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Dietary phosphate restriction suppresses phosphaturia but does not prevent FGF23 elevation in a mouse model of chronic kidney disease

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Abstract

Fibroblast growth factor 23 (FGF23) is a phosphaturic hormone that in end-stage renal disease is markedly increased in serum; however, the mechanisms responsible for this increase are unclear. Here, we tested whether phosphate retention in chronic kidney disease (CKD) is responsible for the elevation of FGF23 in serum using Col4α3 knockout mice, a murine model of Alport disease exhibiting CKD. We found a significant elevation in serum FGF23 in progressively azotemic 8 and 12 week-old CKD mice along with an increased fractional excretion of phosphorus. Both moderate and severe phosphate restriction reduced fractional excretion of phosphorus by 8 weeks, yet serum FGF23 levels remained strikingly elevated. By 12 weeks, FGF23 levels were further increased with moderate phosphate restriction, while severe phosphate restriction led to severe bone mineralization defects and decreased FGF23 production in bone. CKD mice on a control diet had low serum $1,25(OH)_2D$ levels and 3-fold higher renal Cyp24α1 gene expression compared to wild-type mice. Severe phosphate restriction increased $1,25(OH)_2D$ levels in CKD mice by 8 weeks and lowered renal Cyp24α1 gene expression despite persistently elevated serum FGF23. Renal klotho gene expression declined in CKD mice on a control diet, but improved with severe phosphate restriction. Thus, dietary phosphate restriction reduces the fractional excretion of phosphorus independent of serum FGF23 levels in mice with CKD.

INTRODUCTION

Fibroblast growth factor 23 (FGF23) is a phosphaturic and vitamin D regulatory hormone produced by osteocytes in bone and regulated by multiple endocrine pathways.[1-4] Increased circulating levels of FGF23 can be detected in early chronic kidney disease (CKD), rising

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DISCLOSURES

None

exponentially as the underlying disease progresses to end-stage (ESRD).[5,6] While FGF23 has received much attention for its association with adverse clinical outcomes,[7] the pathophysiologic basis underlying the progressive rise in serum levels of this hormone remains undefined.

One potential explanation for the observed rise of FGF23 in CKD is a response to the loss of functional nephron mass and the resulting retention of phosphate.[8,9] In this scenario, transient hyperphosphatemia could signal bone to increase the production of FGF23 resulting in increased urinary excretion of phosphate and decreased intestinal phosphate absorption consequent to increased catabolism and decreased production of $1,25(OH)_2D$. These actions of FGF23, in conjunction with the phosphaturic effects of PTH, would maintain serum phosphorus levels in the normal range until progressive renal injury had rendered the functional nephron mass incompetent to handle the phosphate load. In the context of this hypothesis, dietary phosphate restriction proportionate to the decline in GFR could limit or delay the progressive rise in FGF23. However, rigorous testing of this hypothesis in humans has been difficult owing to the lack of sensitive markers for the early detection of kidney injury, the complexity of performing long-term phosphate restriction studies in human subjects, and the inhomogeneity of disease progression among patients with similar renal disorders.

We recently reported that Col4a3 null mice, the murine homologue of human Alport disease, exhibit a chronic kidney disease-mineral and bone disorder (CKD-MBD) phenotype that recapitulates that found in humans with CKD.[10] In the current study we examined the impact of both moderate and severe dietary phosphate restriction on FGF23 metabolism in $Col4a3^{-/-}$ mice in order to test the hypothesis that phosphate restriction beginning in early CKD could decrease FGF23 levels in CKD. We also determined the impact of phosphate restriction on vitamin D metabolism and renal klotho gene expression in relation to changes in FGF23 and PTH.

RESULTS

Serum and urine mineral metabolism parameters with phosphate restriction

We measured serum and urine mineral metabolism parameters from $Col4a3^{-/-}$ (KO) and wild-type (WT) mice at 8 and 12 weeks-of-age eating either a control (normal phosphate content), 0.2% phosphate, or 0.02% phosphate diet (Table 1). By 8 weeks, KO mice offered the control diet demonstrated elevated serum levels of creatinine (KO 0.44 vs. WT 0.25 mg/dl), intact PTH (KO 703.7 vs. WT 81.3 pg/ml), FGF23 (KO 481.3 vs. WT 169.2 pg/ml) and phosphorus (KO 8.6 vs. 6.5 WT mg/dl), and a modest decline in $1,25(OH)_2D$ levels (KO 136.5 vs. WT 188.4 pmol/l). KO mice receiving the control diet also exhibited an increased fractional excretion of phosphorus (FE_{phos})(KO 33.4 vs. WT 15.1%). After 12 weeks, KO mice demonstrated further increments in serum phosphorus, PTH, FGF23 and creatinine levels, in addition to significant hypocalcemia and $1,25(OH)_2D$ deficiency. The FE_{phos} was similar for KO and WT mice at 12 weeks.

Surprisingly, 8 week-old KO mice consuming either phosphate-restricted diet exhibited serum phosphate levels that were elevated beyond those of KO mice ingesting the control

diet (Table 1). KO mice receiving the phosphate-restricted diets maintained elevations in serum creatinine, FGF23 and PTH above those of WT animals, although PTH levels were decreased in the 0.02% phosphate group compared to control KO mice. Interestingly, KO mice consuming the 0.02% phosphate diet demonstrated a marked increase in serum $1,25(OH)_2D$ levels (1347.5 vs. 136.5 pmol/l for KO control) and suppressed FE_{phos} (1.6% vs. 33.4% for KO control) despite a persistent elevation of FGF23. After 12 weeks, KO mice undergoing moderate phosphate restriction (0.2%) exhibited persistent elevations of FGF23 (Figure 1A). By contrast, many KO mice receiving the 0.02% phosphate diet demonstrated a marked suppression of serum FGF23 levels (Figure 1A), which was confirmed by serum immunoprecipitation studies (supp. Figure 1). PTH levels were persistently elevated in all KO mice, regardless of the degree of phosphate restriction. Serum $1,25(OH)_2D$ levels in the 0.02% phosphate group were increased compared to KO mice eating the control diet (192.0 vs. 64.1 pmol/l for KO control), but were far lower than the levels observed in the phosphate-deficient WT mice (192.0 vs. 969.9 pmol/l for WT control). Furthermore, KO mice receiving either phosphate-restricted diet unexpectedly developed hypercalcemia after 12 weeks. The FE_{phos} in all phosphate-depleted KO mice remained markedly suppressed at 12 weeks.

Effect of phosphate restriction on FGF23 gene and protein expression in bone

Quantitative RT-PCR was performed on calvaria specimens from KO and WT mice at 12 weeks-of-age to evaluate the impact of phosphate restriction on FGF23 gene transcription (Figure 1B). FGF23 gene transcription levels of KO mice consuming the control diet were marginally elevated compared to WT mice (Figure 1B) (P=0.09). Moderate phosphate restriction (0.2%) had no obvious impact on FGF23 gene transcription in KO mice, while severe phosphate restriction (0.02%) led to a marked suppression of FGF23 gene expression in KO mice when compared to KO mice eating the control diet (P=0.001) (Figure 1B). Western blot analysis of calvaria specimens from the 0.02% phosphate group confirmed that the observed gene expression changes translated to reduced FGF23 protein expression (Figure 1C-D).

Severe phosphate restriction attenuates tubular injury

To verify that persistent elevation of circulating FGF23 levels in 8 week-old, phosphate-restricted KO mice did not result from a more rapid progression of renal injury in these groups, we evaluated renal histology at 8 weeks in KO and WT mice consuming all three study diets (Figure 2A). Control KO mice demonstrated early glomerulosclerosis and interstitial inflammation with massive tubular dilation. Interestingly, KO mice consuming the 0.02% phosphate diet exhibited similar degrees of glomerulosclerosis; however, tubulointerstitial injury was strikingly reduced. Quantification of glomerular (Figure 2B) and tubulointerstitial (Figure 2C) injury supported these observations.

Chronic phosphate restriction leads to reduced bone mineralization

We were surprised to find that phosphate restriction resulted in further hyperphosphatemia and frank hypercalcemia in KO mice. We hypothesized that chronic dietary phosphate depletion led to a release of calcium and phosphate from bone in these mice. To confirm this theory, we performed μCT and ash studies on femurs from Col4a3 mice and observed a

stepwise reduction in both cortical (Figure 3A-B) and trabecular (Figure 3C-D) bone mineral density, as well as total bone mineral content (Figure 3E), in mice consuming diets of lower phosphate content. The extent of bone destruction was substantial in animals receiving the 0.02% phosphate diet.

Impact of phosphate restriction on renal Cyp27b1 and Cyp24a1 gene expression

Cyp27b1 and Cyp24a1 are the primary renal enzymes regulating $1,25(OH)_2D$ production and inactivation, respectively. Since WT and KO mice receiving the 0.02% phosphate diet demonstrated a marked elevation of serum $1,25(OH)_2D$ levels by 8 weeks, we used qRT-PCR to study the impact of severe phosphate restriction on renal Cyp27b1 and Cyp24a1 gene expression in KO and WT mice ingesting the control and 0.02% phosphate diets. At baseline, kidneys from KO mice eating a control diet exhibited a 3.5-fold higher Cyp24a1 gene expression compared to WT mice consuming the same diet ($P<0.05$) (Figure 4B). Interestingly, renal Cyp27b1 expression was similar in these two groups (Figure 4A). Severe phosphate restriction stimulated renal Cyp27b1 gene expression in KO and WT mice (Figure 4A) and reversed the over-expression of Cyp24a1 in KO mice ($P<0.01$ compared to control) (Figure 4B). Of note, phosphate depletion in KO mice returned Cyp24a1 gene expression to levels similar to WT mice consuming the control diet.

Effect of phosphate restriction on renal klotho gene expression

Klotho is a co-receptor for FGF23 that may also possess an independent role to regulate phosphate handling in the kidney. We compared whole kidney klotho gene expression at 8 weeks (Figure 5A) and 12 weeks (Figure 5B) in WT and KO mice ingesting the three study diets. Baseline renal klotho gene expression was markedly suppressed in KO mice eating the control diet compared to WT mice at 12 weeks ($P<0.01$). KO mice consuming a 0.02% phosphate diet demonstrated a 2-fold higher renal klotho gene expression compared to control diet KO mice at 12 weeks ($P<0.01$). An increase in klotho gene expression was also observed in WT mice in response to severe phosphate restriction ($P=0.001$ vs. WT control at 8 weeks).

DISCUSSION

Phosphorus plays a key role in fundamental cellular functions including energy metabolism, acid-base regulation, and intracellular signaling; consequently, the circulating pool of this essential mineral is tightly regulated to ensure cellular availability and adequate reserves. Traditionally, PTH and vitamin D were considered the primary regulators of phosphate homeostasis in humans, but the discovery of FGF23 revealed phosphate regulation to be far more complex than once imagined. Since the kidneys have a dominant role in fine tuning plasma levels and the total body content of phosphorus, renal injury would be expected to have a deleterious impact on many vital cellular processes. Not surprisingly, much of the morbidity and mortality observed in patients with CKD associates positively with the abnormal regulation of phosphate homeostasis.[11-13]

While there is growing recognition of the potential contributions of aberrant phosphorus metabolism to excessive morbidity and mortality in patients with CKD, *in vivo*

investigations of the complex pathways regulating phosphorus balance have proven difficult in humans. A significant barrier is a difficulty obtaining adequate tissue samples to examine changes in cellular metabolism and pathophysiology. Likewise, many chronic renal diseases run a course covering years to decades, hindering longitudinal metabolic studies in these patients. Fortunately, the development of genetically altered rodent models that exhibit a consistent pattern of chronic renal injury, such as Col4a3 null mice, affords an opportunity to investigate the pathophysiology of phosphate regulation in the initial stages of renal damage in a relatively predictable disease setting.

Previously, we observed that Col4a3 null mice exhibited a mineral metabolism profile that recapitulates the changes found in humans with CKD.[10] In the current study, we tested a popular hypothesis that chronic phosphate retention accompanying a loss of functioning nephrons is responsible for the progressive rise in circulating FGF23 levels in CKD by studying the impact of moderate and severe phosphate restriction in this model. The hypothesis that early phosphate restriction would delay or prevent the progressive rise in FGF23 in CKD was not supported by the results of this study. Chronic phosphate restriction initiated before the onset of detectable renal dysfunction (~ 5 weeks-of-age in these mice)[14,15] and continued until 8 weeks-of-age did not alter circulating FGF23 levels (Table 1). Similar to humans with a steady decline in GFR, Col4a3 null mice consuming a phosphate replete diet exhibited an increase in the fractional excretion of phosphate (FE_{phos}) compared to wild-type littermates. It is important to note that both phosphate-restricted diets suppressed the FE_{phos} to very low levels (≤3.5% in KO mice consuming phosphate-depleted diets), but did not lead to clear-cut alterations of circulating FGF23 levels by 8 weeks-of-age. Likewise, the failure of phosphate restriction to lower FGF23 levels at this time point is unlikely to have resulted from a more rapid progression of renal injury in phosphate-restricted KO mice, as severe phosphate restriction (0.02% phosphate) resulted in less tubular injury compared to mice ingesting a control diet (Figure 2). Thus, a decrease in renal phosphate load does not lead to a reduction of circulating FGF23 levels in this model of progressive CKD, evidence that weighs against the notion that phosphate retention is the main driver underlying successive increments in FGF23 with progressive renal injury.

Although chronic phosphate restriction failed to alter serum levels of FGF23 in Col4a3 null mice by 8 weeks, serum FGF23 levels were suppressed at 12 weeks in some KO mice consuming the 0.02% phosphate diet (Figure 1A). In support of the idea that circulating FGF23 primarily originates from bone, these low levels of serum FGF23 were accompanied by a suppression of FGF23 mRNA and protein expression in bone (Figure 1B-D). Of note, while chronic consumption of the 0.02% phosphate diet suppressed bone production of FGF23, it was associated with a substantial reduction in bone mineralization and integrity (Figure 3). While the direct cause for suppressed FGF23 levels in these mice is unclear from our studies, we suspect that this observation did not directly result from phosphate restriction alone, but rather from secondary metabolic changes that developed as a consequence of chronic, severe negative phosphate balance. We speculate that alterations in bone physiology may have contributed to this finding, but future studies will be needed to explain this consequence of profound phosphate depletion.

Another unexpected finding in this study was that serum phosphate levels increased further and frank hypercalcemia developed in phosphate-restricted KO mice. The progressive decline in bone mineralization in phosphate-restricted mice implies that a release of calcium and phosphate from bone could explain the abnormal levels of these minerals in serum (Figure 3). However, hypercalcemia and hyperphosphatemia were only observed in KO mice that developed progressive CKD and not WT animals, despite similar reductions in bone mineral content and a comparable urinary fractional excretion of phosphate. An alternative explanation for the elevated serum calcium and phosphate levels could be that the intestinal absorption of dietary phosphate and calcium was higher in the KO mice. Unfortunately, the current studies were not designed to test the impact of phosphate-restriction on intestinal calcium and phosphate transport, so it remains difficult to elucidate the mechanisms responsible for the observed increase in serum levels of these minerals.

In addition to promoting phosphaturia to maintain serum phosphate levels in the normal range, FGF23 has been implicated as a contributor to the development of 25(OH)D and $1,25(OH)_2D$ deficiencies in CKD.[16,17] In support of this hypothesis, Col4a3 null mice consuming a phosphate replete diet exhibited a progressive decline in $1,25(OH)_2D$ levels that appeared to correlate with the rise in serum FGF23. However, we found a substantial rise in serum $1,25(OH)_2D$ levels following severe phosphate restriction in Col4a3 null mice, that occurred despite persistent FGF23 elevations and an apparent decrease in circulating PTH levels at 8 weeks (Table 1). While FGF23 may contribute to alterations in vitamin D metabolism and parathyroid physiology in Col4a3 null mice, it appears that phosphate restriction can override much of the control of FGF23 on these pathways. Our analysis of changes in renal gene expression for vitamin D-regulatory enzymes revealed that Col4a3 null mice ingesting a phosphate-replete diet over-expressed the gene encoding the 24-hydroxylase enzyme that inactivates circulating vitamin D metabolites (Figure 4). Also of importance, Col4a3 null mice did not exhibit a decrease in the Cyp27b1 gene that encodes the 1α-hydroxlase enzyme which is responsible for the conversion of 25(OH)D to the active $1,25(OH)_2D$ hormone. These observations provide support for previous investigations suggesting that failing kidneys retain some capacity for the generation of $1,25(OH)_2D$ and that over-activity of the 24-hydroxylase pathway is a significant contributor to the high prevalence of vitamin D deficiency in CKD.[18,19] While it has been well-documented in past studies that dietary phosphate restriction stimulates $1,25(OH)_2D$ production,[20-22] to our knowledge this is first study to suggest that in CKD this regulation can occur independent of circulating FGF23 levels.

If phosphate retention is not the sole cause for high serum FGF23 concentrations in CKD, as shown by our data, then what elevates FGF23 in this setting? PTH, calcitriol, calcium and increased bone turnover have all been suggested as possible regulators of FGF23 metabolism,[3,4,23] yet evidence identifying a causative relationship between these pathways and FGF23 increments in CKD is lacking. Perhaps klotho, a known co-receptor for FGF23 actions in the kidney, is a promising candidate. Interesting in this regard is the finding that the membrane-bound and secreted forms of klotho appear to progressively decline in CKD.[10,24] Moreover, this decline surfaces in the earliest stages of kidney injury, perhaps prior to increments in FGF23. It is tempting to speculate that such a scenario could lead to an end-organ resistance to FGF23 actions and the subsequent up-regulation of mechanisms

causing the retention of intact FGF23 in the circulation. Recent evidence shows that secreted klotho independently promotes phosphaturia by blocking sodium-phosphate co-transporters in the proximal tubule,[25] leading to the possibility that renal klotho expression could also be directly regulated by phosphate balance in the body. As a secondary analysis, we tested whether chronic phosphate restriction in Col4a3 null mice could alter the progressive decline in renal klotho gene expression. We observed a significant increase in klotho gene expression in both WT and KO mice consuming the 0.02% phosphate diet (Figure 5). Similar to a previous report that phosphate restriction increases renal klotho expression in klotho-hypomorphic mice,[26] our data implies that phosphate restriction can increase klotho gene expression in CKD. Since prior studies in transgenic mice with normal renal function and FGF23 over-expression exhibit low levels of renal klotho gene expression,[27] it is conceivable that the observed increase in renal klotho gene expression with severe phosphate restriction resulted from the suppression of circulating FGF23, as opposed to a direct effect of phosphate itself. Although we have insufficient information to implicate an underlying mechanism, our data may be interpreted to indicate that severe phosphate restriction may improve, but not normalize, renal klotho gene expression in CKD.

Although the major findings in this study seem relatively clear-cut, there are several limitations. First, we acknowledge that our conclusions are based on a single rodent model of CKD and that studies in other CKD models are necessary to verify a universal application of our results to all CKD states. Second, while $1,25(OH)_2D$ levels significantly increased in association with severe phosphate restriction in this model and changes in renal gene expression indicate this observation to be largely due to alterations in 24-hydroxylase activity, we reserved measurements of renal 24-hydroxylase and 1α-hydroxylase enzyme activities to future studies. Third, measurement of serum FGF23 levels in older Col4a3 null mice required significant dilution of serum, which may have contributed to the observed variability in serum FGF23 levels in these mice. Lastly, while phosphate restriction does appear to increase renal klotho gene expression in Col4a3 null mice, it is unclear if this translates to changes in klotho protein expression or soluble klotho levels, as these were not quantified here.

In summary, observations in Col4a3 mice demonstrate that dietary phosphate restriction reduces urinary phosphate excretion independent of serum FGF23 levels in CKD. Long-term, severe phosphate restriction may ultimately suppress FGF23 production in CKD, although this is likely an indirect effect of chronic negative phosphate balance and cellular phosphate deficiency. Evidently phosphate restriction can raise serum$1,25(OH)_2D$ levels independent of effects on FGF23, and increase renal klotho gene expression in CKD. Although FGF23 is a powerful phosphaturic hormone, it appears that phosphate retention is not the primary driver for increments in circulating FGF23 with progressive kidney injury.

METHODS

Animal Preparation and Study Protocol

All mice were maintained in accordance with recommendations in the “Guide for Care and Use of Laboratory Animals,” from the Institute on Laboratory Animal Resources, National Research Council (National Academy Press, 1996), and all animal protocols were reviewed

and approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee prior to the commencement of this research. Mice containing a targeted deletion of the NC1 domain of the α3(IV) collagen chain[28] were originally obtained from Jackson Laboratory (strain 129-Col4a3^{tm1Dec}, Bar Harbor, ME, USA). Wild-type littermates of the Col4a3 null mice were used as the control groups for all experiments in this study. Mouse diets were created by Harlan Teklad (Madison, WI) and consisted of purified, phytate-free ingredients containing equal amounts of calcium (0.6%), vitamin D (3000 IU), and protein (200g) per kg of diet. The phosphate content of the diets was 0.54% for the control diet, 0.2% for the phosphate-depleted diet, and 0.02% for the phosphate-deficient diet. While both the control and 0.2% phosphate diet utilized a casein-based protein source, an egg white solid protein source was required to reduce the phosphorus content to the 0.02% level in the phosphate-deficient diet. Study mice were generated by mating Col4a3$^{+/-}$ males and females to obtain the desired genotypes. Col4a3 null and wild-type mice were enrolled at weaning (3 weeks-of-age) and placed on one of 2 separate diets (control or phosphate-deficient diet) and were sacrificed at one of two predetermined collection time points of 8 and 12 weeks-of-age. Since the measured serum parameters and observed phenotype were identical between Col4a3$^{+/-}$ and Col4a3$^{+/+}$, their values were combined for data analysis. Mice were housed in metabolic cages for urine collection beginning 12 hours prior to blood and tissue collection. All mice were anesthetized with pentobarbital (50mg/kg) prior to euthanasia by exsanguination.

Tissue Processing

Kidneys were fixed in 4% paraformaldehyde for 24 hours, embedded in paraffin, and cut into 4-Im sections. Kidney images were acquired from mid-sagittal sections of whole kidneys stained by the periodic-acid Schiff (PAS) method (Spot v5.0; Diagnostic Instruments, Sterling Heights, MI). Total area and area of tubulointerstitial injury (microcysts, tubular necrosis and atrophy) were measured using ImageJ64 (http://rsb.info.nih.gov). The tubulointerstitial injury index was calculated by dividing injured area by the total area. Glomerular injury (including sclerosis, necrosis, and crescent formation) was assessed by light microscopy and reported as the percentage of total glomeruli in a mid-sagittal section showing light microscopic evidence of injury. Kidney specimens for gene expression analysis were snap frozen in liquid nitrogen and stored at −80°C until further processing.

Serum and Urine Biochemistries

Serum and urine calcium (Ca) were measured using the Liquicolor kit (Stanbio Lab, Boerne, TX) and phosphorus (Pi) was measured by the phosphomolybdate–ascorbic acid method. Fractional excretion of phosphorus was calculated using the formula: [(urine phosphorus in mg/dl)/(serum phosphorus in mg/dl)]/[(urine creatinine in mg/dl)/(serum creatinine in mg/dl)] × 100. Intact FGF23 was measured by ELISA (Kainos Lab, Tokyo), serum 1,25$(OH)_2$D by the IDS EIA (Tyne and Wear, UK), PTH by the Mouse Intact PTH ELISA Kit (Alpco Diagnostics, Salem, NH), and urine and serum creatinine by the University of Texas Southwestern Mouse Phenotyping Core using a Vitros 250 analyzer (Ortho-Clinical Diagnostics, Rochester, NY).

Quantitative Real-time PCR and Western Blot Analyses

Quantitative RT-PCR analyses were performed using previously described methods.[10] See supplemental methods for gene primer sequences, as well as a detailed description of Western blot and immunoprecipitation protocols.

Bone Ash and μCT Studies

For ash studies, whole femurs were dried at 100°C for 24 hours prior to obtaining dry weight. Samples were next baked at 600°C for 24 hours. Inorganic ash was weighed and % mineral content was calculated by the formula: ash weight/dry weight ×100. Micro-CT analysis of whole femurs was performed using the μCT40 analyzer (Scanco Medical) as previously described.[29]

Statistical Analysis

Differences between multiple groups were evaluated by one-way ANOVA, while differences between two groups were evaluated by two-sided Student's t-test (for data with a Gaussian distribution) or Mann-Whitney test (for data with a non-Gaussian distribution). Computations were performed using Prism 5 software (GraphPad Software, San Diego, CA) and presented as mean ± SEM unless otherwise specified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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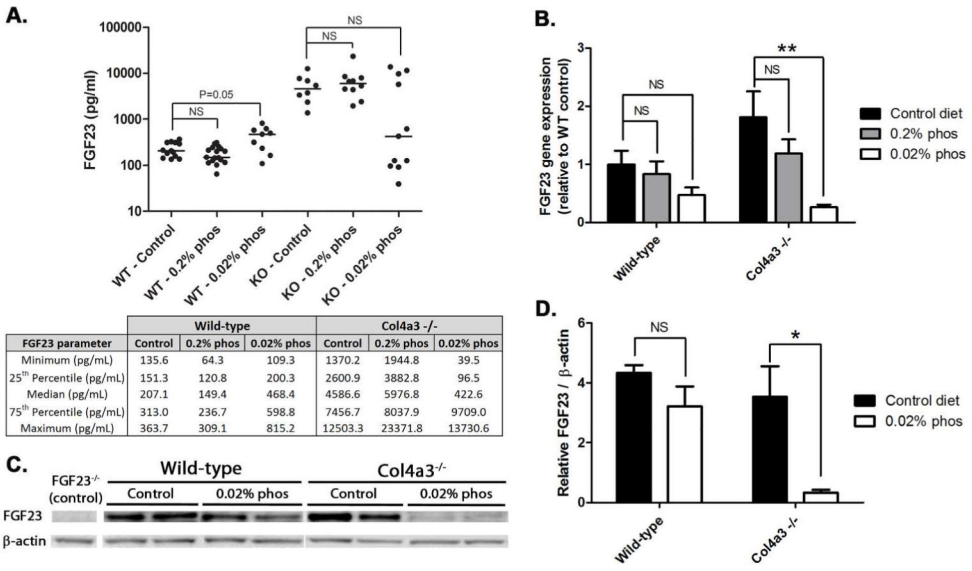

FGF23 parameter	Wild-type			Col4a3 -/-		
	Control	0.2% phos	0.02% phos	Control	0.2% phos	0.02% phos
Minimum (pg/mL)	135.6	64.3	109.3	1370.2	1944.8	39.5
25th Percentile (pg/mL)	151.3	120.8	200.3	2600.9	3882.8	96.5
Median (pg/mL)	207.1	149.4	468.4	4586.6	5976.8	422.6
75th Percentile (pg/mL)	313.0	236.7	598.8	7456.7	8037.9	9709.0
Maximum (pg/mL)	363.7	309.1	815.2	12503.3	23371.8	13730.6

Figure 1. Effects of chronic phosphate restriction on calvarial FGF23 gene and protein expression in Col4a3 null mice

(A) Graphical representation and statistical data of serum FGF23 levels for individual wild-type (WT) and Col4a3 null (KO) mice at 12 weeks demonstrating persistent elevation of serum FGF23 in KO mice consuming both a control and 0.2% phosphate diet; conversely, many KO mice receiving the 0.02% diet demonstrated a marked suppression of serum FGF23 levels at 12 weeks. **(B)** Quantitative RT-PCR was performed on calvaria from KO and WT mice at 12 weeks-of-age on all three diets. KO mice consuming a control diet exhibited an approximate 2-fold increase in FGF23 gene transcription compared to WT mice on the same diet (P=0.09). Severe dietary phosphate restriction in KO mice suppressed FGF23 gene expression to levels below those found in the WT mice on a similar diet (**P=0.001; n≥5 per group). **(C)** Representative images of Western blot for FGF23 protein expression in calvaria from KO and WT mice receiving the control and 0.02% phosphate diets (2 per group). **(D)** Quantification of calvarial FGF23 protein expression from Western blot analysis demonstrates a marked reduction in FGF23 expression in KO mice consuming the 0.02% phosphate diet (*P<0.05; n=4 per group).

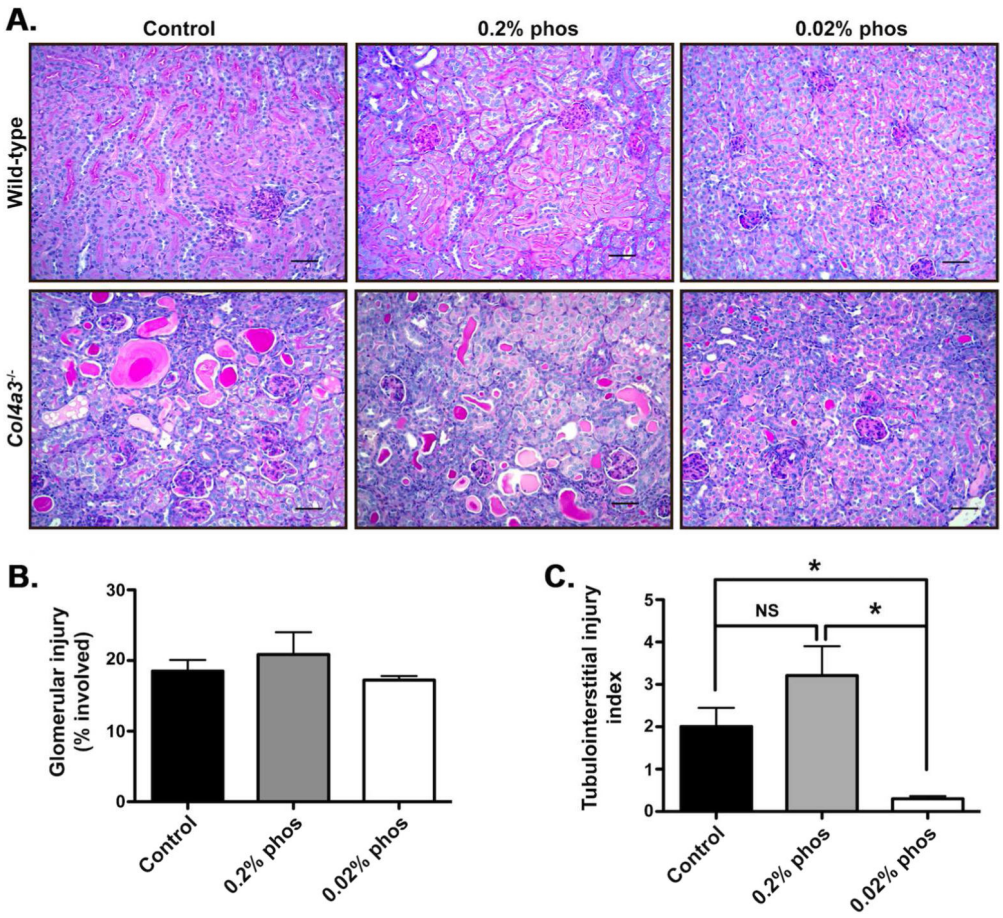

Figure 2. Phosphate deficient diet prevents microcystic tubular injury in Col4a3 null mice

(A) PAS staining of mid-sagittal kidney sections from Col4a3 null (KO) and wild-type (WT) mice on control, 0.2% or 0.02% phosphate diets. KO mice consuming the control and 0.2% phosphate diet exhibited interstitial inflammation, tubular dilation and cast formation, while KO mice receiving the 0.02% phosphate diet demonstrated less severe inflammation and tubular injury. **(B)** Glomerular injury scoring in KO mice fed either a control, 0.2% or 0.02% phosphate diet (P=NS, n=3 per group for all measurements). **(C)** Tubulointerstitial injury index (see Methods) in KO mice fed a control, 0.2% or 0.02% phosphate diet (*P<0.05).

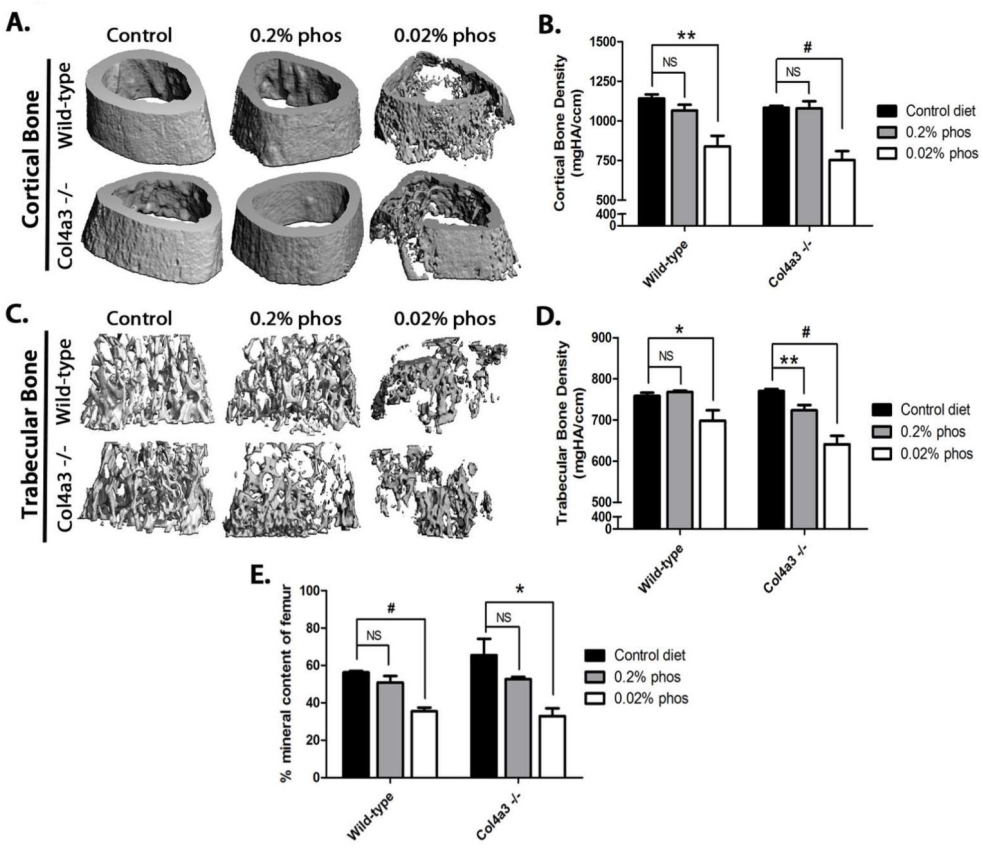

Figure 3. Chronic phosphate restriction leads to bone mineralization defects in Col4a3 mice

Micro-CT (μCT) images of femurs from Col4a3 wild-type (WT) and null (KO) mice reveal a step-wise decrease in both cortical **(A-B)** and trabecular **(C-D)** bone mineral density with increased extent of phosphate restriction (n=6 per group; *P<0.05, **P<0.01, #P<0.001). **(E)** Bone ash studies of whole femurs from Col4a3 mice confirm total mineral content is decreased in mice consuming phosphate-restricted diets (n=3 per group; *P<0.05, #P<0.001).

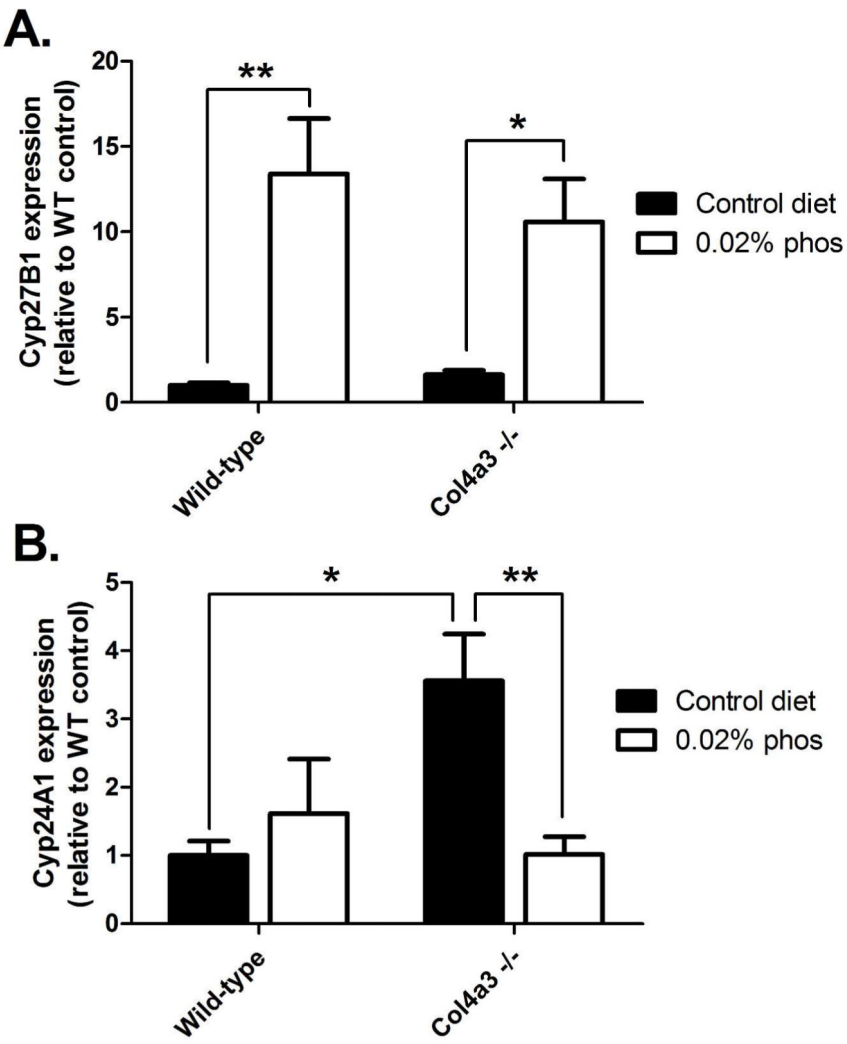

Figure 4. Changes in renal Cyp27b1 and Cyp24a1 expression in Col4a3 mice in response to chronic phosphate restriction

Kidney specimens from 8 week-old Col4a3 null (KO) and wild-type (WT) mice receiving either a control or 0.02% phosphate diet were analyzed by qRT-PCR for differences in Cyp27b1 and Cyp24a1 gene expression. **(A)** On the control diet, KO mice exhibited Cyp27b1 gene expression comparable to WT mice. Chronic phosphate restriction markedly increased Cyp27b1 gene expression in both KO and WT mice (*P<0.05, **P<0.01). **(B)** Cyp24a1 gene expression was 3.5-fold higher in KO mice compared to WT mice on the control diet (*P<0.05). Severe phosphate restriction suppressed Cyp24a1 gene expression to levels comparable to those observed in WT mice on the phosphate-deficient diet (**P<0.01; n≥5 per group for each analysis).

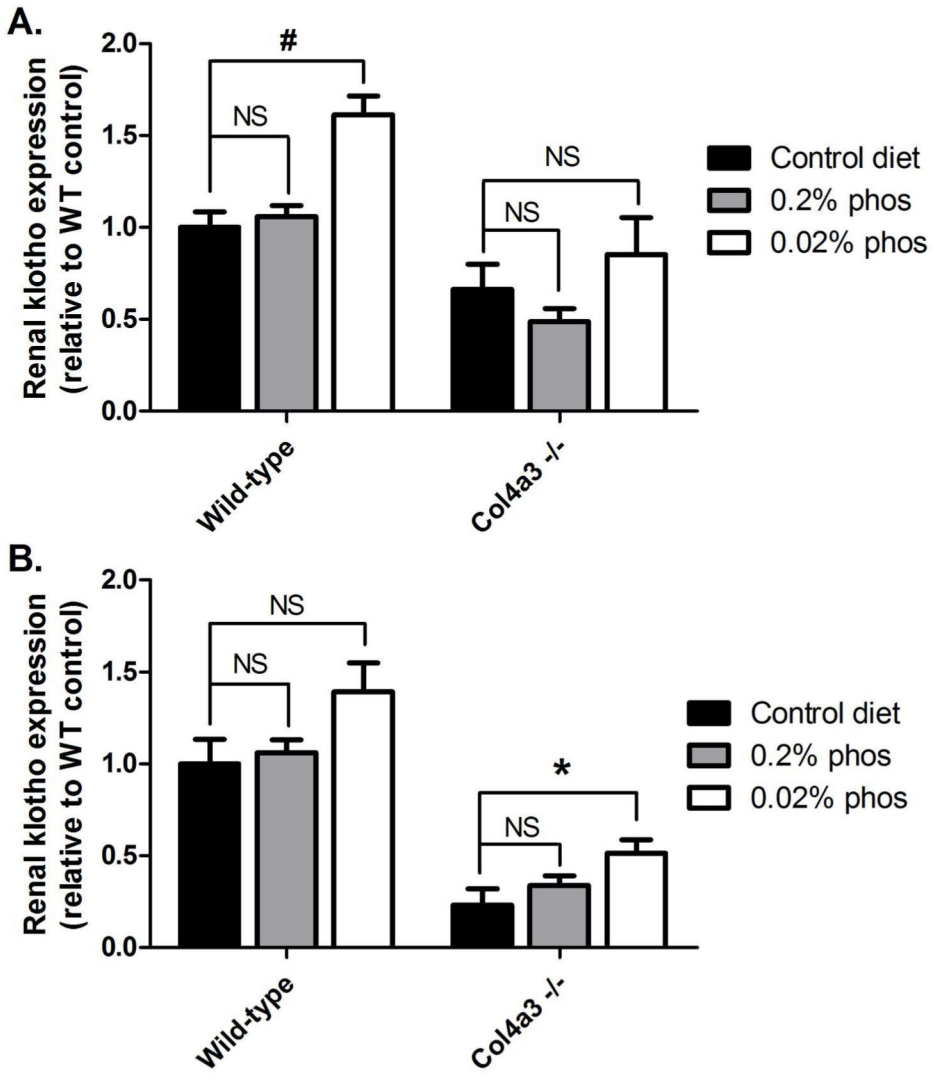

Figure 5. Effect of phosphate restriction on renal klotho gene expression in Col4a3 mice

Kidney specimens from Col4a3 null (KO) and wild-type (WT) mice consuming the control and phosphate-restricted diets were analyzed by qRT-PCR for differences in klotho gene expression at **(A)** 8 weeks and **(B)** 12 weeks of age. At baseline, KO mice on a control diet exhibited 40% lower levels of klotho gene expression compared to WT littermates on the same diet at 8 weeks (P=0.06) and a 77% lower klotho gene expression by 12 weeks (P<0.001). Moderate phosphate restriction (0.2%) had no obvious impact on renal klotho gene expression in WT or KO mice at either 8 or 12 weeks of age. On the other hand, severe phosphate restriction (0.02%) was associated with a 60% greater renal klotho gene expression in WT mice at 8 weeks when compared to control diet WT mice ($^{\#}$P=0.001) and a 2-fold greater klotho gene expression in KO mice at 12 weeks in relation to control diet KO mice (*P=0.05). Despite an apparent increase in renal klotho gene expression in KO mice with severe phosphate restriction, klotho gene expression remained suppressed

compared to WT mice on the same diet. ($P<0.01$ for KO 0.02% phos vs. WT 0.02% phos; $n\geq4$ per group for all groups).

Table 1

Serum & urine biochemistries in Wild-Type & Col4a3$^{-/-}$ mice

	Control Diet		Phos-restricted Diet (0.2%)		Phos-deficient Diet (0.02%)	
	WT	**KO**	**WT**	**KO**	**WT**	**KO**
8 weeks old						
Serum Phos (mg/dl)	6.5 ± 0.4	8.6 ± 0.4^{a}	6.6 ± 0.3	13.1± 1.5^{ab}	7.9 ± 0.5	10.9 ± 0.7^{ab}
Serum Ca (mg/dl)	8.2 ± 0.1	9.0 ± 0.2^{a}	7.8 ± 0.5	9.2 ± 0.2^{a}	8.2 ± 0.1	8.6 ± 1.0
PTH (pg/ml)	81.3 (22.5 - 205.6)	703.7 (211.9 - 1985.0)^{a}	129.4 (51.4 - 434.7)	795.3 (552.5 - 1260.0)^{a}	45.36 (10.1 - 213.5)	327.2 (104.4 - 500.4)^{ab}
1,25$(OH)_2$D (pmol/l)	188.4 ± 25.8	136.5 ± 12.0	102.4 ± 15.1	121.1 ± 23.6	943.4 ± 304.3	1347.5 ± 407.0^{b}
FGF23 (pg/ml)	169.2 (134.3 - 238.0)	481.3 (186.9 - 3663.4)^{a}	322.8 (150.3 - 553.6)	1472.5 (277.2 – 3003.0)^{a}	147.5 (62.3 – 290.8)	1349.1 (116.3 - 2190.2)^{a}
Serum Cr (mg/dl)	0.25 ± 0.1	0.44 ± 0.2^{a}	0.29 ± 0.02	0.36 ± 0.01^{a}	0.28 ± 0.2	0.82 ± 0.25
Urine FE_{phos} (%)	15.1 ± 4.0	33.4 ± 6.1^{a}	0.8 ± 0.1	1.24 ± 0.3^{a}	0.7 ± 0.2	1.6 ± 0.3^{ab}
12 weeks old						
Serum Phos (mg/dl)	6.7 ± 0.5	17.8 ± 2.4^{a}	6.1 ± 0.2	11.8 ± 1.0^{ab}	7.5 ± 0.9	8.9 ± 1.8^{b}
Serum Ca (mg/dl)	8.7 ± 0.2	7.3 ± 0.5^{a}	7.9 ± 0.1	10.3 ± 0.4^{ab}	8.0 ± 0.6	11.6 ± 1.1^{ab}
PTH (pg/ml)	207.1 (10.2 -1103.5)	5117.7 (1127.7 – 6400.0)^{a}	123.1 (28.7 – 294.1)	786.2 (122.9 – 3237.3)^{ab}	100.8 (9.0 – 286.9)	2037.6 (745.2 – 3816.0)^{ab}
1,25$(OH)_2$D (pmol/l)	175.7 ± 31.9	64.1 ± 29.8^{a}	156.5 ± 56.1	34.2 ± 12.0^{a}	969.9 ± 256.2	192.0 ± 71.7^{a}
FGF23 (pg/ml)	207.1 (135.6 - 363.7)	4586.6 (1370.2 - 12503.3)^{a}	149.5 (64.3 - 309.1)	5976.8 (1944.8 – 23371.8)^{a}	468.4 (109.3 - 815.2)	422.6 (39.5 – 13730.6)
Serum Cr (mg/dl)	0.44 ± 0.04	1.27 ± 0.23^{a}	0.27 ± 0.01	0.61 ± 0.04^{ab}	0.47 ± 0.12	1.43 ± 0.48
Urine FE_{phos}	40.7 ± 6.9	45.2 ± 4.9	0.8 ± 0.1	1.8 ± 0.2^{ab}	1.2 ± 0.3	3.5 ± 1.8^{b}

FGF23 and PTH are presented as median (min - max) values; all other measurements presented as mean ± SEM

n ≥ 8 per group for FGF23 measurements, n ≥ 5 mice per group for all other parameters

Abbreviations: Phos, phosphorus; Ca, calcium; PTH, parathyroid hormone; FGF23, fibroblast growth factor 23; Cr, creatinine; FEphos, fractional excretion of phosphorus, KO, Col4a3$^{-/-}$; WT, wild-type

^{a}Statistically different (P<0.05) compared to WT on same diet at same age

[b] Statistically different (P<0.05) compared to KO on control diet at same age