In Vivo Genetic Evidence for Suppressing Vascular and Soft-Tissue Calcification Through the Reduction of Serum Phosphate Levels, Even in the Presence of High Serum Calcium and 1,25-Dihydroxyvitamin D Levels

Mutsuko Ohnishi, MD, PhD; Teruyo Nakatani, PhD; Beate Lanske, PhD; M. Shawkat Razzaque, MD, PhD

Background—Klotho-knockout mice (klotho<sup>-/-</sup>) have increased renal expression of sodium/phosphate cotransporters (NaPi2a), associated with severe hyperphosphatemia. Such serum biochemical changes in klotho<sup>-/-</sup> mice lead to extensive soft-tissue anomalies and vascular calcification. To determine the significance of increased renal expression of the NaPi2a protein and concomitant hyperphosphatemia and vascular calcification in klotho<sup>-/-</sup> mice, we generated klotho and NaPi2a double-knockout (klotho<sup>-/-</sup>/NaPi2a<sup>-/-</sup>) mice.

Methods and Results—Genetic inactivation of NaPi2a activity from klotho<sup>-/-</sup> mice reversed the severe hyperphosphatemia to mild hypophosphatemia or normophosphatemia. Importantly, despite significantly higher serum calcium and 1,25-dihydroxyvitamin D levels in klotho<sup>-/-</sup>/NaPi2a<sup>-/-</sup> mice, the vascular and soft-tissue calcifications were reduced. Extensive soft-tissue anomalies and cardiovascular calcification were consistently noted in klotho<sup>-/-</sup> mice by 6 weeks of age; however, these vascular and soft-tissue abnormalities were absent even in 12-week-old double-knockout mice. Klotho<sup>-/-</sup>/NaPi2a<sup>-/-</sup> mice also regained body weight and did not develop the generalized tissue atrophy often noted in klotho<sup>-/-</sup> single-knockout mice.

Conclusion—Our in vivo genetic manipulation studies have provided compelling evidence for a pathological role of increased NaPi2a activities in regulating abnormal mineral ion metabolism and soft-tissue anomalies in klotho<sup>-/-</sup> mice. Notably, our results suggest that serum phosphate levels are the important in vivo determinant of calcification and that lowering serum phosphate levels can reduce or eliminate soft-tissue and vascular calcification, even in presence of extremely high serum calcium and 1,25-dihydroxyvitamin D levels. These in vivo observations have significant clinical importance and therapeutic implications for patients with chronic kidney disease with cardiovascular calcification. (Circ Cardiovasc Genet. 2009;2:583-590.)

Key Words: klotho ■ vitamin D ■ NaPi2a ■ calcification

Understanding the molecular regulation of phosphate homeostasis has enormous clinical and biological importance because it is involved in numerous essential biochemical reactions, including cell signaling process and energy metabolism. Adequate bone mineralization is closely dependent on the status of phosphate metabolism. Abnormal regulation of phosphate homeostasis can cause myopathy, cardiac dysfunctions, hematologic abnormalities, and vascular and soft-tissue calcifications.<sup>1-3</sup> Recent studies have found that klotho, a transmembrane protein, is actively involved in regulation of mineral ion metabolism by affecting the functionality of ion channels and cotransporter proteins in the kidney.<sup>4,5</sup> The in vivo importance of klotho in regulation of mineral ion metabolism is further evident in klotho-knockout mice because these mice have severely impaired mineral ion homeostasis.<sup>5,6</sup>

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The klotho<sup>-/-</sup> mice develop severe hyperphosphatemia by 3 weeks of age and remain hyperphosphatemic throughout their life.<sup>7,8</sup> The hyperphosphatemia in klotho<sup>-/-</sup> mice is associated with increased renal expression of the NaPi2a cotransporter protein in the proximal tubular epithelial cells.<sup>3,7,9</sup> To assess the significance of increased renal expression of NaPi2a in soft-tissue anomalies and vascular calcification in klotho<sup>-/-</sup> mice, we have generated a new mouse model by genetically ablating both the klotho and the NaPi2a genes.
Gross phenotype of wild-type (WT), klotho 

Hyperphosphatemia and reduced serum levels of 1,25
dihydroxyvitamin D are the major biochemical changes

Methods

Generation of Double-Mutant Mice
We have crossbred heterozygous klotho mutants (Lexicon Genetics; Mutant Mouse Regional Resource Centers, University of California, Davis, Calif) with heterozygous NaPi2a mutants to obtain compound heterogeneous animals, which were then interbred to generate the desired double-homozygous mutants (klotho 

Gross Phenotype and Body Weight
The total body weight of each of wild-type, klotho 

Biochemical Measurements
Blood was obtained by cheek-pouch bleeding of wild-type, klotho 

Figure 2. Biochemical analysis of serum phosphate and calcium. The serum phosphate (upper panel) and calcium (lower panel) levels are higher in klotho 

Figure 1. Macroscopic phenotype of klotho 

Body weight curves (lower panel) for all 4 genotypes showing DKO mice (n=23), suggesting that inactivation of NaPi2a function

Routine polymerase chain reaction was used to identify the genotypes of various mice (supplemental Figure I). All studies performed were approved by the institutional animal care and use committee at Harvard Medical School (Boston, Mass).

Figure 2. Microscopic phenotype of klotho 

The average body weight of the mice (n=34) are smaller than WT mice (n=22) but larger than klotho 

Mice 

Mice 

mice. The average body weight of the NaPi2a 

mice (n=42) is more than that of the DKO mice. The statistical analyses among the groups were compared using Student's unpaired 2-tail t test (P<0.0001 at all time points for WT versus klotho 

mice; P<0.0001 at all time points for klotho 

mice versus NaPi2a 

mice; P<0.0001 at all time points for klotho 

versus DKO mice).

The current treatment approach of reducing serum phosphate levels and providing vitamin D analogs in patients with CKD often poses a dilemma because studies have linked vitamin D treatment to subsequent vascular calcification. Importantly, ~50% of mortalities in patients with CKD undergoing dialysis treatment are due to the cardiovascular complication of vascular calcification. Our current study provides the in vivo beneficial effects of reducing serum phosphate levels on preventing vascular calcification, even in the presence of extremely high serum 1,25-dihydroxyvitamin D levels.

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To determine the effects of hyperphosphatemia on soft-tissue and vascular calcification in klotho−/− mice, sections were prepared from the heart, lung, kidney, liver, spleen, aorta, and gastrointestinal tract and were stained with von Kossa to visualize mineralized tissues by light microscopy. The von Kossa–stained sections were subsequently embedded in paraffin. Four- to 6-µm paraffin sections of various tissues were mounted on SuperFrost Plus slides. The sections were then routinely stained with hematoxylin and eosin and von Kossa.

Histological Analyses
Soft tissues obtained from wild-type, klotho−/−, NaPi2a−/−, and klotho−/−/NaPi2a−/− mice at 9 to 12 weeks were fixed with 4% paraformaldehyde, 10% buffered formalin, or Carnoy’s solution and were subsequently embedded in paraffin. Four- to 6-µm paraffin sections of various tissues were mounted on SuperFrost Plus slides. The sections were then routinely stained with hematoxylin and eosin and von Kossa. Histological changes were observed by light microscopy.

Calcification Analyses
To determine the effects of hyperphosphatemia on soft-tissue and vascular calcification in klotho−/− mice, sections were prepared from the heart, lung, kidney, liver, spleen, aorta, and gastrointestinal tract and were stained with von Kossa to visualize mineralized tissues by light microscopy. The von Kossa–stained sections of klotho−/−/NaPi2a−/− mice were compared with similarly stained sections from wild-type, klotho−/−, and NaPi2a−/− mice. The von Kossa staining procedure was detailed in an earlier publication.

Immunofluorescence Staining
Immunostaining was performed as described previously. In brief, kidneys obtained from wild-type and klotho−/− mice were embedded in Optimal Cutting Temperature (OCT) compound and stored at −80°C. Frozen sections were incubated in a blocking solution for 30 minutes and then with polyclonal anti-NaPi2a antibody (dilution 1:100; Alpha Diagnostic, San Antonio, Tex) at 4°C for overnight. The slides were washed with PBS and incubated with fluorescein isothiocyanate-labeled anti-rabbit secondary antibody (dilution 1:100) for 30 minutes. After washing with PBS, cover slips were placed on slides using 4,6-diamidino-2-phenylindole—containing mounting media. The expression of NaPi2a was visualized using an immunofluorescence microscopy. Rabbit serum, in place of primary antibody, was used as a negative control. Kidney sections prepared from NaPi2a−/− mice were simultaneously stained for NaPi2a and used as additional negative control.

Quantitative Real-Time Polymerase Chain Reaction
Total RNA isolated from the 3 or more kidneys and aortas of wild-type, klotho−/−, NaPi2a−/−, and klotho−/−/NaPi2a−/− mice was used to detect the relative expression of Emp-1, ANK, Pit-1, and RUNX2 mRNA, as described previously. Real-time polymerase chain reaction was performed in duplicate. The quantity of mRNA was calculated by normalizing the threshold cycle value of Emp-1, ANK, Pit-1, or RUNX2 to the threshold cycle value of the housekeeping gene GAPDH. The sequences of the primers used to detect expression patterns of various genes are reported in our earlier publications.
hyperphosphatemia. To determine whether hyperphosphatemia is a NaPi2a-dependent process, we generated klotho−/−/NaPi2a−/− mice and compared their physical, morphological, and biochemical phenotype with klotho−/− mice.

As mentioned, genetic deletion of klotho results in hyperphosphatemia. To determine whether hyperphosphatemia in klotho−/− mice is a NaPi2a-dependent process, we generated a new mouse model that is deficient in both klotho and NaPi2a genes (klotho−/−/NaPi2a−/−) by interbreeding heterozygous klotho mice with heterozygous NaPi2a mice. The klotho−/−/NaPi2a−/− mice were viable and larger in size to klotho−/− mice. At birth, klotho−/−/NaPi2a−/− mice were indistinguishable from their littermates of other genotypes. At 3 weeks of age, klotho−/−/NaPi2a−/− mice were slightly larger in size than klotho−/− mice (12.8±0.5 g versus 10.4±0.5 g) but smaller than wild-type mice (16.0±0.5 g). Double-knockout mice were similar in size to NaPi2a−/− animals (13.4±0.27 g). At 9 weeks of age, klotho−/−/NaPi2a−/− mice were still smaller than their wild-type littermates (20.2±0.85 g versus 28.8±1.1 g), but their body weight was significantly higher than that of klotho−/− mice (11.3±0.9 g). At 9 weeks of age, the average body weight of NaPi2a−/− mice was 25.7±0.76 g (Figure 1). Furthermore, klotho−/− mice had a maximum survival of around 15 weeks, whereas all the klotho−/−/NaPi2a−/− mice were capable of survival beyond 15 weeks and were alive until 20 weeks of observation period.

Next, we measured serum phosphate and calcium levels in 3-, 6-, and 9-week-old wild-type, klotho−/−, NaPi2a−/−, and klotho−/−/NaPi2a−/− mice. The double-knockout mice were slightly hypophosphatemic by 6 weeks of age (7.2±0.41 mg/dL, n=10) compared with wild-type mice (8.5±0.69 mg/dL, n=5). The low serum phosphate levels were also noted in age-matched NaPi2a−/− mice (4.6±0.14 mg/dL, n=10). Serum phosphate levels were high in klotho−/− mice (12.1±0.64 mg/dL, n=11; Figure 2). Significant reduction of serum phosphate levels was also noted in 9-week-old double-knockout mice compared with klotho−/− mice. Collectively, these findings suggest that inactivation of NaPi2a function can reverse hyperphosphatemia to hypophosphatemia or normophosphatemia at 3, 6, and 9 weeks of age.

Serum calcium levels were higher in the klotho−/− mice at around 6 weeks of age, as also noted in both NaPi2a−/− and klotho−/−/NaPi2a−/− mice (Figure 2). At around 6 weeks of age, serum calcium in klotho−/−/NaPi2a−/− mice (11.4±0.44 mg/dL, n=12) was significantly higher than in wild-type mice (7.6±0.46 mg/dL, n=6). The higher serum levels of calcium were also noted in klotho−/− mice (9.8±0.34 mg/dL, n=10) and NaPi2a−/− mice (10.0±0.12 mg/dL, n=10) of similar age (Figure 2).

Despite reversal of serum phosphate level from severe hyperphosphatemia to mild hypophosphatemia or normophosphatemia in klotho−/−/NaPi2a−/− mice, the serum 1,25(OH)2D3 levels in klotho−/−/NaPi2a−/− mice were extremely high. Statistically significant increased serum levels
of 1,25(OH)\textsubscript{2}D\textsubscript{3} were also detected in klotho\textsuperscript{-/-} and NaPi2a\textsuperscript{-/-} mice (Figure 3). In contrast to 1,25(OH)\textsubscript{2}D\textsubscript{3}, serum PTH levels were markedly reduced in klotho\textsuperscript{-/-} and klotho\textsuperscript{-/-}/NaPi2a\textsuperscript{-/-} mice (Figure 3); serum PTH was also extremely low in NaPi2a\textsuperscript{-/-} mice. Of relevance, compared with the control (176 pg/mL), serum FGF23 levels were extremely high in the klotho\textsuperscript{-/-} mice (7107 pg/mL) at 6 weeks of age.\textsuperscript{22} Markedly increased serum levels of FGF23 were also noted in the klotho\textsuperscript{-/-}/NaPi2a\textsuperscript{-/-} mice (7440 pg/mL); the serum FGF23 levels were low in NaPi2a\textsuperscript{-/-} mice (53 pg/mL; Figure 4).

The major finding of our in vivo genetic manipulation studies is that NaPi2a regulates systemic phosphate homeostasis in klotho\textsuperscript{-/-} mouse. Inactivation of the NaPi2a gene from klotho\textsuperscript{-/-} mice reversed severe hyperphosphatemia to hypophosphatemia or normophosphatemia, despite significantly higher serum levels of calcium and 1,25(OH)\textsubscript{2}D\textsubscript{3}.

We then examined the effects of reducing serum phosphate from klotho\textsuperscript{-/-} mice on vascular and soft-tissue calcification. Extensive vascular and soft-tissue calcifications were present in the heart, lung, kidney, aorta, and other organs in klotho\textsuperscript{-/-} mice, as determined by von Kossa staining (Supplemental Figure III). The extensive calcification observed in klotho\textsuperscript{-/-} mice by 6 weeks was significantly reduced or eliminated in klotho\textsuperscript{-/-}/NaPi2a\textsuperscript{-/-} mice, even at 12 weeks of age (Figures 5 and 6). These results indicate that high serum phosphate is an important determinant of calcification and that lowering
serum phosphate levels can reduce or eliminate calcification, even in the presence of higher serum calcium and 1,25(OH)₂D₃ levels (Figure 3).

Lowering serum phosphate levels in klotho/H₁1002/H₁1002 mice significantly reduced soft-tissue anomalies, helped restore fertility, and markedly reduced extensive soft-tissue and vascular calcifications in klotho/H₁11002/H₁1002/NaPi2a/H₁1002 mice (Table). These results suggest that the phenotypes of klotho/H₁1002/H₁1002 mice are mostly derived from high serum phosphate levels. Despite significantly high serum levels of calcium and 1,25(OH)₂D₃, the opposing phenotypes of klotho/H₁1002/H₁1002 and klotho/H₁11002/NaPi2a/H₁1002 mice suggest that such dissimilarities are due to phosphate toxicity. Our results provide compelling genetic evidence of the in vivo importance of NaPi2a in the regulation of systemic phosphate homeostasis in klotho⁻/⁻/NaPi2a⁻/⁻ mice and show that abnormal regulation of NaPi2a cotransporters can lead to phosphate toxicity to induce severe soft-tissue anomalies, including general tissue atrophy and reduction of skeletal muscle mass. Of relevance, hyperphosphatemia and severe muscle wasting are common clinical problems encountered in patients with CKD.

Extensive vascular and soft-tissue calcifications were widely present in the lung, kidney, aorta, and other organs in klotho⁻/⁻ mice, as detected by von Kossa staining (Figure 5, Supplemental Figure III). The extensive calcification noted in klotho⁻/⁻ mice by 6 weeks of age was completely eliminated in klotho⁻/⁻/NaPi2a⁻/⁻ mice and was not detected even in 12-week-old double-mutant mice. These results indicated that high serum phosphate is an important determinant of calcification and that lowering serum phosphate levels can reduce or eliminate calcification, even in the presence of higher serum 1,25(OH)₂D₃ levels (Figure 3).

Phosphate retention and subsequent hyperphosphatemia, together with reduced circulating levels of 1,25-dihydroxyvitamin D, are the major biochemical changes detected in patients with CKD. Coronary calcification is the single most important pathological condition that influences the mortality of patients with CKD undergoing dialysis treatment. Hyperphosphatemia is believed to be an important risk factor for such cardiovascular calcification. The current approach of reducing serum phosphate levels and correcting vitamin D insufficiency or deficiency in patients with CKD often poses a dilemma because high doses of vitamin D or analog treatment are believed to affect the subsequent vascular calcification process. Our in vivo genetic manipulation study suggests that minimizing phosphate toxicity can reduce vascular calcification, even in the presence of extremely high serum 1,25-dihydroxyvitamin D and calcium levels. One limitation of animal study is that the mechanisms of human vascular diseases in renal insufficiency may be different from those in vascular lesions in klotho⁻/⁻ mice, and established

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E indicates extremely; M, moderately; S, slightly; D, diffuse; F, focal.
medial and atherosclerotic lesions may persist even after molecular manipulations because advanced stages of calcification may not be reversible. We believe that lowering serum phosphate levels can delay the progression of vascular lesions but may not always reverse the established lesions.

Hyperphosphatemia in klothoΔ/Δ mice is associated with extensive soft-tissue calcification (Figure 5, Supplemental Figure III).1,9,22 Imbalance between phosphate and pyrophosphate usually determines the ectopic calcification process.26,27 We have found that the expression of Enpp-1 (pyrophosphate generator) and ANK (pyrophosphate transporter) is slightly increased in the kidneys and aortas of klothoΔ/Δ mice and that such increase might be a compensatory response in these mutant mice (supplemental Figures IV and V). Because increase of pyrophosphate-regulating molecules cannot suppress calcification process, it seems likely that extensive calcification in klothoΔ/Δ mice is primarily associated with high serum phosphate levels.

In summary, the phenotypes of klothoΔ/Δ/NaPi2aΔ/Δ mice suggest that (1) increased NaPi2a activity is the main cause for the severe hyperphosphatemia and ectopic calcification observed in klothoΔ/Δ mice; and (2) NaPi2a-mediated renal phosphate homeostasis is independent of serum 1,25(OH)2D3 levels in mice deficient for klotho. Notably, lowering phosphate burden, by reducing serum phosphate levels, can modulate vascular and soft-tissue calcification, despite the presence of extremely high serum 1,25(OH)2D3 levels. These results provide compelling genetic evidence of the importance of NaPi2a in regulating renal phosphate homeostasis in klothoΔ/Δ mice, and more importantly, suggest that reducing “phosphate toxicity” should be the single most important therapeutic priority in minimizing the risk of vascular calcification and eventual disease progression.25,28–30

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Disclosures

None.

References

In this study, we have shown that serum phosphate levels are an important in vivo determinant of vascular calcification and that lowering serum phosphate levels can reduce or eliminate soft-tissue and vascular calcification, even in the presence of extremely high serum calcium and 1,25-dihydroxyvitamin D levels. Hyperphosphatemia and reduced serum levels of 1,25-dihydroxyvitamin D are the major biochemical changes detected in patients with chronic kidney disease. The current treatment approach of reducing serum phosphate levels and providing vitamin D analogs in patients with chronic kidney disease often poses a dilemma because vitamin D treatment is often linked to subsequent vascular calcification. Of relevance, ≈50% of mortality in patients with chronic kidney disease undergoing dialysis treatment is due to cardiac complications, including coronary calcification. Our current study provides evidence for the in vivo beneficial effects of reducing serum phosphate levels that prevented or reduced vascular calcification, even in the presence of extremely high serum calcium and 1,25-dihydroxyvitamin D levels. These results provide compelling genetic evidence that suggests that reducing "phosphate toxicity" should be a critical therapeutic priority for minimizing the risk of vascular calcification and disease progression.
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SUPPLEMENTAL MATERIALS

In vivo genetic evidence for suppressing vascular and soft tissue calcification through the reduction of serum phosphate levels, even in the presence of high serum calcium and 1,25-dihydroxyvitamin-D levels

Mutsuko Ohnishi MD, PhD, Teruyo Nakatani PhD, Beate Lanske PhD, and M. Shawkat Razzaque MD, PhD
Supplementary Figure 1
PCR amplification of wild-type (WT), mutant klotho, and mutant NaPi2a alleles to identify desired genotypes of WT, klotho<sup>−/−</sup>, klotho<sup>−/−</sup>/NaPi2a<sup>−/−</sup> (DKO), and NaPi2a<sup>−/−</sup> mice to study biochemical, molecular, and morphological phenotypes of different mice.
Supplementary Figure 2
Expression of NaPi2a

Immunostaining of NaPi2a in the kidney sections prepared from the wild-type (WT) and klotho−/− mice using a polyclonal antibody, as detailed in material and methods section. In contrast to WT kidney, there is markedly increased expression of NaPi2a in kidney sections prepared from klotho−/− mice (magnification x20).
Supplementary Figure 3
Vascular calcification in various organs of klotho<sup>−/−</sup> mice. Heart, lung, aorta, and kidney sections prepared from klotho<sup>−/−</sup> mice and stained with von Kossa, showing vascular calcification (arrows) in all these organs (magnification: Heart x10; Lung x20; Kidney x20; Aorta x60).
Supplementary Figure 4

Real-time PCR analysis of RUNX2, Ennp-1, ANK and Pit-1 in the kidney. The relative expression of RUNX2, Ennp-1, ANK and Pit-1 in the kidneys obtained from wild-type (WT), klotho−/−, klotho−/−/NaPi2a−/− (DKO), and NaPi2a−/− mice. Compared to the WT, the expression of RUNX2, Ennp-1, ANK and Pit-1 is slightly higher in klotho−/− mice. Data presented as RUNX2, Ennp-1, ANK and Pit-1 mRNA expression relative to WT mice, normalized with GAPDH (*: p < 0.05, vs. WT).
Supplementary Figure 5

Real-time PCR analysis of RUNX2, Ennp-1, ANK and Pit-1 in the aorta. The relative expression of RUNX2, Ennp-1, ANK and Pit-1 in the aortas obtained from wild-type (WT), klotho<sup>-/-</sup>, klotho<sup>-/-</sup>/NaPi2<sup>a/-</sup> (DKO), and NaPi2<sup>a/-</sup> mice. Compared to the WT, the expression of RUNX2, Ennp-1, ANK and Pit-1 is slightly higher in klotho<sup>-/-</sup> mice. Data presented as RUNX2, Ennp-1, ANK and Pit-1 mRNA expression relative to WT mice, normalized with GAPDH. (*: p < 0.05, vs. WT; #: p < 0.01, vs. klotho<sup>-/-</sup>)