked and the effect of NH4Cl is blunted by NFAT5 overexpression in HAOsMCs. (A–D) Arithmetic means±SEM (n=6; arbitrary units [a.u.]) of NFAT5 (A), Sox9 (B), CBFA1 (C), and ALPL (D) relative mRNA expression in HAOsMCs transfected for 48 hours with empty vector (white bar) or with a construct encoding human NFAT5 (black bar) without (control) or with treatment for 24 hours with 2 mM β-glycerophosphate alone (Pi) or with 2 mM β-glycerophosphate and 500 μM NH4Cl (Pi+NH4Cl). *P<0.01, **P<0.01, and ***P<0.001, indicates statistically significant differences from empty vector–transfected untreated HAOsMCs (ANOVA); #P<0.05, ##P<0.01, ###P<0.001, statistically significant difference from empty vector–transfected HAOsMCs treated with 2 mM β-glycerophosphate alone (ANOVA); $P<0.05, $$$P<0.01, and $$$$P<0.001, statistically significant difference from respective empty vector–transfected HAOsMCs (t test). (E) Arithmetic means±SEM (n=6; arbitrary units) of NFAT5 relative mRNA expression in untreated HAOsMCs (control) and in HAOsMCs after 24 hours of treatment with 10 ng/ml of human TGFβ1 alone, with 2 mM β-glycerophosphate alone (Pi), with 2 mM β-glycerophosphate and 500 μM NH4Cl (Pi+NH4Cl) and with 2 mM β-glycerophosphate, 500 μM NH4Cl, and 10 ng/ml of human TGFβ1 (Pi+NH4Cl+TGFβ1). *P<0.01 and $$$P<0.001, statistically significant differences from untreated HAOsMCs (ANOVA); #P<0.05 and ###P<0.001, statistically significant differences from HAoSMCs treated with 2 mM β-glycerophosphate and 500 μM NH4Cl (ANOVA).
showed that NH₄Cl was not able to reduce the phosphate-induced increase of NFAT5 expression in the presence of active TGFβ1. Accordingly, NH₄Cl is at least partially effective by downregulating TGFβ1. By alkalinizing lysosomes, NH₄Cl presumably disrupts the processing of TGFβ1 and thus its detrimental effects on NFAT5, CBEA1, and ALPL expression.

Excessive ammonia concentrations are toxic particularly to the brain and impaired ammonia detoxification by the liver is a major cause of hepatic encephalopathy.⁴⁵–⁴⁷ Apparently, toxic ammonia concentrations have not been reached by the treatment, because the treated mice lived an almost normal life span in contrast with the severe reduction of life span in untreated mice. Moreover, long-term treatment of wild-type mice did not lead to obvious alterations of behavior. Apparently, an increase of ammonia to toxic levels was prevented by urea formation in the liver, leading to the respective increase of renal urea excretion.

Similar to vascular calcification in \( kl/kl \) mice,¹⁷ vascular calcification in patients with CKD is an active process as part of the mineral bone disorder.¹⁷,²⁰ Vascular calcification increases the risk for cardiovascular events,²¹ which are the leading cause of death in patients with CKD.²² The impaired renal phosphate elimination in CKD is followed by hyperphosphatemia, which is paralleled by strong reduction of klotho expression¹⁷ and increased expression of osteochondrogenic reprogramming markers²³ including CBFA1. Prevention of osteogenic reprogramming in vascular tissue could thus provide a benefit to patients with CKD.²³ However, our observations cannot be translated without reservations into treatment of CKD. Unlike in patients with CKD, the kidney is not a priori defective in \( kl/kl \) mice and the treated mice have no difficulties excreting the excessive \( Cl^- \), \( NH_4^+ \), or urea produced from NH₄Cl.

To the extent that NH₄Cl disrupts osteogenic signaling by alkalinizing lysosomal pH, it may be effective in CKD without acidosis. Although some observations point to a beneficial effect of acidosis in experimental renal disease in rodents,²⁸,³²,³³ the bulk of observations indicate that alkali treatment has beneficial and acidosis exacerbaratory effects in experimental CKD.³⁴–³⁷ Moreover, clinical studies in humans demonstrate that acidosis accelerates and alkali treatment retards the progression of CKD.³⁴,³⁹,⁷⁴–⁷⁶ Along those lines, decreased bicarbonate concentrations are associated with decline of eGFR in community-living older persons.⁷⁷

Our observations may shed light on vascular calcification in further clinical conditions. For instance, NFAT5 is upregulated by hyperglycemia²⁸ and NFAT5-dependent osteoinduction may thus contribute to triggering vascular calcification in diabetes.⁷⁹ Moreover, NFAT5 is upregulated by dehydration,⁸⁰ inflammation,⁸¹ hypoxia,⁸¹ and ischemia.⁸¹

In conclusion, our observations uncover a powerful effect of NH₄Cl treatment on plasma aldosterone and ADH levels, vascular and soft tissue calcification, osteoinductive signaling, and survival in klotho-hypomorphic mice. NH₄Cl treatment is effective despite continued increases of plasma 1,25(OH)₂D₃, Ca²⁺, and phosphate concentrations. The observations further disclose a decisive role of Tgfb1 as well as TGFβ1-sensitive and osmosensitive transcription factor NFAT5 in the triggering of osteogenic signaling.

**CONCISE METHODS**

Detailed methods are available in the Supplemental Methods. Animal experiments were conducted according to German law. Where not otherwise indicated, male mice were analyzed. NH₄Cl treatment (0.28 M in tap water) started with mating of the parental generation. In plasma obtained from blood drawn from retro-orbital plexus ammonia, phosphate and calcium concentrations were determined photometrically; plasma aldosterone, 1,25(OH)₂-vitamin D₃, C-terminal FGF23, intact FGF23, and PTH were determined by ELISA; and plasma ADH concentration was determined by enzyme immuno assay. Blood pH, pCO₂, and electrolytes were measured by a blood analyzer (EDAN care lab 115; EDAN Instruments, China). For histology, tissues were embedded in paraffin, cut in 2- to 3-μm sections, and stained with von Kossa and hematoxylin and eosin stain.

Primary HAoSMCs (Invitrogen) were grown to confluence in Waymouth’s MB 752/1 medium and Ham’s F-12 nutrient mixture (1:1) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with 2 μg DNA encoding human NFAT5 in pcDNA6V5-HisC vector or with 2 μg DNA of empty pcDNA6V5-HisC vector using X-tremeGENE HP DNA transfection reagent and/or treated for 24 hours with 2 mM β-glycerophosphate (Sigma-Aldrich), with 500 μM NH₄Cl (Sigma-Aldrich) or with 10 ng/ml human TGFβ1 (R&D Systems).

For quantitative RT-PCR, total RNA was isolated from aortic tissues and HAoSMCs using TriFast Reagent (Peqlab). RT and quantitative RT-PCR were performed as described in the Supplemental Methods.

**Statistical Analyses**

Data are provided as means±SEM. Significance was tested with ANOVA or unpaired t tests. P<0.05 was considered statistically significant.

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**DISCLOSURES**

None.

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Rescue of Klotho-Hypomorphic Mice by NH4Cl

2433
SUPPLEMENTAL MATERIALS:

Renal function

Average kidney weight was 5.8 ± 0.2 mg/g bw. in untreated wild-type mice and 5.7 ± 0.2 mg/g bw. in untreated kl/kl mice. NH₄Cl treatment increased the kidney mass significantly (p < 0.001) to 7.1 ± 0.2 mg/g bw. in wild-type mice and to 7.0 ± 0.1 mg/g bw. in kl/kl mice. Neither before nor after NH₄Cl treatment the ratio of kidney mass over body weight was significantly different between wild-type mice and kl/kl mice. Measurements in metabolic cages were performed in wild-type mice, NH₄Cl treated wild-type mice and NH₄Cl treated kl/kl mice. Because untreated kl/kl mice do not survive this procedure, in a fourth group of NH₄Cl treated kl/kl mice the treatment was stopped at the age of seven weeks. After a 3 week wash-out phase the animals were housed in the metabolic cages. According to the measurements (Supplemental Figures 1-3) body weight, food and fluid intake, urinary volume and pH, fecal mass, plasma creatinine concentration, creatinine excretion, creatinine clearance, plasma protein, plasma albumin, urinary albumin excretion, plasma cystatin C, urinary cystatin C excretion, urinary urea excretion and urinary ammonia excretion were similar in kl/kl mice and wild-type mice. Plasma urea concentration was, however, significantly higher and plasma ammonia concentration tended to be higher in the previously NH₄Cl treated kl/kl mice than in wild-type mice. In both, kl/kl mice and wild-type mice NH₄Cl treatment significantly decreased urinary pH, and significantly increased plasma ammonia concentration, urinary urea excretion, urinary ammonia excretion and, in wild-type mice, plasma urea concentration. All other measured parameters were not significantly modified by the NH₄Cl treatment.

Detailed description of 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 (Regierungspräsidium Tübingen). Male klotho-hypomorphic mice (kl/kl) were compared to male wild-type mice (WT). Origin, breeding and genotyping of the mice were described previously. The mice had access to either tap water or a solution of NH₄Cl (15 g/l) in tap water ad libitum and were fed a standard chow diet. The lifelong NH₄Cl treatment started with the mating of the parental generation and was maintained from pregnancy until the offspring was sacrificed. Where not otherwise indicated, blood was taken and the animals sacrificed for histology and quantitative RT-PCR at the age of 7-8 weeks.

**Blood chemistry**

Blood specimens were obtained from male klotho-hypomorphic mice (kl/kl) and male wild-type mice (WT) by puncturing the retro-orbital plexus. Ammonia was determined enzymatically using glutamate dehydrogenase with NADPH as cofactor. The reaction was photometrically followed and quantified using an ADVIA 1650 analyser (Siemens, Fernwald, Germany). The plasma phosphate and calcium concentrations were determined utilizing a photometric method (FUJI FDC 3500i, Sysmex, Norsted, Germany). The plasma aldosterone concentrations (Alpha Diagnostics International, Texas; USA), 1,25(OH)₂-vitamin D₃ concentrations (IDS, Boldon, UK), C-terminal FGF23 (ImmunDiagnostics, Boston, USA), intact FGF23 (Kainos Laboratories, Tokyo, Japan) and PTH (Immutopics, San Clemente, USA) were determined by ELISA. The plasma ADH concentration was determined utilizing a commercial EIA-Kit (AVP EIA Kit, Phoenix Europe, Karlsruhe, Germany). For the determination of blood pH, blood CO₂ and electrolytes a blood gas analyzer was used (EDAN care lab i15, EDAN Instruments, China)

**Metabolic cage studies**

To assess metabolic parameters male wild-type mice and klotho-hypomorphic mice were housed in metabolic cages at the age of 10 weeks. The wild-type mice were either untreated or
lifelong treated with NH₄Cl solution (0.28 M). Due to their strong phenotype untreated klotho-hypomorphic mice cannot be kept in metabolic cages. For this reason only klotho-hypomorphic mice with lifelong NH₄Cl treatment (0.28 M) and klotho-hypomorphic mice with NH₄Cl treatment until the age of 7 weeks and subsequent 3 weeks without treatment were used for this experiment. After a three day training phase specimens were collected in a four day acquisition phase. Bodyweight, fluid intake, food intake as well as urine volume and fecal mass were measured daily at the same time. At the last day of the experiment blood was drawn by puncturing the retro-orbital plexus. Urinary pH was measured by a pH microelectrode (Mettler-Toledo, Greifensee, Schweiz). Plasma creatinine was measured by an enzymatic procedure (Mouse Creatinine Kit, Crystal Chem, Downers Grove, USA). Urinary creatinine concentrations were determined with a urinary creatinine detection kit (Arbor Assays, Ann Arbor, USA) utilizing the Jaffe method. Plasma urea and total protein were measured photometrically (FUJI FDC 3500i, Sysmex, Norsted, Germany). ELISA was employed for the measurement of plasma and urinary albumin (ICL, Portland, USA) as well as plasma and urinary cystatin C (R&D Systems, Minneapolis, USA). Urinary urea was determined by a colorimetric detection kit (DetectX Urea Nitrogen, Arbor Assays, Ann Arbor, USA). Urinary ammonia was measured with the EnzyChrom Ammonia/Ammonium Assay Kit (BioAssay Systems, Hayward, USA).

**Histology**

To elucidate trachea, lung, renal, gastric and vascular pathology, tissues from male klotho-hypomorphic mice with or without treatment with NH₄Cl (0.28 M in tap water) have been embedded in paraffin, cut in 2–3 µm sections and stained with von Kossa and H&E.

**Cell culture of HAoSMCs**

Primary human aortic smooth muscle cells (Invitrogen) were routinely cultured in Waymouth’s MB 752/1 medium and Ham’s F-12 nutrient mixture (1:1, Gibco, Life Technologies)
supplemented with 10% FBS (Gibco, Life Technologies) and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies). HAoSMCs were grown to confluency and used in all experiments from passages 4 to 11. HAoSMCs were transfected with 2 µg DNA encoding human NFAT5 in pcDNA6V5-HisC vector or with 2 µg DNA of empty pcDNA6V5-HisC vector using X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer’s protocol. The cells were used 48 hours after transfection. Transfection efficiency was verified by quantitative RT-PCR. HAoSMCs were treated for 24 hours with 2 mM β-glycerophosphate (Sigma-Aldrich), with 500 µM NH₄Cl (Sigma-Aldrich) or with 10 ng/ml human TGFB1 (R&D Systems).

The pH of the culture media was measured before and after 24 hours treatment of HAoSMCs with 2 mM β-glycerophosphate and/or 500 µM NH₄Cl. At the beginning of the treatment, the pH was 7.83 ± 0.03 in culture media from untreated HAoSMCs, 7.82 ± 0.03 in culture media from HAoSMCs treated with 2 mM β-glycerophosphate and 7.83 ± 0.03 in culture media from HAoSMCs treated with 2 mM β-glycerophosphate and 500 µM NH₄Cl. After 24 hours of treatment, the pH was 7.68 ± 0.07 in culture media from untreated HAoSMCs, 7.58 ± 0.04 in culture media from HAoSMCs treated with 2 mM β-glycerophosphate and 7.63 ± 0.04 in culture media from HAoSMCs treated with 2 mM β-glycerophosphate and 500 µM NH₄Cl.

Quantitative RT-PCR

After sacrificing the animals, tissues were immediately snap frozen in liquid nitrogen. Total RNA was isolated from aortic tissues of male klotho-hypomorphic mice (kl/kl) and male wild-type mice (WT) by using Trifast Reagent (Peqlab) according to the manufacturer’s instructions. HAoSMCs were washed with PBS and total RNA was isolated using Trifast Reagent (Peqlab) according to the manufacturer’s instructions. Reverse transcription of 2 µg RNA was performed using oligo(dT)₁₂-₁₈ primers (Invitrogen) and SuperScriptIII Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed with the iCycler iQ™ Real-Time PCR Detection System
(Bio-Rad Laboratories) and iQ™ Sybr Green Supermix (Bio-Rad Laboratories) according to the manufacturer’s instructions. The following mouse primers were used (5’→3’ orientation):

**TN alkaline phosphatase**
- fw: TTGTGCCAGAGAAGAGAGAGA;
- rev: GTTTCAGGGCATTTTTCAAGGT;

**Cbfa1**
- fw: AGAGTCAGATTACAGATCCCAGG;
- rev: AGGAGGGGAAGACTGTCATA;

**Gapdh**
- fw: AGGTCGGTGTGAACGGATTTG;
- rev: TGTAGACCATGTAGTTGAGGTCA;

**Glb1**
- fw: TGGCTGGGCTGAATGCTATC;
- rev: TGGTTGGGGTTCATGGAAGTT;

**Nfat5**
- fw: CTGTAGGCGATCTGTTGGGG;
- rev: CTGGTGCTCATGTTACTGAAGTT;

**Sox9**
- fw: AGTACCCGCATCTGCACAAC;
- rev: ACGAAGGGTCTCTTCTCGCT.

**p21**
- fw: CCTGGTGATGTCCGACCTG;
- rev: CCATGAGCGCATCGCAATC;

**Pai-1**
- fw: TTCAGCCCTTGCTTGCCCTC;
- rev: ACACTTTTACTCCGAAATCGGT;

**Tgfb1**
- fw: CTCCCGTGCTCTGTAGTGC;
- rev: GCCTTAGTTTGGACAGGATCTG.

The following human primers were used (5’→3’ orientation):

**TN alkaline phosphatase**
- fw: GGGACTGGTACTCAGACAACG;
- rev: GTAGGCGATGTCCTTACAGCC;

**CBFA1**
- fw: GGAAGGGCTTTGATGCGGT;
- rev: CAGAAACAAACATAGCACTGACT;

**GAPDH**
- fw: GAGTCAACGGATTTGGTCGT;
GAPDH rev: GACAAGCTTCCGTTCCTCAG;
GLB1 fw: TATACCTGGCCTGGCTAGATCACTG;
GLB1 rev: GGAACGACTGGTCGCCTATAA;
NFAT5 fw: GGGTCAAACGACGAGATTGTG;
NFAT5 rev: GTCCGTGGTAAGCTGAGAAAG;
SOX9 fw: AGCGAACGCACATCAAGAC;
SOX9 rev: CTGTAGGCGATCTGTTGGGG.
p21 fw: TGTCCCGTCAGAACCAGTGC;
p21 rev: AAAGTGACAGTCCATCGCTC;
PAA1-1 fw: ACCGCAACGTGGTTTCTCA;
PAA1-1 rev: TTGAATCCCATAGCTGTTGAAT;
TGFB1 fw: CAATTCCTGGCGATACCTCAG;
TGFB1 rev: GCACAACCTCCGGTGACATCAA.

The specificity of the PCR products was confirmed by analysis of the melting curves and in addition by agarose gel electrophoresis. All PCRs were performed in duplicate, and mRNA fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method using GAPDH as internal reference.

Statistics

Data are provided as means ± SEM, $n$ represents the number of independent experiments. All data were tested for significance using ANOVA followed by posthoc analysis or unpaired t-test, where appropriate. For the life span experiments, SAS Jmp version 10.0.2 (SAS Institute Inc., Cary, NC, USA) was used. Only results with $p < 0.05$ were considered statistically significant.
Supplemental Figures

Supplemental Figure 1:

*Basic metabolic parameters of wild-type mice with or without NH₄Cl treatment as well as kl/kl mice with NH₄Cl treatment or discontinued NH₄Cl treatment.*

Arithmetic means ± SEM (n=5) of (A) bodyweight, (B) food intake, (C) fluid intake, (D) urine excretion, (E) urinary pH and (F) fecal mass of wild-type mice (WT, white bars) with (NH₄Cl) and without (Control) NH₄Cl treatment and klotho-hypomorphic mice (kl/kl, black bars) with NH₄Cl treatment (NH₄Cl) and discontinued NH₄Cl treatment (Control). Animals were kept in metabolic cages to obtain the specimens. #(p < 0.05) indicate statistically significant differences from untreated mice.

Supplemental Figure 2:

*The effect of NH₄Cl administration on kidney function of wild-type mice as well as kl/kl mice with NH₄Cl treatment or discontinued NH₄Cl treatment.*

Arithmetic means ± SEM (n=5) of (A) plasma creatinine concentrations, (B) urinary creatinine excretion, (C) GFR, (D) plasma protein concentration, (E) plasma albumin concentration, (F) urinary albumin excretion, (G) plasma cystatin C concentration and (H) urinary cystatin C excretion of wild-type mice (WT, white bars) with (NH₄Cl) and without (Control) NH₄Cl treatment and klotho-hypomorphic mice (kl/kl, black bars) with NH₄Cl treatment (NH₄Cl) and discontinued NH₄Cl treatment (Control). Animals were kept in metabolic cages to obtain the specimens.

Supplemental Figure 3:

*The effect of NH₄Cl on nitrogen metabolism of wild-type mice as well as kl/kl mice with NH₄Cl treatment or discontinued NH₄Cl treatment.*

Arithmetic means ± SEM (n=5) of (A) plasma urea concentrations, (B) urinary urea excretion, (C) plasma ammonia concentrations and (D) urinary ammonia excretion of wild-type mice (WT, white
bars) with (NH₄Cl) and without (Control) NH₄Cl treatment and klotho-hypomorphic mice (kl/kl, black bars) with NH₄Cl treatment (NH₄Cl) and discontinued NH₄Cl treatment (Control). Animals were kept in metabolic cages to obtain the specimens. # (p < 0.05) indicate statistically significant differences from untreated mice.

Supplemental References


Supplemental Fig. 1

A) Bodyweight [g]

B) Food intake [µg/g bw]

C) Fluid intake [µl/g bw]

D) Urine excretion [µl/g bw]

E) Urinary pH

F) Faeces [g/g bw]

- WT
- kl/kl
Supplemental Fig. 2

(A) Plasma creatinine [mg/dl]:
- Control: [data]
- NH₄Cl: [data]

(B) Creatinine excretion [µg/24h/g bw]:
- Control: [data]
- NH₄Cl: [data]

(C) GFR [µl/min/g bw]:
- Control: [data]
- NH₄Cl: [data]

(D) Plasma total protein [g/dl]:
- Control: [data]
- NH₄Cl: [data]

(E) Plasma albumin [mg/ml]:
- Control: [data]
- NH₄Cl: [data]

(F) Albumin excretion [µg/24h/g bw]:
- Control: [data]
- NH₄Cl: [data]

(G) Plasma cystatin C [ng/ml]:
- Control: [data]
- NH₄Cl: [data]

(H) Cystatin C excretion [µg/24h/g bw]:
- Control: [data]
- NH₄Cl: [data]

Legend:
- WT
- kl/kl
Supplemental Fig. 3

A. Plasma urea [mg/dl] for Control and NH₄Cl conditions.
B. Urea excretion [mg/24h/g bw] for Control and NH₄Cl conditions.
C. Plasma ammonia [µg/dl] for Control and NH₄Cl conditions.
D. Ammonia excretion [µg/24h/g bw] for Control and NH₄Cl conditions.

Legend:
- WT
- kl/kl

Key:
- *: Significant difference from Control group
- #: Significant difference from WT group