Interactions between calcium and phosphorus in the regulation of the production of fibroblast growth factor 23 in vivo

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[1]*Division of Endocrinology, Diabetes, and Hypertension, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts;* [2]*Department of Molecular Drug Research, Faculty of Pharmaceutical Sciences, University of Copenhagen, Copenhagen, Denmark;* [3]*Renal Division, Beth Israel-Deaconess Medical Center, Boston, Massachusetts;* and [4]*Calcium Research Laboratory, Department of Medicine, McGill University, Montreal, Quebec, Canada*

Quinn SJ, Thomsen AR, Pang JL, Kantham L, Bräuner-Osborne H, Pollak M, Goltzman D, Brown EM. Interactions between calcium and phosphorus in the regulation of the production of fibroblast growth factor 23 in vivo. *Am J Physiol Endocrinol Metab* 304: E310–E320, 2013. First published December 11, 2012; doi:10.1152/ajpendo.00460.2012.—Calcium and phosphorus homeostasis are highly interrelated and share common regulatory hormones, including FGF23. However, little is known about calcium's role in the regulation of FGF23. We sought to investigate the regulatory roles of calcium and phosphorus in FGF23 production using genetic mouse models with targeted inactivation of PTH (PTH KO) or both PTH and the calcium-sensing receptor (CaSR; PTH-CaSR DKO). In wild-type, PTH KO, and PTH-CaSR DKO mice, elevation of either serum calcium or phosphorus by intraperitoneal injection increased serum FGF23 levels. In PTH KO and PTH-CaSR DKO mice, however, increases in serum phosphorus by dietary manipulation were accompanied by severe hypocalcemia, which appeared to blunt stimulation of FGF23 release. Increases in dietary phosphorus in PTH-CaSR DKO mice markedly decreased serum 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] despite no change in FGF23, suggesting direct regulation of $1,25(OH)_2D_3$ synthesis by serum phosphorus. Calcium-mediated increases in serum FGF23 required a threshold level of serum phosphorus of about 5 mg/dl. Analogously, phosphorus-elicited increases in FGF23 were markedly blunted if serum calcium was less than 8 mg/dl. The best correlation between calcium and phosphorus and serum FGF23 was found between FGF23 and the calcium × phosphorus product. Since calcium stimulated FGF23 production in the PTH-CaSR DKO mice, this effect cannot be mediated by the full-length CaSR. Thus the regulation of FGF23 by both calcium and phosphorus appears to be fundamentally important in coordinating the serum levels of both mineral ions and ensuring that the calcium × phosphorus product remains within a physiological range.

fibroblast growth factor 23; calcium × phosphorus product; 1,25-dihydroxyvitamin D_3; parathyroid hormone; calcium-sensing receptor

CALCIUM AND PHOSPHORUS HOMEOSTASIS are highly dependent on one another, and changes in the serum level of either calcium or phosphorus will in many cases lead to secondary changes in the serum level of the other. Fibroblast growth factor 23 (FGF23) is a newly discovered hormone that is implicated in phosphorus homeostasis, and dysregulation of FGF23 may result in several disorders of phosphorus homeostasis (1, 24, 38). FGF23 is expressed in and released from osteoblasts and osteocytes in response to hyperphosphatemia and 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] (19, 20, 39). In the renal tubule, FGF23 binds to FGF receptors and their cofactor Klotho, causing inhibition of the expression of the sodium-dependent phosphate cotransporters Npt2a and Npt2c, thereby resulting in less renal phosphorus reabsorption (11, 21, 35, 40, 41). In addition, FGF23 inhibits expression of the 1α-hydroxylase enzyme, leading to less formation of $1,25(OH)_2D_3$ (28, 35, 37, 38).

Although a tight relationship between calcium and phosphorus homeostasis exists, possible regulation of FGF23 by calcium or vice versa is still poorly understood. Ablation of the FGF23 gene in mice causes high serum levels of phosphorus due to lack of inhibition of the Npt2a and Npt2c phosphate cotransporters in the renal proximal tubule but also produces elevated levels of serum calcium (37). These elevated levels of serum calcium are thought to be mediated by the lack of inhibition of 1α-hydroxylase expression by FGF23 in the renal proximal tubule, which increases the production and serum concentration of $1,25(OH)_2D_3$ (28, 37, 38). High serum $1,25(OH)_2D_3$ levels subsequently lead to increased intestinal absorption of calcium and can also enhance bone resorption. The opposite effect is seen in transgenic mice that overexpress an FGF23 mutant resistant to degradation (R176Q), where both serum calcium and phosphorus concentrations are low (3). In this case the low serum values can be explained by an inappropriate inhibition of Npt2a, Npt2c, and 1α-hydroxylase despite hypophosphatemia, which causes less renal reabsorption of phosphorus and less intestinal absorption of calcium. Additionally, it has been shown that FGF23 decreases parathyroid hormone (PTH) expression and secretion from the parathyroid glands, thereby affecting another calcium regulatory mechanism (4).

Shimada et al. (39) showed that wild-type (WT) and vitamin D receptor knockout mice kept on a high-calcium diet for 7 days exhibited an elevated level of serum calcium, and interestingly, they showed elevated FGF23 expression and release from bone. These effects were independent of $1,25(OH)_2D_3$ and serum phosphorus. However, a similar study using mice of the same genotype could not confirm these findings (14). The reason(s) underlying the differing results in these two studies is unknown. Of interest, a recent study demonstrated that FGF23 release is positively modulated by calcium in rats and that FGF23 release stimulated by phosphorus, PTH, or $1,25(OH)_2D_3$ is dependent on a normal calcium concentration (34).

Thus, there are a limited number of studies that indicate that FGF23 regulates serum calcium through interaction with calcium-elevating hormones and that FGF23 itself might be regulated by calcium. Since the calcium-sensing receptor (CaSR) is the major sensor of the extracellular calcium concentration in various tissues, including the parathyroid glands (5, 6, 12, 29), thyroidal C cells (15, 23, 26), and kidneys (9, 32, 33), it is possible that the receptor is involved in regulating the expression and release of FGF23. Recently, it has been shown that CaSR mice with conditional knockout of the CaSR in osteoblasts have impaired bone growth and mineralization (6), adding to the evidence that the receptor has essential functions in osteoblasts (7, 8, 43). It is possible that the CaSR in osteoblasts or in subsequently formed osteocytes is involved in the regulation of FGF23. Therefore, the objective of this study was to investigate the importance of the CaSR in regulating FGF23 release as well as to examine interactions between calcium and phosphorus homeostasis that might be important for FGF23 regulation using wild-type (WT), PTH knockout (PTH KO), and PTH-CaSR double-knockout (PTH-CaSR DKO) mice. In addition to CaSR, PTH is an important regulator of calcium and phosphorus homeostasis, and therefore, the usage of these PTH-CaSR KO genotypes also allows effective control of serum concentrations of calcium and phosphorus.

MATERIALS AND METHODS

Materials. $1,25(OH)_2D_3$ and primers for genotyping experimental mice were obtained from Sigma-Aldrich (St. Louis, MO). Commercial kits were used for measuring serum and urinary calcium levels (Eagle Diagnostics, De Soto, TX). Urinary and serum phosphorus concentrations were measured as phosphorus using the Phosphorus Liqui-UV Test kit (Stanbio Laboratory, Boerne, TX). Serum and urinary creatinine levels were measured using the Stanbio Creatinine Liquiclear Test (Endpoint/Enzymatic; Stanbio Laboratory), which had a sensitivity of 0.04 mg/dl. The sensitivity of the assay could be enhanced by increasing the sample volume and making appropriate adjustments in the volumes of the standard and zero wells. The mean values measured in our studies for each of the genotypes before and after a phosphate load by intraperitoneal (ip) injection or by adding phosphate to the drinking water (0.121–0.207 mg/dl) were very similar to those in a study of the normal serum creatinine levels in 12 different strains of mice (42). Serum $1,25(OH)_2D_3$ levels were determined using a competitive enzyme immunoassay (Immunodiagnostic Systems, Fountain Hills, AZ). Serum full-length intact FGF23 levels were determined using an FGF23 ELISA Kit (Kainos Laboratories).

Knockout mice. The experimental utility of WT, PTH KO, and PTH-CaSR DKO mice has been documented previously (9, 15). The use of the PTH KO and PTH/CaSR DKO mice allowed us to study a broad range of calcium and phosphorus concentrations (over an ∼3-fold range for each) and to modulate serum calcium largely independently of phosphorus and vice versa by the use of calcium or phosphate injections, which is very hard to achieve in WT mice. Furthermore, the absence of PTH in the knockout genotypes is an advantage since PTH can both respond to and modulate serum phosphorus concentration when CaSR-regulated PTH is intact. Thus these models allow us to isolate the effects of calcium or phosphorus on FGF23 independent of concomitant changes in the other mineral ion, PTH, and full-length CaSR, all of which could directly or indirectly modulate FGF23.

All animals were maintained in microisolator cages in a Brigham and Women's Hospital animal facility. We routinely genotyped and screened the mice biochemically to ensure the authenticity and stability of their genetic backgrounds and phenotypes. The PCR primers and genotyping protocols were the same as described previously (12, 27). All animals were fed a regular chow diet [0.8% calcium (wt/wt); Harlan Teklad, Madison, WI], a low-calcium diet [0.01% calcium (wt/wt); Harlan Teklad], or a low-phosphorus diet [0% phosphorus (wt/wt); Harlan Teklad] and plain water ad libitum. A high-phosphorus diet was defined as a combination of low-phosphorus diet and drinking water containing 25–100 mM phosphate ad libitum.

In vivo experiments. Animal protocols were approved by the Institutional Animal Care and Use Committee at Harvard Medical School and were in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. Mice were housed in microisolator cages in a pathogen-free facility according to the regulations of the Harvard Medical School Center for Animal Resources and Comparative Medicine.

Several sets of 4- to 8-mo-old male mice of all three genotypes (each "set" comprised 4 mice of each genotype) were kept on a phosphorus-deficient diet for 1 wk. Different dietary phosphorus loads were provided by adding 25, 50, or 100 mM sodium monobasic phosphate (pH 7) to the drinking water. Serum samples of hormones and minerals in mice that had been maintained for seven days on the various dietary phosphorus diets were obtained by cheek bleeding. Spot urine samples were likewise obtained.

Several sets of 4- to 8-mo-old male mice of all three genotypes were kept on a phosphorus-deficient diet for 1 wk. To raise serum phosphorus, the mice received a dose of 100 or 200 mM sodium monobasic phosphate (10 µl/g body wt ip) in the morning at 9 AM and again 8 h later for 7 consecutive days while being maintained on a phosphorus-deficient diet. Baseline serum and spot urine samples were obtained 1 wk prior to the start of the injections to allow the mice to recover. Serum samples and spot urines were likewise obtained after 7 days of ip injection.

Several sets of male mice of the three genotypes were maintained on a normal chow diet for 1 wk. To raise serum calcium, the mice received a dose of 50 mM calcium gluconate (17 µl/g body wt ip) in the morning at 9 AM and again 8 h later for 7 consecutive days while being maintained on the normal chow diet. As a control, the mice received two doses of 50 mM sodium gluconate (17 µl/g body weight ip) every day for 7 consecutive days. Baseline serum and spot urine samples were obtained 1 wk prior to the start of the injections to allow the mice to recover. Serum samples and spot urines were likewise obtained after 7 days of ip injection.

Several sets of male mice of all three genotypes were kept on a low-calcium diet. Serum samples were obtained from these mice at baseline after ≥1 wk on the low-calcium diet. A single daily dose of $1,25(OH)_2D_3$ (0.5 ng/g body wt) was administrated ip for 2 days. Serum samples were obtained 24 h after the first dose of $1,25(OH)_2D_3$ and again 24 h after the second dose of $1,25(OH)_2D_3$.

Several sets of male WT mice were kept on a phosphorus-deficient diet. Serum samples for determination of baseline PTH levels were obtained by cheek bleeding. In addition, the mice were gavaged with 300 µl of 0.4 M sodium monobasic phosphate, and serum samples of PTH were obtained 1 h later.

Statistics. All values are presented as means ± SE. Graphical illustrations and statistical analyses were performed using Microsoft Office Excel 2003. A value of $P < 0.05$ was considered to be a statistically significant difference.

RESULTS

Effect of high-phosphorus diet on FGF23 production. To investigate whether CaSR is involved in regulating FGF23 production, the ability of a high-phosphorus diet to increase the serum concentration of FGF23 was tested in male mice of the three different genotypes that we have used previously: WT, PTH KO, and PTH-CaSR DKO (9, 15). All three genotypes were maintained on a phosphorus-deficient diet for 7 days.

Phosphate was added to the diet using three concentrations of monobasic sodium phosphate in the drinking water (25, 50, and 100 mM) together with the phosphorus-deficient diet. On *day 7*, blood samples were drawn for serum analyses of calcium, phosphorus, creatinine, $1,25(OH)_2D_3$, and FGF23. Simultaneously, spot urines were obtained and analyzed for calcium, phosphorus, and creatinine. Serum phosphorus concentrations in all genotypes maintained with phosphate added to the drinking water were significantly elevated compared with the phosphorus-deficient diet (Fig. 1*A*). Urinary phosphorus normalized to urinary creatinine was significantly elevated with the 50 and 100 mM concentrations of monobasic sodium phosphate (Fig. 1*B*). Serum calcium concentrations were significantly reduced on all high-phosphorus diets for the PTH KO and PTH-CaSR DKO mice, whereas the WT mice showed

a reduced serum calcium concentration only at the 100 mM phosphate load (Fig. 1*C*). In addition, serum calcium levels were greatly reduced in PTH KO and PTH-CaSR DKO mice compared with the WT at the 50 and 100 mM phosphate loads. Moreover, serum calcium was substantially higher in the PTH-CaSR DKO mice vs. the other genotypes when receiving the phosphorus-deficient diet. These differences in serum calcium can be explained by changes in the bioavailable calcium load with increasing dietary phosphate loads as well as the effects of phosphorus depletion per se on the calcium homeostatic system (15). Urinary calcium normalized to urinary creatinine was significantly reduced at all phosphate loads (Fig. 1*D*).

FGF23 levels were significantly higher on the phosphorus-deficient diet for the CaSR-PTH DKO mice compared with the WT and PTH KO mice (Fig. 1*E*). FGF23 levels in serum were
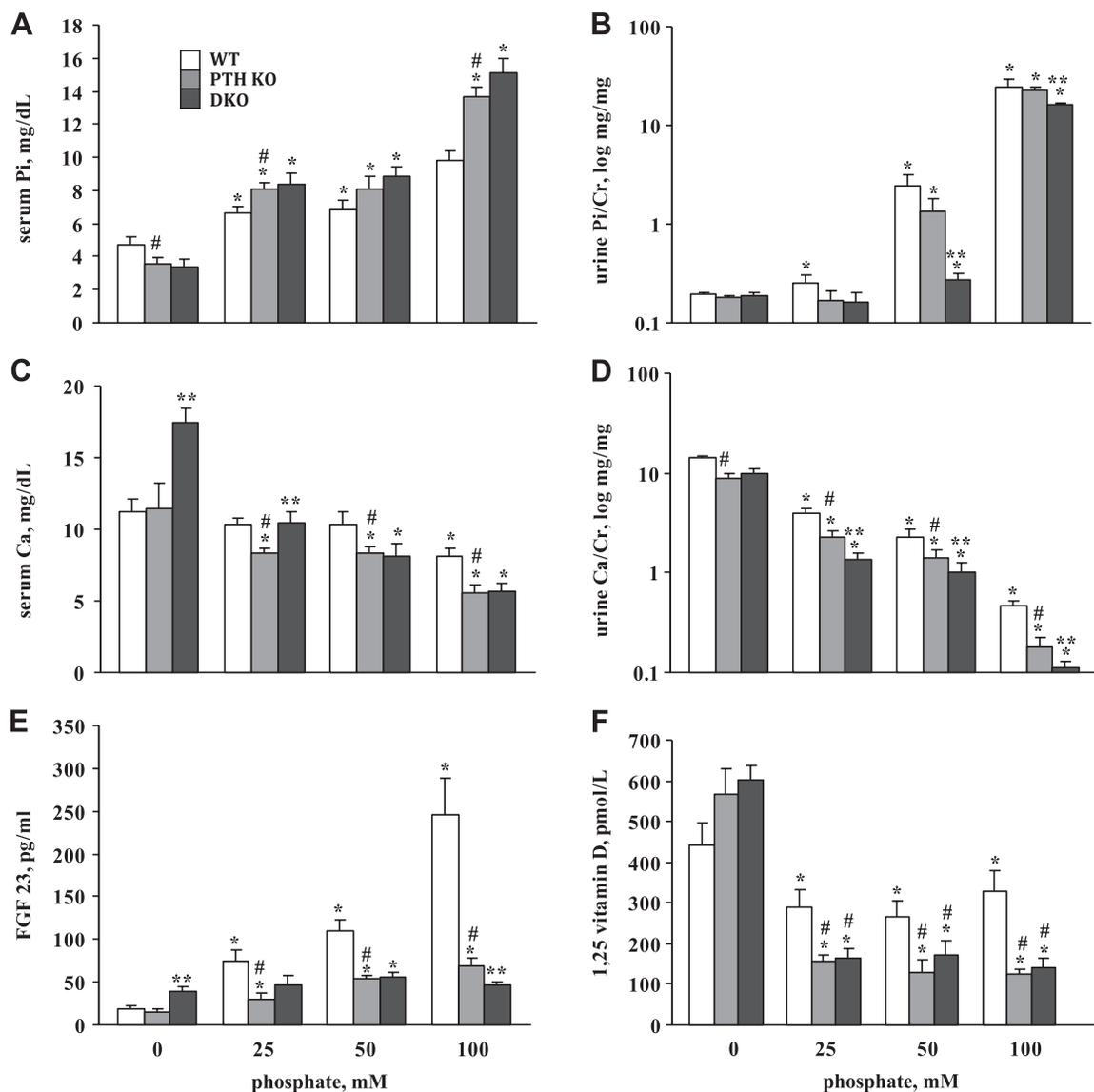

Fig. 1. Concentrations of calcium (Ca) and phosphorus (Pi) in sera and spot urines, serum fibroblast growth factor 23 (FGF23), and serum 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] on different dietary phosphate loads. All diets were maintained for 7 days for wild-type (WT; open bars), parathyroid hormone knockout (PTH KO; gray bars), and PTH calcium-sensing receptor double-knockout (PTH-CaSR DKO; black bars) mice. Urinary Ca and Pi were normalized to urinary creatinine (Cr). Values are means ± SE ($n = 6$–10/genotype). *$P < 0.05$ for comparison of values for phosphorus-deficient diet vs. different dietary phosphorus loads; #$P < 0.05$ for comparison of values for different dietary phosphorus loads of WT mice vs. PTH KO mice. **$P < 0.05$ for comparison of values of PTH KO vs. PTH-CaSR DKO mice. WT and PTH KO mice are the genetic controls for statistical comparisons with PTH KO and PTH-CaSR DKO mice, respectively.

significantly elevated at all phosphate loads for the WT and PTH KO mice. For the PTH-CaSR DKO mice, there was a small but significant increase in FGF23 only with the 50 mM phosphate load. FGF23 levels for WT mice were substantially elevated compared with PTH KO mice at all phosphate loads. Serum $1,25(OH)_2D_3$ concentrations were substantially reduced at all phosphate loads for the three genotypes relative to the baseline low-phosphorus condition (Fig. 1F). In addition, $1,25(OH)_2D_3$ levels for both PTH KO and PTH-CaSR DKO mice were significantly lower compared with WT mice at all phosphate loads. Furthermore, $1,25(OH)_2D_3$ concentrations at the three different phosphate loads were similar for each of the mouse genotypes. Thus the maximal effect of phosphate loading on serum $1,25(OH)_2D_3$ was already achieved with the lowest phosphate dose. Together, these results suggest that the full-length CaSR is involved in dietary phosphate-induced FGF23 production in some manner that is independent of concomitant changes in $1,25(OH)_2D_3$. However, the hypocalcemia found in the PTH KO and PTH-CaSR DKO mice could be a confounding factor in understanding the role of CaSR in the regulation of FGF23 production under these conditions, since the full-length CaSR is absent in the PTH-CaSR DKO mice and would be expected to be largely inactive with hypocalcemia in the PTH KO mice. Finally, serum creatinines were in the normal range (mean values between 0.121 and 0.207 mg/dl) in all groups of mice and did not differ significantly before and after oral phosphate loading as well as after the ip injections of phosphate (see next section), ruling out alterations in renal function as a cause of changes in FGF23.

Raising serum phosphorus concentration by ip injection of sodium monobasic phosphate. To better demonstrate the role of serum phosphorus per se in the control of FGF23 production in PTH KO and PTH-CaSR DKO mice and to better reveal the role of CaSR in FGF23 regulation, serum phosphorus levels were increased in mice of all three genotypes by ip injection of a solution of sodium monobasic phosphate. As described in MATERIALS AND METHODS, all mice were kept on the phosphorus-deficient diet, and serum phosphorus concentration was increased by giving 2 ip injections of sodium monobasic phosphate daily for 7 days using concentrations of 100 and 200 mM. Preliminary experiments revealed that injections of sodium monobasic phosphate produced an early peak (data not shown) and later a sustained elevation of serum phosphorus. Figure 2A shows that there was a chronic, significant increase of serum phosphorus in all three genotypes when measured on *day 7* on the morning after injection of the last dose. There was no consistent effect of phosphate injection on serum calcium levels in each genotype, which remained at or above the baseline levels (Fig. 2B). The approximately normal and substantially elevated serum calcium concentrations in the PTH KO and PTH-CaSR DKO mice, respectively, are likely due to the large bioavailable calcium load in the phosphorus-deficient diet, owing to the absence of calcium chelation typically found with phosphorus-replete diets, as noted earlier.

The PTH-CaSR DKO mice had a higher basal serum FGF23 concentration compared with the WT mice and the PTH KO mice on the phosphorus-deficient diet (Figs. 1E and 2C). Injection with sodium monobasic phosphate for 7 days caused the
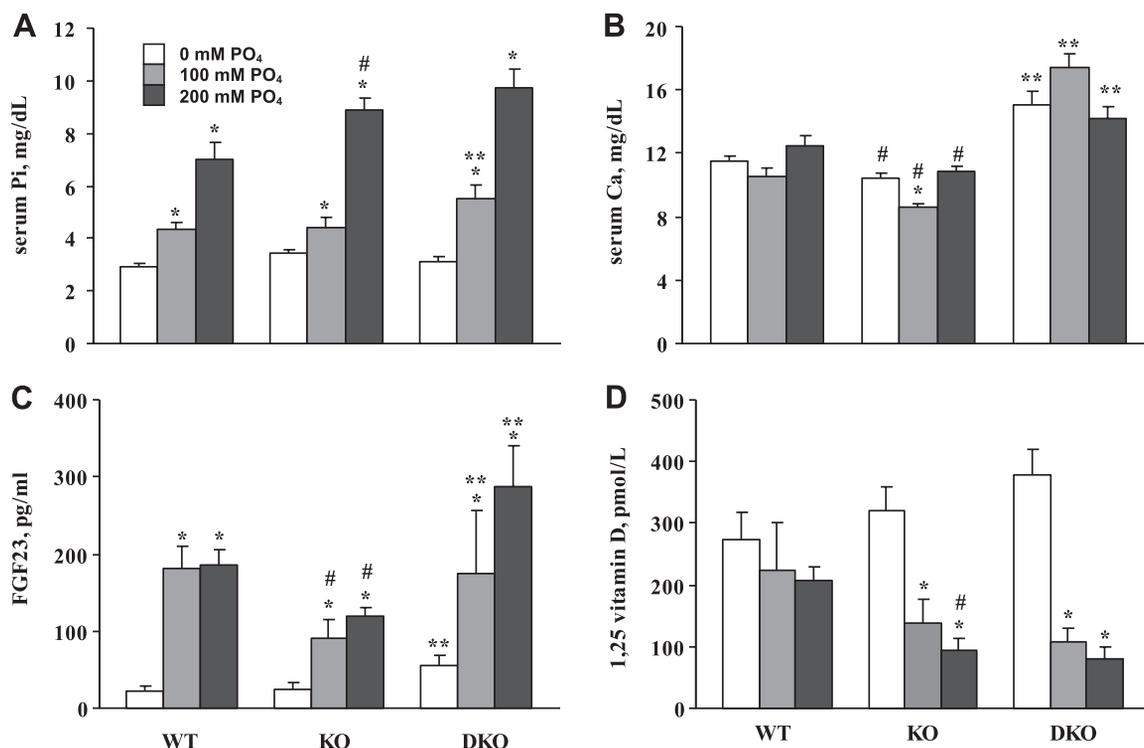

Fig. 2. Serum concentrations of Ca, Pi, FGF23, and $1,25(OH)_2D_3$ after intraperitoneal (ip) injection of sodium monobasic phosphate. Serum from WT, PTH KO, and PTH-CaSR DKO mice was collected at baseline (open bars) and after 7 days of treatment with 100 (gray bars) or 200 mM sodium monobasic phosphate (black bars). Values are means ± SE ($n = 6$–8/genotype). *$P < 0.05$ for comparison of values at baseline and following treatment; #$P < 0.05$ for comparison of values at baseline or following treatment of WT mice vs. PTH KO mice; **$P < 0.05$ for comparison of values of PTH KO vs. PTH-CaSR DKO mice.

serum concentrations of FGF23 to increase significantly in all three genotypes (Fig. 2*C*). Furthermore, serum FGF23 levels were significantly higher in the PTH-CaSR DKO than in the PTH KO mice, similar to the response of the WT mice. Baseline levels of 1,25(OH)$_2$D$_3$ were somewhat higher in the PTH-CaSR DKO mice compared with those in the WT and PTH KO mice (Fig. 2*D*). In addition, the phosphate injections caused significant reductions in serum 1,25(OH)$_2$D$_3$ concentrations in the PTH KO and PTH-CaSR DKO mice, with no significant change in WT mice (Fig. 2*D*). These reductions in 1,25(OH)$_2$D$_3$ were consistent with the observed increases in FGF23. Therefore, our results show that elevating serum phosphorus by ip phosphate injection causes FGF23 release independent of full-length CaSR, PTH, and 1,25(OH)$_2$D$_3$ when serum calcium levels are at or above physiological concentrations.

Fractional excretion of phosphorus in wild-type, PTH KO, and CaSR-PTH DKO. Both PTH and FGF23 regulate phosphorus excretion through the control of renal sodium-dependent phosphate cotransporters. In the presence of elevated levels of either PTH or FGF23, the levels of expression of these cotransporters are diminished and they are internalized, thus decreasing phosphorus reabsorption and increasing phosphorus loss in the urine. To further investigate the regulation of renal phosphorus handling in the PTH KO and PTH-CaSR DKO mice, urinary phosphorus and fractional excretion of phosphorus were determined in mice on a low-phosphorus diet following addition of 100 or 200 mM phosphate to the drinking water. On the phosphorus-deficient diet, urinary phosphorus was very low, and the fractional excretion of phosphorus in the urine of all three genotypes was <1% (Fig. 3). When 100 mM phosphate was added to the drinking water, fractional excretion of phosphorus increased greatly in all three genotypes (Fig. 3*B*). The dietary phosphate-induced increase in fractional excretion was significantly blunted in the PTH KO mice compared with the WT mice and was further decreased in the PTH-CaSR DKO mice. The additional decline in phosphorus excretion in the PTH-CaSR DKO mice could be explained by the loss of the FGF23 response with hypocalcemia. With injection of 200 mM sodium monobasic phosphate, urinary phosphorus and its fractional excretion were substantially increased above the baseline levels (Fig. 3). Fractional excretion was blunted in the PTH KO mice compared with the WT mice and was further decreased significantly in the PTH-CaSR DKO mice compared with the PTH KO mice. Thus the loss of both PTH and the full-length CaSR appears to compromise the ability of the kidney to excrete phosphorus.

Raising serum calcium concentration by ip injection of calcium gluconate. One of the effects of phosphate addition to the diet of the mice was a decline in serum and urinary calcium, which was due at least in part to a reduction in bioavailable calcium in the diet. This decline in calcium bioavailability is likely due to the chelation of calcium in the gastrointestinal tract by the added phosphate. A recent publication suggests that normal serum calcium levels are needed for stimulation of FGF23 secretion (34). Consistent with that report, we found a blunted stimulation of FGF23 in PTH KO mice and no FGF23 response in PTH-CaSR DKO mice with similarly low serum calcium levels (Fig. 1*E*). Therefore, we sought to investigate the roles of serum calcium and the CaSR in FGF23 regulation by elevating the serum level of calcium by ip injection of a
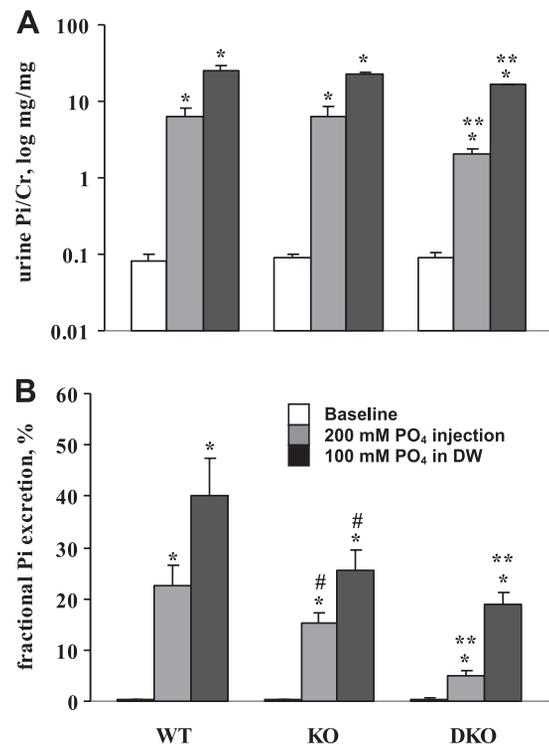

Fig. 3. Changes in urinary concentration of Pi (*A*) and fractional Pi excretion (*B*) with increased phosphate load. WT, PTH KO, and PTH-CaSR DKO mice were given ip injections of 200 mM sodium monobasic phosphate (gray bars) or 100 mM phosphate in the drinking water (DW) for 7 days (black bars). All mice were maintained on a phosphate-deficient diet during the entire experiment. Note the log scale on the vertical axis in *A*. Values are means ± SE (*n* = 6–10/genotype). *$P < 0.05$ for comparison of baseline values to phosphate injection or 100 mM phosphate in DW; #$P < 0.05$ for comparison of values for different dietary phosphorus loads in WT vs. PTH KO mice; **$P < 0.05$ for comparison of values in PTH KO vs. PTH-CaSR DKO mice.

calcium gluconate solution, as described in MATERIALS AND METHODS. Injection of calcium gluconate produced an early peak (not shown) and later a sustained elevation of serum calcium in the PTH KO and PTH-CaSR DKO mice, whereas WT mice did not show a persistent sustained phase (data not shown). Thus we were able to raise the serum calcium concentration significantly in the PTH KO and PTH-CaSR DKO mice despite their supranormal serum phosphorus concentrations (Fig. 4, *A* and *B*), whereas serum calcium remained tightly controlled in the WT mice. The serum concentration of phosphorus in WT mice was unaffected by calcium gluconate treatment, whereas it was reduced somewhat in PTH KO and the PTH-CaSR DKO mice but remained at physiological levels or above (Fig. 4*A*).

On the regular chow diet, the WT mice had a higher basal FGF23 concentration than the PTH KO and PTH-CaSR DKO mice. Calcium gluconate injections elevated the serum concentration of FGF23 significantly in all three genotypes (Fig. 4*C*), although the responses were less pronounced in the KO genotypes. The basal level of 1,25(OH)$_2$D$_3$ was significantly higher in WT mice compared with those in the PTH KO and PTH-CaSR DKO mice, which was likely due to the presence of PTH in the WT mice (Fig. 4*D*). In addition, calcium gluconate treatment caused significant, marked reductions of serum 1,25(OH)$_2$D$_3$ concentration in all three genotypes, consistent with the observed increase in serum FGF23. Vehicle-treated
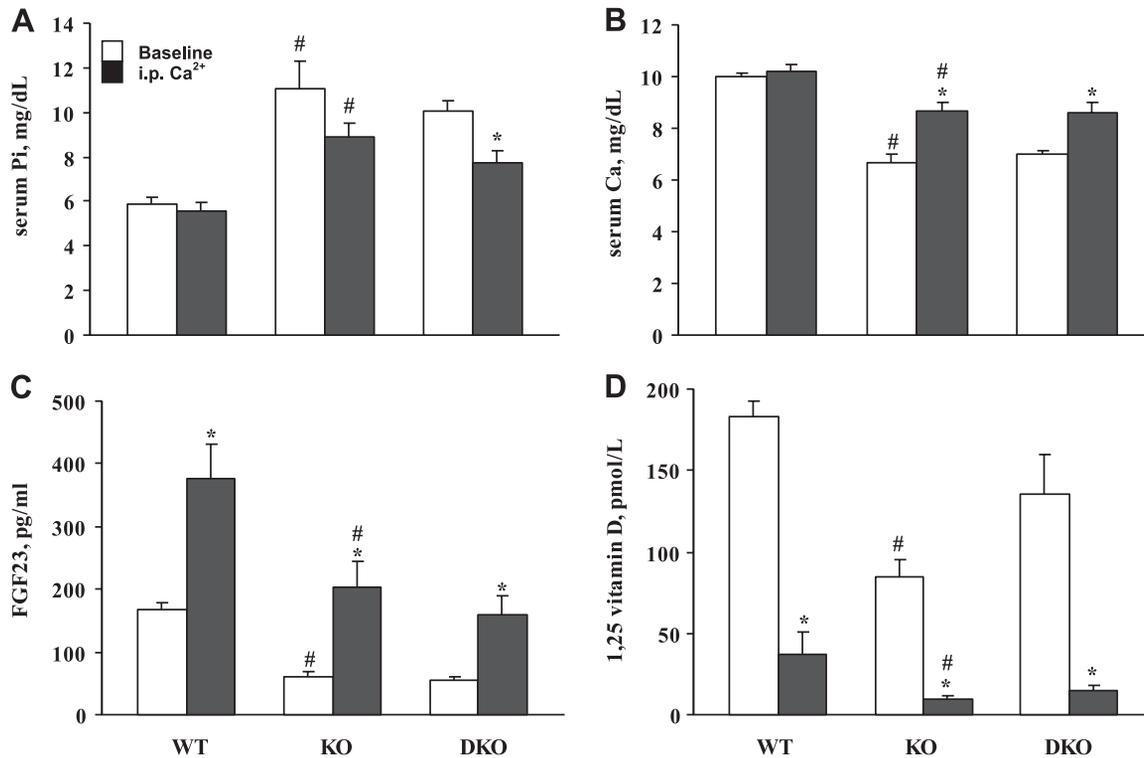
Fig. 4. Serum concentrations of Pi (*A*), Ca (*B*), FGF23 (*C*), and 1,25(OH)$_2$D$_3$ (*D*) after ip injection with calcium gluconate. Sera from WT, PTH KO, and PTH-CaSR DKO mice were collected at baseline (open bars) and after 7 days of treatment with calcium gluconate (black bars). Values are means ± SE (*n* = 6–8/genotype). *$P < 0.05$ for comparison of values at baseline or following calcium gluconate; #$P < 0.05$ for comparison of values at baseline or following treatment of WT vs. PTH KO mice.

mice injected with sodium gluconate showed no changes in serum calcium, serum phosphorus, FGF23, or 1,25(OH)$_2$D$_3$ (data not shown). Therefore, our results show that calcium causes FGF23 release independent of full-length CaSR and PTH and in the presence of reduced serum 1,25(OH)$_2$D$_3$ and phosphorus levels.

Effect of ip injection of 1,25(OH)$_2$D$_3$ on FGF23 release. To investigate any role of full-length CaSR in the 1,25(OH)$_2$D$_3$-elicited stimulation of FGF23 production, we gave 2 ip injections of 1,25(OH)$_2$D$_3$ separated by 24 h, which had resulted in dramatic elevations of serum FGF23 concentration in mice in a previous study (19). To avoid lethal 1,25(OH)$_2$D$_3$-induced hypercalcemia and to maintain physiological parameters at as similar levels as possible among the three genotypes, the mice were kept on a low-calcium diet during the entire experiment. In WT mice, 1,25(OH)$_2$D$_3$ administration had no significant effect on the serum calcium or phosphorus concentration during the entire experiment. In the PTH KO mice, serum calcium rose from 5.4 to 6.9 mg/dl at 24 h after the first injection and to 7.9 mg/dl after the second injection, whereas serum calcium increased from 5.6 to 7.6 mg/dl after the first injection and to 9.5 mg/dl after the second injection in PTH-CaSR DKO mice. Serum phosphorus remained unchanged following the injection in all three genotypes. Baseline serum FGF23 levels were lower in the PTH KO and PTH-CaSR DKO mice than in the WT mice, which may reflect the reduced levels of 1,25(OH)$_2$D$_3$ and/or serum calcium observed in the absence of PTH. FGF23 concentration increased significantly after 1,25(OH)$_2$D$_3$ administration in all three genotypes; however, the PTH KO mice showed a significantly blunted FGF23 response to 1,25(OH)$_2$D$_3$ compared

with the other two genotypes (Fig. 5), perhaps as a result of mild hypocalcemia in this genotype. Therefore, 1,25(OH)$_2$D$_3$ administration can stimulate FGF23 production in the absence of PTH and full-length CaSR.

Time course of phosphorus effects on serum PTH and FGF23 levels. It has been shown previously that increased dietary phosphate leads to enhanced PTH secretion and elevations in
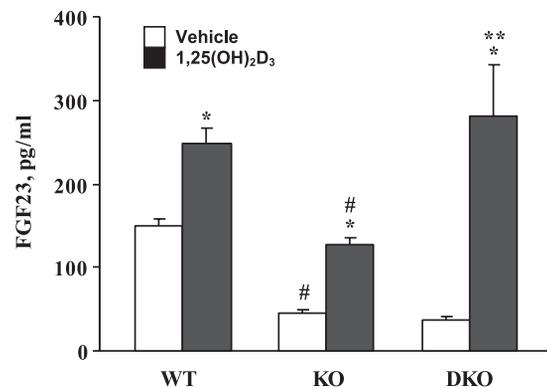

Fig. 5. Changes in serum concentrations of FGF23 in WT, PTH KO, and PTH-CaSR DKO mice after 2 injections of 0.5 ng/g body wt 1,25(OH)$_2$D$_3$. Measurements were taken at baseline (open bars) and 24 h after the 2nd injection (black bars). During the entire experiment, all mice were kept on a low-calcium diet starting 72 h before the experimental protocol. Values are means ± SE (*n* = 6–8/genotype). *$P < 0.05$ for comparison of values at baseline and following calcium gluconate; #$P < 0.05$ for comparison of values at baseline or following treatment of WT mice vs. PTH KO mice; **$P < 0.05$ for comparison of values of PTH KO vs. PTH-CaSR DKO mice.

serum PTH. Our WT mice exhibited extremely low serum PTH concentrations (8.5 ± 3 pg/ml) when fed a phosphorus-deficient diet for 7 days. Serum PTH levels rose sharply to 491 ± 68 pg/ml at 1 h following gavage with 300 μl of 0.4 M sodium monobasic phosphate. Furthermore, serum PTH levels were chronically increased to 225 ± 49 mg/ml after 7 days of phosphate supplementation with 150 mM sodium monobasic phosphate in the drinking water. In contrast, a gavage with 300 μl of 0.4 M sodium monobasic phosphate produced no change in baseline serum FGF23 levels after 1 h (data not shown), whereas phosphate loads in the drinking water for 7 days resulted in significant increases in serum FGF23 (Fig. 1*E*).

Relationship between serum calcium, serum phosphorus, the calcium × *phosphorus product, and serum FGF23 levels.* To better appreciate the relationships between serum phosphorus, calcium, and FGF23 levels, serum FGF23 was plotted as a function of serum phosphorus, serum calcium, and the calcium × phosphorus product (Fig. 6), using data from all the experimental conditions used in this study (e.g., in Figs. 1–4), except for the data from injection of 1,25(OH)$_2$D$_3$ (i.e., Fig. 5). Serum phosphorus was plotted vs. serum FGF23 for experimental conditions in which serum calcium concentrations were >8 mg/dl. FGF23 levels rose sharply above a serum phosphorus concentration of 5 mg/dl, showing an exponential relationship with an *r* value of 0.58 (Fig. 6*A*). For experiments with serum calcium levels <8 mg/dl, there was a flat linear relationship between serum phosphorus and FGF23, suggesting that serum phosphorus was not regulating serum FGF23 when serum calcium was low. Similarly, serum FGF23 rose sharply when serum calcium was >8 mg/dl (*r* = 0.65) as long as serum phosphorus was >5 mg/dl (Fig. 6*B*). However, in experiments with serum phosphorus levels <5 mg/dl, there was a flat linear relationship between serum calcium and FGF23. The loss of responsiveness of FGF23 to increases in serum calcium or serum phosphorus when serum phosphorus or serum calcium levels, respectively, were low suggests that there may be thresholds for these ions, which must be surpassed in order for the reciprocal ion to regulate FGF23.

The calcium × phosphorus product is used clinically as an indicator of dysregulation of the calcium and/or phosphorus axes. The calcium × phosphorus product was plotted against serum FGF23 concentrations for all of our experimental conditions, except for injection of 1,25(OH)$_2$D$_3$ (Fig. 6*C*). FGF23 levels rose exponentially (*r* = 0.70) for calcium × phosphorus products >50 mg^2/dl^2 for all three genotypes (Fig. 6*C*), showing a tighter correlation with the calcium × phosphorus product than observed with changes in serum calcium or phosphorus individually.

DISCUSSION

To investigate the roles of calcium and the CaSR in the FGF23/phosphorus homeostatic pathway, we used mice of three genotypes: WT, PTH KO, and PTH-CaSR DKO. The global CaSR KO mouse was not viable, so the PTH-CaSR DKO mouse was used to examine the role of CaSR, and the PTH KO mouse was its genetic control. Since both PTH and CaSR are key regulators of serum calcium, an additional advantage of using these mice was that it enabled us to study broad ranges of serum calcium and phosphorus concentrations, which is very difficult to accomplish in WT mice. On a phosphorus-
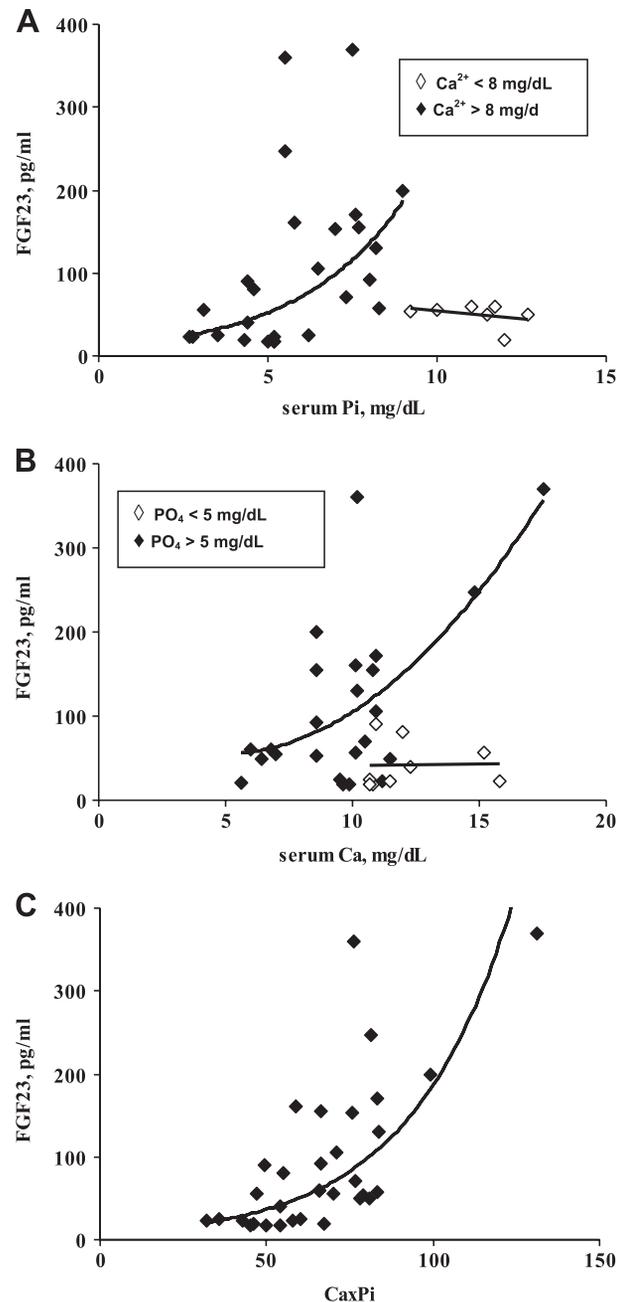

Fig. 6. Serum FGF23 concentration plotted as a function of serum Pi, serum Ca, and the calcium × phosphorus (CaxPi) product. *A*: serum FGF23 concentration is plotted as a function of serum phosphorus for all 3 genotypes. ◆, Values for serum phosphorus and FGF23 when serum calcium was >8 mg/dl. These data fit an exponential function with an *r* value of 0.58. ◇, Serum phosphorus and FGF23 values when serum calcium was <8 mg/dl, showing a nearly flat linear fit. *B*: serum FGF23 concentration is plotted as a function of serum Ca for all 3 mouse genotypes. ◆, Serum Ca and FGF23 values when serum Pi was >5 mg/dl. These data fit an exponential function with an *r* value of 0.65. ◇, Serum calcium and FGF23 values when serum Pi was <5 mg/dl, showing a nearly flat linear fit. *C*: serum FGF23 concentration as a function of the CaxPi product in all 3 genotypes. These data fit an exponential function with an *r* value of 0.70.

deficient diet, all three genotypes showed low serum phosphorus levels, with the PTH KO and PTH-CaSR DKO mice having significantly lower serum phosphorus levels compared with the WT mice (Fig. 1). Serum calcium levels were slightly elevated

in the WT and PTH KO mice, whereas the PTH-CaSR DKO mice showed significant hypercalcemia. These serum calcium values are consistent with our understanding of each genotype's ability to regulate serum calcium with a large dietary calcium load (15). In the case of the phosphorus-deficient diet, an enhanced calcium load is a result of the lack of phosphate chelation of calcium in the gastrointestinal tract. The WT mouse uses the PTH axis to tightly control serum calcium levels close to normal values. Under normal dietary conditions, the PTH KO mouse combats hypocalcemia poorly due to the loss of PTH. However, the PTH KO mouse can still defend against hypercalcemia by increasing calcitonin secretion and enhancing urinary calcium excretion. However, the PTH-CaSR DKO mouse controls serum calcium poorly due to the lack of both the PTH and calcitonin pathways, combined with an inability to upregulate renal calcium excretion appropriately in the face of hypercalcemia. FGF23 is low with a phosphorus-deficient diet for all three genotypes, with the PTH-CaSR DKO mice having slightly but significantly higher serum FGF23 levels compared with the WT and PTH KO mice, perhaps due to their higher serum calcium concentration. Serum $1,25(OH)_2D_3$ concentrations were high in all three genotypes, consistent with the low serum FGF23 levels even in the face of the low level of serum PTH in the WT mice and the absence of PTH in the two KO genotypes.

When maintained on a high-phosphorus diet through the addition of phosphate to the drinking water, all three genotypes had significantly higher levels of serum and urinary phosphorus than mice maintained on a phosphorus-deficient diet. Serum calcium concentrations declined with increasing dietary loads of phosphate, which was probably due in part to the chelation of dietary calcium by phosphate in the gastrointestinal tract with a resultant decrease in bioavailable calcium. WT and PTH KO mice showed significant decreases in serum calcium with the 100 mM phosphate load and smaller declines at intermediate phosphate loads. For PTH-CaSR DKO mice, the decline in serum calcium levels was more marked, showing a significant decrease at each phosphate load. With 100 mM phosphate in the drinking water, both the PTH KO and PTH-CaSR DKO mice had significantly lower serum calcium levels than the WT mice. This is consistent with the inability of the KO mice to regulate their serum calcium concentration when faced with a smaller dietary calcium load (15).

WT and PTH KO mice showed a direct concentration-response relationship between serum phosphorus and serum FGF23, although the response of the PTH KO mice was significantly blunted compared with that of the WT mice. This blunted FGF23 response in the PTH KO mice could be explained by the absence of PTH, which has been suggested to stimulate FGF23 production (22, 25, 31). The PTH-CaSR DKO mice showed little change in FGF23 level, with a small, statistically significant increase only at 50 mM phosphate. The serum $1,25(OH)_2D_3$ levels declined significantly, with 25 mM phosphate for all three genotypes, and there were no further decreases in serum $1,25(OH)_2D_3$ levels with greater phosphate loads despite further increases in serum phosphorus and FGF23 in the WT and PTH KO mice. Serum $1,25(OH)_2D_3$ levels were significantly lower in the PTH KO and PTH-CaSR DKO mice compared with the WT mice with each phosphate load. The modest decline in serum $1,25(OH)_2D_3$ levels in the WT mice might be explained by the sharp increase in serum PTH with the addition of phosphate, whose stimulatory effects on $1,25(OH)_2D_3$ production should counterbalance the inhibitory effects of elevated serum FGF23. In the PTH KO and PTH-CaSR DKO mice, there were substantial additional decreases in serum $1,25(OH)_2D_3$ despite little change in serum FGF23. These data suggest that $1,25(OH)_2D_3$ production is more sensitive to the dietary phosphate load than to FGF23 levels. In the KO mouse genotypes, there is no PTH to activate $1,25(OH)_2D_3$ production, leaving the regulation of $1,25(OH)_2D_3$ production under the control of FGF23 and other inhibitory factors, including the possibility of a direct effect of phosphorus. A previous report has suggested that there is an unidentified gastrointestinal factor that helps to regulate phosphorus handling in the kidney (20), which may also be responsible for the decline in serum $1,25(OH)_2D_3$ found here in the two KO mouse genotypes subjected to dietary phosphate loads.

The PTH-CaSR DKO mice showed little FGF23 response to elevated serum phosphorus during phosphate loading, suggesting that the CaSR might be involved in phosphorus-induced FGF23 production. However, a confounding factor involving changes in dietary phosphorus is the profound hypocalcemia observed with high-phosphorus diets, which is likely due to reduced bioavailability of dietary calcium. Both the PTH KO and PTH-CaSR DKO mice, which require supplemental dietary calcium to maintain normocalcemia (15), exhibit significant hypocalcemia on a high-phosphorus diet. Indeed, it has been found recently that hypocalcemia inhibits the FGF23 response to a phosphate load in rats (34). Therefore, the hypocalcemia associated with phosphate loads may help to explain the reduced production of FGF23 in PTH KO mice and the lack of the FGF23 response in the PTH-CaSR DKO mice. To directly test the ability of PTH KO and PTH-CaSR DKO mice to modulate FGF23 release during changes in serum phosphorus independent of dietary phosphorus, we elevated serum phosphorus through ip injections of sodium monobasic phosphate, which allowed us to sustain increases in serum phosphorus in all three genotypes, with little change in serum calcium concentration. In addition, serum calcium levels were all at or above the normal range due to the use of a phosphorus-deficient diet that provided ample bioavailable dietary calcium. The administration of sodium monobasic phosphate and the resultant increase in serum phosphorus produced a significant elevation of serum FGF23 in all three genotypes, with the PTH-CaSR DKO mice generating greater FGF23 production than the PTH KO mice. This was accompanied by a dramatic reduction in $1,25(OH)_2D_3$ in the PTH KO and PTH-CaSR DKO mice. There were more modest decreases in $1,25(OH)_2D_3$ in the WT mice, which was likely due to the increase in PTH with elevation in serum phosphorus levels. Therefore, our results clearly indicate that serum phosphorus can be sensed by the PTH KO and PTH-CaSR DKO mice, leading to regulation of serum FGF23 when serum calcium levels are at or above normal levels despite a concomitant decrease in $1,25(OH)_2D_3$.

Previous studies have indicated that serum calcium has a modulatory role in the regulation of FGF23 production. Little is known about the role of CaSR in the effects of serum calcium on this pathway. To directly test the effects of increased serum calcium level on FGF23 production, mice were injected ip with calcium gluconate, which will not alter bioavailability of phosphorus in the gastrointestinal tract and will bypass any intestinal factor that might be altered with dietary

manipulations (30, 44). There was a sustained increase in serum calcium for PTH KO and PTH-CaSR DKO mice, whereas there was only a slight, insignificant elevation in WT mice. Time course studies following ip injection of calcium indicated that serum calcium rose transiently in all three genotypes, whereas a sustained phase was maintained in only the KO mice (data not shown). There was a modest reduction in serum phosphorus for the two KO genotypes, which nevertheless remained within the normal range and above 6 mg/dl. The calcium gluconate injections elevated serum FGF23 concentrations significantly in all three genotypes but not those in vehicle-injected mice. These elevations of FGF23 were accompanied by dramatic reductions in $1,25(OH)_2D_3$, presumably caused by FGF23 activation of the FGFR-Klotho complex in the renal proximal tubule, leading to a reduction in 1α-hydroxylase expression (28, 37, 38). Our results clearly indicate that calcium regulates FGF23 release in vivo. Although the mechanism underlying calcium-induced FGF23 release is unknown, it cannot involve PTH, $1,25(OH)_2D_3$, full-length CaSR, or serum phosphorus.

The role of the CaSR in this calcium/phosphorus homeostatic pathway is somewhat elusive. CaSR is clearly important for the regulation of serum calcium levels through its actions on the parathyroid gland, thyroidal C cells, bone, and kidney (5–9, 12, 15, 23, 26, 29, 32, 33, 43). On the other hand, full-length CaSR does not appear to be critically involved in the regulation of serum FGF23, although a modest modulatory role cannot be excluded by our results. Further studies would be needed to determine whether the isoform of CaSR lacking exon 5 is capable of stimulating FGF23 when serum calcium rises or whether complete KO of the CaSR in osteoblasts/osteocytes can modify FGF23 regulation (6). KO of full-length CaSR in chondrocytes and osteoblasts has been shown to have substantial effects on skeletal development, which are not observed with our CaSR KO lacking exon 5 (6). Thus it is possible that an isoform of CaSR lacking exon 5 may permit chondrocytes and osteoblasts to function in a more normal manner, allowing skeletal development and mineralization to proceed. In the kidney, the CaSR appears to facilitate phosphorus handling, since the PTH-CaSR DKO mouse is less able to excrete phosphorus in the urine than the WT or PTH KO mice. This is best seen with sodium monobasic phosphate injections, where FGF23 levels are highest in the PTH-CaSR DKO mice yet the excretion of phosphorus is the lowest of the three mouse genotypes. The CaSR plays a similar role in calcium handling in the kidney, where it promotes loss of calcium in the urine when serum calcium rises (9).

One striking observation in our results is that both serum calcium and phosphorus are involved in the control of FGF23. Both PTH KO and PTH-CaSR DKO mice respond with increased serum FGF23 levels when serum calcium is elevated from hypocalcemic levels with calcium gluconate injection (if serum phosphorus is maintained at normal levels) or when serum phosphorus is elevated from hypophosphatemic levels with phosphate injections (if calcium is kept at normal levels or above) (Fig. 6). Furthermore, low serum concentrations of either calcium or phosphorus appear to block FGF23 production in response to elevations of the other mineral ion. This phenomenon is seen with the PTH KO and PTH-CaSR DKO mice, in which extremes of serum calcium or phosphorus can be achieved. When the mice were kept on a low-calcium diet,

serum levels of phosphorus were high in PTH KO and PTH-CaSR DKO mice, but the FGF23 concentration remained low (Fig. 5). In contrast, when these mice were maintained on a low-phosphorus diet, the serum calcium level was high, but the serum FGF23 concentration remained low (Figs. 1 and 2). These experimental conditions led to an inability to increase FGF23 production in response to an elevation in the other ion (Fig. 6). For WT mice, serum calcium is tightly controlled, allowing phosphorus levels to change without accompanying changes in serum calcium. It appears that some threshold levels for both calcium and phosphorus are required to stimulate FGF23 production. We have not precisely identified these threshold levels, but they appear to be close to the lower range of normal serum levels for mice with serum phosphorus levels below 5 mg/dl and serum calcium levels below 8 mg/dl, resulting in an unresponsive FGF23 pathway. Another way to look at this shared control of FGF23 production is the relationship between the calcium × phosphorus product and FGF23 production.

The strongest correlation coefficient was found for the relationship between the calcium × phosphorus product and serum FGF23 compared with changes in FGF23 resulting from alterations in serum calcium or phosphorus. Interestingly, the steep rise in the serum FGF23 levels began in the range of 55 mg^2/dl^2, a value for the calcium × phosphorus product that is considered to be an indicator of dysregulation of calcium-phosphorus homeostasis in clinical conditions such as chronic kidney disease (16). In hypocalcemic and hyperphosphatemic patients with hypoparathyroidism, the reduction in serum phosphorus that usually occurs during treatment of hypocalcemia with vitamin D may result from calcium-stimulated increases in FGF23 that promote phosphaturia. However, the serum phosphorus concentration commonly does not completely normalize, presumably because both PTH and FGF23 are needed for normal phosphorus homeostasis. Nevertheless, a calcium-induced increase in FGF23 in this setting may contribute to a relatively normal and constant calcium × phosphorus product as serum calcium rises toward normal and to the avoidance of overly high levels of the calcium × phosphorus product despite the absence of PTH, which might increase the risk of ectopic calcification.

The molecular mechanisms for serum calcium and phosphorus regulation of FGF23 levels are not known. One possibility is that FGF23 secreted by osteoblasts and osteocytes can independently sense serum calcium and phosphorus through cell surface receptors that in turn stimulate FGF23 production. The CaSR would be a candidate receptor for this calcium sensing if the CaSR lacking exon 5 can function as a calcium sensor in osteoblasts and osteocytes. Otherwise, an additional calcium sensor must be hypothesized. In the case of phosphorus, there is no identified phosphorus-sensing receptor to date. Interestingly, serum phosphorus changes are sensed in minutes by the parathyroid gland, leading to stimulation of PTH secretion, whereas FGF23 secretion from osteoblasts and osteocytes takes many hours to days before elevation of serum FGF23 is observed (34). That may provide a mechanism to ensure the availability of at least one phosphaturic hormone following a phosphate load, even at very early time points. One possibility for this difference in response time could be the more rapid access to serum changes by the parathyroid cells vs. the greater diffusion barrier experienced by osteoblasts and osteocytes.

Another possibility is that serum calcium and phosphorus can alter bone cell activity, leading to the regulation of FGF23 production. Both calcium and phosphorus stimulate osteoblasts to form bone matrix and to mineralize it, thereby extracting both minerals from the extracellular fluid and lowering the calcium-phosphorus product (8, 17, 43, 46). Furthermore, it has been shown that this mineralization and bone formation by osteoblasts are associated with an increased expression and release of FGF23 (45). It has also been demonstrated that phosphorus-dependent stimulation of the expression of miner-alization-associated proteins (e.g., matrix Gla protein and osteopontin) in osteoblasts is modulated by the extracellular calcium via the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway (17). In addition, the concomitant presence of phosphorus and calcium has been shown to form calcium-phosphorus precipitates, which can stimulate ERK1/2 activity (17). In this way, FGF23 secretion might communicate the state of "mineral balance". Excessive calcium and/or phosphorus in serum and/or skeletal compartments in the vicinity of osteoblasts and osteocytes, sensed as soluble ions and/or following the initiation of calcium-phosphorus precipitation, could then lead to elevated FGF23 levels, which could promote excretion of phosphorus in the urine. This could potentially reduce the risk of inappropriate or excessive mineral ion precipitation. Through its actions to lower $1,25(OH)_2D_3$ and PTH, FGF23 would also reduce the calcium load entering through the intestines and promote greater excretion of calcium in the urine (e.g., via the CaSR). FGF23 and PTH can act in concert to combat elevations in serum calcium and phosphorus. PTH increases acutely when serum phosphorus is elevated, leading to an immediate PTH-stimulated loss of phosphorus in the urine (2, 10, 18). On the other hand, FGF23 appears to require hours/days to respond to increases in serum phosphorus; thus FGF23 is more likely to be a more chronic regulator of serum phosphorus (13, 19). The interplay among FGF23, PTH, and $1,25(OH)_2D_3$ will lead to a new hormonal balance that is better suited to controlling the serum levels of both calcium and phosphorus.

In summary, the key findings of this study are as follows: *1*) phosphate loading inhibits $1,25(OH)_2D_3$ synthesis independent of changes in FGF23 in the PTH-CaSR DKO genotype; *2*) the full-length CaSR is needed for efficient excretion of phosphorus during phosphate loading; *3*) serum calcium stimulates FGF23 through a mechanism that does not involve the full-length CaSR; *4*) there are thresholds for serum calcium and phosphorus that must be exceeded in order for phosphorus or calcium to stimulate FGF23 (these thresholds contribute to maintaining a nearly constant calcium × phosphorus product by limiting FGF23 production when one or the other ion is below its threshold); and *5*) the strongest correlation between calcium and phosphorus for stimulation of FGF23 is not with the individual mineral ions but with the calcium × phosphorus product (5). Thus osteoblasts and osteocytes are capable of responding via changes in FGF23 production to increases in the serum calcium concentration in the context of the serum phosphorus level and vice versa, thereby defending against an excessive "total mineral ion load".

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