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. TGFB1 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 TGFB1 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 activity⁴ and lack of klotho leads to extracellular volume depletion with secondary increase of antiuretic hormone (ADH) and aldosterone release.⁴ Klotho-hypomorphic mice (kl/kl) carrying a disruption in the promoter sequence...
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 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 alkalinizes 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. The average life span of female kl/kl mice significantly lower than the body weight of wild-type mice. The body weight of kl/kl mice was significantly lower than the body weight of wild-type mice. 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. 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) in male kl/kl mice.

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. Treatment with NH₄Cl increased plasma ammonia concentration in both wild-type and kl/kl mice. Plasma phosphate and Ca²⁺ 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 concentration was significantly higher in kl/kl mice than in wild-type mice, a difference that again was not significantly
BASIC RESEARCH


Rescue of Klotho-Hypomorphic Mice by NH4Cl

EFFECT OF NH4Cl TREATMENT ON SURVIVAL OF KLOTHO-HYPOMORPHIC MICE

Figure 1. Effect of NH4Cl and NaCl treatment on body weight and survival of kl/kl mice. (A) Photograph of wild-type mice (WT) as well as klotho-hypomorphic mice (kl/kl) without treatment (left), with NH4Cl treatment (0.28 M in tap water) (middle), and with NaCl treatment (0.28 M in tap water) (right). (B) Arithmetic means±SEM of body weight (n=8–9) of wild-type mice (white bars) and klotho-hypomorphic mice (black bars) without treatment (control, left bars), with NH4Cl treatment (0.28 M in tap water) (NH4Cl, middle bars), and with NaCl treatment (0.28 M in tap water) (NaCl, right bars). ***P<0.001, statistically significant differences from respective klotho-hypomorphic mice; $$$P<0.001, statistically significant differences from respective untreated mice; $$$$P<0.001, statistically significant differences from respective NH4Cl-treated mice. (C) Percentage of surviving male klotho-hypomorphic mice maintained on control diet without treatment (closed circles), with NH4Cl treatment (0.28 M in tap water, open circles), and with NaCl treatment (0.28 M in tap water, open triangles) as a function of age. Survival of kl/kl mice is significantly extended by NH4Cl treatment (log-rank, Wilcoxon; P<0.001; n=7–9).

Table 1. NH4Cl treatment restores fertility of male kl/kl mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>H2O</th>
<th>NH4Cl</th>
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<tr>
<td></td>
<td>σ+/hm</td>
<td>σ hm/hm</td>
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<tr>
<td>Offspring number</td>
<td>20.4±1.9</td>
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Data are presented as the average number of puppies (±SD) born from six to eight breedings with three heterozygous female mice (+/hm) and either heterozygous male mice (+/hm) or hypomorphic male mice (hm/hm) drinking plain tap water (H2O) or an aqueous 0.28 M NH4Cl solution (NH4Cl).

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 lower in kl/kl mice than in wild-type mice (Figure 2G). NH4Cl treatment tended to decrease the PTH levels in both genotypes; however, the difference did not reach statistical significance. Plasma ADH (Figure 2H) and aldosterone (Figure 2I) concentrations were both significantly higher in kl/kl mice than in wild-type mice. NH4Cl treatment significantly decreased the plasma ADH (Figure 2H) and aldosterone (Figure 2I) concentrations in kl/kl mice but not in wild-type mice. Accordingly, after NH4Cl treatment, plasma ADH (Figure 2H) and aldosterone (Figure 2I) concentrations were similar in kl/kl mice and wild-type mice (Figure 2, H and I).

Effect of NH4Cl Treatment on Acid/Base Parameters and Plasma Electrolyte Concentrations

As shown in Figure 3, blood pH is lower in untreated kl/kl mice than in untreated wild-type mice. NH4Cl treatment significantly decreased blood pH levels in wild-type mice (Figure 3A) but only tended to decrease blood pH levels in kl/kl mice (Figure 3A). Blood pCO2 values were significantly increased in kl/kl mice but decreased to normal values under treatment with NH4Cl (Figure 3B). Plasma HCO3− levels were significantly higher in untreated kl/kl mice than in untreated wild-type mice, a difference that again was reversed by NH4Cl treatment (Figure 3, C and D). 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 Cbfa1 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 HAoS MCs**

Because vascular calcification is fostered by cellular senescence of vascular smooth muscle cells, the senescence-associated molecules Tgfb1, Pai-1, p21, and SA-β-galactosidase (Glb1) were measured in aortic tissue of kl/kl mice and in HAoS MCs. As illustrated in Figure 7, A–D, the transcript levels of Tgfb1, 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 Tgfb1, Pai-1, p21, and Glb1 in the aortic tissue of kl/kl mice and virtually cotreatment with 500 μM NH4Cl. Treatment of HAoS MCs with β-glycerophosphate significantly increased NFAT5, SOX9, CBFA1, and ALPL mRNA expression (Figure 6), effects that were significantly suppressed by cotreatment with NH4Cl.

**Effect of NH4Cl Treatment on Phosphate-Induced Osteoinductive Signaling in Primary Human Aortic Smooth Muscle Cells**

To further define the effects of NH4Cl on phosphate-induced osteoinductive signaling, primary human aortic smooth muscle cells (HAoS MCs) were treated for 24 hours with 2 mM β-glycerophosphate with or without additional cotreatment with 500 μM NH4Cl. Treatment of HAoS MCs 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 (iFGF23), 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 TGFβ1, 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 TGFβ1 on NFAT5 Expression in HAoSMCs**

Additional experiments were performed to test whether the regulation of SOX9, CBFA1, and ALPL transcript levels is sensitive to NFAT5 in HAoSMCs. To this end, HAoSMCs were transfected for 48 hours with a construct encoding NFAT5 with or without subsequent treatment with 2 mM β-glycerophosphate and/or 500 μM NH4Cl for 24 hours. Transfection efficiency was verified by quantitative RT-PCR (Figure 8A). As illustrated in Figure 8, B–D, overexpression of NFAT5 in HAoSMCs was followed by marked and significant increase of SOX9, CBFA1, and ALPL mRNA levels. In empty vector–transfected HAoSMCs, the mRNA levels of SOX9, CBFA1, and ALPL were increased after treatment with β-glycerophosphate to similarly high levels as in NFAT5-overexpressing HAoSMCs (Figure 8, B–D), an effect completely reversed by additional
treatment with NH₄Cl. By contrast, neither β-glycerophosphate nor NH₄Cl significantly modified the transcript levels of SOX9, CBFA1, and ALPL in NFAT5-overexpressing HAoSMCs. Accordingly, in the absence of β-glycerophosphate and in the presence of both β-glycerophosphate and NH₄Cl, the SOX9, CBFA1, and ALPL transcript levels were significantly higher in NFAT5-transfected HAoSMCs than in empty vector–transfected HAoSMCs.

In a further series of experiments, the influence of TGFβ1 on NFAT5 mRNA expression in HAoSMCs was tested. Treatment of HAoSMCs for 24 hours with 10 ng/ml of human TGFβ1 significantly increased NFAT5 relative mRNA expression to similarly high values as treatment with β-glycerophosphate (Figure 8E). NH₄Cl treatment significantly decreased NFAT5 mRNA expression in HAoSMCs treated with β-glycerophosphate, an effect completely abrogated by additional treatment with TGFβ1 (Figure 8E).

**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 wt 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

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**Figure 5.** 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, ##P<0.01 and ###P<0.001 indicate statistically significant differences from respective untreated mice.

**Figure 6.** NH₄Cl interferes with phosphate-induced osteoinductive signaling in HAoSMCs. Arithmetic means±SEM (n=6–8; arbitrary units [a.u.]) of NFAT5 (A), Sox9 (B), CBFA1 (C), and ALPL (D) relative mRNA expression (exp.) 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<0.01 and ****P<0.001, statistically significant differences from untreated HAoSMCs; #P<0.05 and ###P<0.001, statistically significant difference from HAoSMCs treated with 2 mM β-glycerophosphate alone.
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 differences from respective wild-type mice; P<0.05, #P<0.01, and ###P<0.001, statistically significant differences from respective untreated 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.

Figure 7. 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 wild-type mice; P<0.05, #P<0.01, and ###P<0.001, statistically significant difference respective untreated 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. It is triggered by increased extracellular phosphate concentration, which is known to inhibit calcification in uremic rats. 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, 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. 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. However, the survival benefit of aldosterone receptor blockade by spironolactone is only modest in kl/kl mice. 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 euvoletic 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, 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 Cbfa1, a transcription factor decisive for the stimulation of osteoblastic differentiation. Cbfa1 stimulates the expression of alkaline phosphatase Alpl, which in turn degrades pyrophosphate and thus fosters precipitation of calcium phosphate. The vascular osteoinduction is closely associated with vascular smooth muscle cell senescence, a key factor in vascular aging and injury. NH₄Cl treatment blunted the expression of senescence indicators Pai-1, p21, and Gli1. Excessive extracellular phosphate concentration promotes Tgfb1 production. Tgfb1 stimulates cellular senescence, thus contributing to aging and vascular osteoinduction. Disruption of Tgfb1–Pai-1 signaling ameliorates vascular calcification. We observed enhanced Tgfb1 mRNA expression in untreated kl/kl mice and in HAOsMCs after phosphate treatment. The increased Tgfb1 transcript levels were significantly decreased by NH₄Cl treatment. NH₄Cl has previously been shown to hinder the maturation of Tgfb1. 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 CBFA1 and ALPL. NFAT5 further upregulates the transcript levels of Sox9, a downstream mediator of NFAT5-induced CBFA1 expression. Sox9 is involved in the chondrogenic gene expression in vascular smooth muscle cells. Sox9 is upregulated in vascular tissue of uremic rats. 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
showed that NH₄Cl was not able to reduce the phosphate-induced increase of NFAT5 expression in the presence of active TGFBI. Accordingly, NH₄Cl is at least partially effective by downregulating TGFBI. By alkalinizing lysosomes, NH₄Cl presumably disrupts the processing of TGFBI 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. 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, vascular calcification in patients with CKD is an active process as part of the mineral bone disorder. Vascular calcification increases the risk for cardiovascular events, which are the leading cause of death in patients with CKD. The impaired renal phosphate elimination in CKD is followed by hyperphosphatemia, which is paralleled by strong reduction of klotho expression and increased expression of osteochondrogenic reprogramming markers including CBFA1. Prevention of osteogenic reprogramming in vascular tissue could thus provide a benefit to patients with CKD. 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⁻, NH₄⁺, 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, the bulk of observations indicate that alkali treatment has beneficial and acidosis exacerbaratory effects in experimental CKD. Moreover, clinical studies in humans demonstrate that acidosis accelerates and alkali treatment retards the progression of CKD. Along those lines, decreased bicarbonate concentrations are associated with decline of eGFR in community-living older persons.

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

REFERENCES


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