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Vitamin D receptor-independent FGF23 actions in regulating phosphate and vitamin D metabolism

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Shimada, Takashi, Yuji Yamazaki, Motoo Takahashi, Hisashi Hasegawa, Itaru Urakawa, Takeshi Oshima, Kaori Ono, Makoto Kakitani, Kazuma Tomizuka, Toshiro Fujita, Seiji Fukumoto, and Takeyoshi Yamashita. Vitamin D receptor-independent FGF23 actions in regulating phosphate and vitamin D metabolism. *Am J Physiol Renal Physiol* 289: F1088–F1095, 2005. First published July 5, 2005; doi:10.1152/ajprenal.00474.2004.—FGF23 suppresses both serum phosphate and 1,25-dihydroxyvitamin D [1,25D] levels in vivo. Because 1,25D itself is a potent regulator of phosphate metabolism, it has remained unclear whether FGF23-induced changes in phosphate metabolism were caused by a 1,25D-independent mechanism. To address this issue, we intravenously administered recombinant FGF23 to vitamin D receptor (VDR) null (KO) mice as a rapid bolus injection and evaluated the early effects of FGF23. Administration of recombinant FGF23 further decreased the serum phosphate level in VDR KO mice, accompanied by a reduction in renal sodium-phosphate cotransporter type IIa (NaPi2a) protein abundance and a reduced renal 25-hydroxyvitamin D-1 α -hydroxylase (1 α OHase) mRNA level. Thus FGF23-induced changes in NaPi2a and 1 α OHase expression are independent of the 1,25D/VDR system. However, 24-hydroxylase (24OHase) mRNA expression remained undetectable by the treatment with FGF23. We also analyzed the regulatory mechanism for FGF23 expression. The serum FGF23 level was almost undetectable in VDR KO mice, whereas dietary calcium supplementation significantly increased circulatory levels of FGF23 and its mRNA abundance in bone. This finding indicates that calcium is another determinant of FGF23 production that occurs independently of the VDR-mediated mechanism. In contrast, dietary phosphate supplementation failed to induce FGF23 expression in the absence of VDR, whereas marked elevation in circulatory FGF23 was observed in wild-type mice fed with a high-phosphate diet. Taken together, FGF23 works, at least in part, in a VDR-independent manner, and FGF23 production is also regulated by multiple mechanisms involving VDR-independent pathways.

VDR; phosphate metabolism; sodium phosphate cotransporter

FGF23 HAS BEEN IDENTIFIED as a common causative factor for two types of hypophosphatemic rickets/osteomalacia, namely, autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR) and tumor-induced osteomalacia (TIO) (1, 19). Recent studies showed that administration of FGF23 resulted in increased renal phosphate excretion caused by reduction in renal sodium-phosphate cotransporters, indicating that FGF23 is a novel phosphate-regulating factor (3, 16, 17, 19, 28). In addition, it has been shown that FGF23 is a potent suppressor

of 1,25-dihydroxyvitamin D (1,25D) levels, as demonstrated by reduced renal 1 α -hydroxylase (1 α OHase) and increased 24-hydroxylase (24OHase) mRNA expression in mice administered with recombinant FGF23 (2, 3, 8, 17, 19, 21). Furthermore, FGF23 knockout (KO) mice exhibited hyperphosphatemia and abnormally elevated 1,25D levels accompanied by enhanced renal 1 α OHase mRNA expression (18). Therefore, these lines of evidence suggest an essential role for FGF23 in the regulation of both phosphate and 1,25D levels.

On the other hand, as we previously reported, the serum 1,25D level decreased within 3 h after a rapid bolus injection of recombinant FGF23, whereas reduction in serum phosphate concentration first appeared at 8–9 h after the injection (17). This finding is a mirror image of the changes induced by the rapid reduction in the serum FGF23 level after excision of a causative tumor from a patient with TIO, who showed rapid recovery of the serum 1,25D level followed by an elevation of the serum phosphate level (29). In addition to the different time courses of changes in serum 1,25D and phosphate levels, the minimal doses of FGF23 required for reduction in serum 1,25D and phosphate levels were also different (17). Serum phosphate concentration is mainly determined by a balance of intestinal phosphate absorption and renal phosphate reabsorption (24). One of the potent regulators of phosphate balance is 1,25D because its deficient action caused hypophosphatemia in 1 α OHase null mice and vitamin D receptor (VDR) KO mice (10, 14, 30). Thus in animals administered recombinant FGF23, the reduction in the serum 1,25D level potentially affected the serum phosphate level. In other words, based on previous observations on the regulation of phosphate and 1,25D levels by FGF23 action, two possibilities may be proposed: one is that FGF23 stimulates two independent pathways for regulating phosphate and vitamin D metabolism, respectively, and the other is that the FGF23-induced change in the phosphate level is a secondary phenomenon due to prior alteration of the serum 1,25D level by FGF23.

In the present study, we investigated whether FGF23 has a VDR-independent action by examining the effects of FGF23 in VDR null mice. In addition, we analyzed FGF23 production in VDR null mice so as to obtain insights into the possible feedback regulation of the FGF23–1,25D pathway and VDR-independent regulation of FGF23.

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MATERIALS AND METHODS

Establishment of VDR null mice. Genomic DNA corresponding to exon 2, including the translation start site codon, 2.2 kb of the 5'-intronic sequence, and 5.0 kb of the following 3'-intronic sequence, in the *Vdr* gene was isolated from a C57BL/6 genomic BAC library. The targeting vector was constructed by subcloning these DNA fragments into the 5'- and 3'-sites of a neomycin resistance gene (Fig. 1A). This targeting vector was introduced into TT2F embryonic stem (ES) cells (26) by electroporation to replace the 83-bp DNA fragment of exon 2. To select the ES clones carrying the targeted allele, the genomic DNA of these cells was extracted using a PUREGENE kit (Gentra) and digested with *EcoT22I*. The digested DNA was then blotted onto a nylon membrane and hybridized with the ³²P-labeled probe obtained from the genomic DNA of parental ES cells by PCR with the following primers: 5'-GCAGCTAAGTCAGGACCCTTTTCTA-3' and 5'-ACCGTCTAGGAGTCGAAGGAATCTT-3' (Fig. 1, A and B). The selected ES clones were microinjected into ICR 8-cell embryos. The chimeric mice with germline transmission of the targeted allele were mated with C57BL/6 mice to obtain heterozygous offspring. Interbreeding of heterozygous mice generated homozygous mice. Genotyping of the offspring was performed by a combination of genomic PCRs with the following primers: 5'-GTCCCCCTCGAATCAAGCTTAG-3' and 5'-CCCCCGTTGCTCTCAGTAACC-3' for the neomycin resistance gene and primers 5'-ggatgtGGTGTGATCCTGAGCTTTG-3' and 5'-cctgactgacctaacgaatgtg-3' for the deleted region of the *Vdr* gene. To check for the absence of the VDR

transcript, the kidney and femur were isolated from homozygous and wild-type littermates. Total RNA was extracted using Isogen reagent (Nippon Gene), and this RNA was treated with DNase I (Invitrogen) to digest contaminating genomic DNA. Using 300 ng of RNA as a template, RT-PCR was performed using the SuperScript One-Step RT-PCR system (Invitrogen) with the following primer set for amplifying the *Vdr* gene: 5'-TCGGATCTGTGGAGTGTGTGGAGACCGAGCC-3' and 5'-GTAAAAGACTGGTTGGAGCG-3'. Similarly, GAPDH mRNA was monitored as an internal standard. Roentgenograms were taken by an X-ray imaging system (Fuji Film), and total bone mineral densities were calculated by the Latheta computed tomography system (Aloka). All studies using animals were reviewed and approved by the institutional animal care and use committee at the Pharmaceutical Research Laboratories, Kirin Brewery.

Diet. All mice were fed on commercially available rodent solid chow (CE-2, Clea), containing 1.17% calcium and 0.96% phosphate, and tap water ad libitum; however, the diet was changed during each experiment as follows. From 1 wk before to the administration of recombinant FGF23, mice were fed a diet containing 0.5% calcium and 0.6% phosphorus based on AIN-93G (Oriental Bioservice Kanto) throughout the experiments. Other specified diets were also based on AIN-93G (Oriental Bioservice Kanto), and several modifications were made as follows. The high-calcium diet contained 2.0% calcium, 1.25% phosphate, 20% lactose, and 1,000 IU/kg vitamin D; the corresponding control diet contained 0.5% calcium, 0.6% phosphate, 20% lactose, and 1,000 IU/kg vitamin D. This recipe is based on a previous report by Li et al. (9) except that calcium phosphate was substituted with calcium lactate to enhance intestinal calcium absorption. The high-phosphate diet contained 0.5% calcium and 1.2% phosphate, and the corresponding control diet contained 0.5% calcium and 0.6% phosphate. During all experiments, all mice were allowed access to the diet and tap water ad libitum.

Measurement of serum parameters. Blood samples were collected from the orbital cavity or heart under anesthesia, and they were centrifuged to obtain sera. Serum phosphate and calcium concentrations were determined using P-test Wako and Ca-test Wako, respectively (Wako). Serum parathyroid hormone (PTH) and 1,25(OH)₂D levels were determined using a Rat Intact PTH RIA Kit (Immutopics) and an RIA Kit (TFB), respectively. The serum FGF23 level was measured by sandwich ELISA (Kainos Laboratory), which can quantify the intact form of FGF23 using human recombinant FGF23 as a standard (29).

Immunoblot analysis of sodium-phosphate cotransporter type IIa. The renal brush-border membrane (BBM) fraction was prepared from isolated kidney cortexes by a previously reported method (6). The protein concentration of the suspended BBM fraction was determined by Bradford's standard method. Twenty micrograms of the BBM protein were separated by SDS-PAGE and subjected to Western blot analysis using an anti-sodium-phosphate cotransporter type IIa (NaPi2a) polyclonal antibody that was affinity purified from rabbit antisera raised using a synthetic peptide corresponding to the COOH-terminal sequence of NaPi-2a, as previously reported (17). The signals were detected by the ECL system (Amersham Bioscience).

Northern blot analysis. Total RNA was isolated from the frozen kidney using the Isogen reagent (Nippon Gene). RNA samples (20 μg each) were electrophoresed and transferred to Hybond N⁺ (Amersham Bioscience). The probe DNA fragments for 1αOHase, 24OHase, and GAPDH mRNAs were prepared as previously reported (17), and these were radiolabeled using the Megaprime labeling system (Amersham Bioscience). Hybridization was performed overnight in PerfectHyb reagent (Toyobo) at 65°C, and stringent washing was carried out for 30 min in a solution of 0.1× SSC and 0.5% SDS at 65°C. The signals were visualized with a Typhoon system (Amersham Bioscience).

Real-time PCR. Total RNA was isolated from the frozen tissues using Isogen reagent (Nippon Gene); this was followed by DNase I

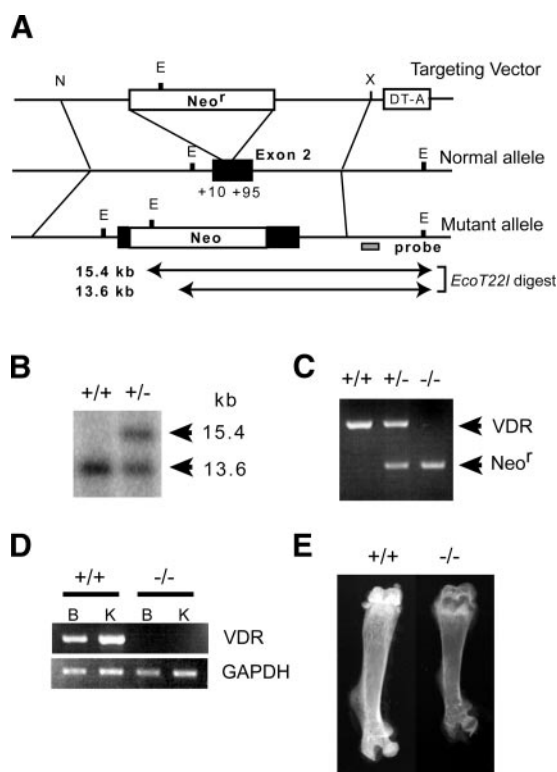


Fig. 1. Establishment of vitamin D receptor (VDR) knockout mice. *A*: targeting strategy for the *Vdr* gene. *E*, *EcoT22I*. *B*: Southern blot analysis of *EcoT22I*-digested genomic DNA isolated from embryonic stem (ES) cells transformed with a targeted allele. Successful targeting of the *Vdr* gene generated an additional 15.4-kb fragment. *C*: genotyping for wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mice. *D*: RT-PCR for the VDR and GAPDH mRNAs in bone (B) and kidney (K). *E*: soft X-ray analysis of the femurs isolated from a 7-wk-old homozygous mouse and wild-type littermate.

treatment (Invitrogen). Three micrograms of total RNA were reverse transcribed into first-strand cDNA with an oligo-dT primer using a SuperScript III first-strand synthesis kit (Invitrogen), and 100 ng of first-strand cDNA were used in the PCR reaction as the template. Real-time quantitative PCR for FGF23 was performed on an ABI 7700 system using the primers 5'-ACAAGGACACCTAAACCGAACAC-3' and 5'-AGCTACTGACTGGTCTATCACAGAA-3' and a QuantiTect SYBR Green RT-PCR kit (Qiagen). The results were corrected by normalization with the corresponding levels of the internal control GAPDH, which was amplified using the primers 5'-GCAAAGTGGAGATTGTTGCCA-3' and 5'-AATTTGCCGTGAGTGGAGTCA-3'.

Statistical analyses. Statistical significance was evaluated either by Student's *t*-test or by one-way analysis of variance followed by Dunnett's method for comparison of multiple means. An unadjusted *P* value of <0.05 was considered to be significant.

RESULTS

Generating VDR KO mice. We generated a new line of VDR KO mouse that lacked the 83-bp internal sequence of exon 2 (Fig. 1, A–C). RT-PCR analysis confirmed successful targeting of the *Vdr* gene; this confirmation was based on the observation that no VDR transcripts were amplified from the kidney and bone isolated from an offspring judged as a homozygote (Fig. 1D). The homozygous mice developed hypocalcemia, hypophosphatemia, and growth retardation by 6 wk of age (Table 1). The serum 1,25D level was abnormally higher than that in heterozygous or wild-type littermates, and the PTH level was drastically elevated in VDR KO mice (Table 1). These VDR KO mice showed undetectable serum levels of FGF23 (<3 pg/ml, the lowest detection limit of ELISA) at 6 wk of age (Table 1). Compared with their wild-type littermates, femurs isolated from homozygotes were short and had enlarged metaphysis and significantly increased X-ray translucency (Fig. 1E). The total bone mineral density of the femur significantly decreased in homozygotes (wild-type, 574.9 ± 22.4 mg/cm³ vs. homozygotes, 277.9 ± 10.5 mg/cm³; *P* < 0.001; *n* = 3). Additionally, histological analysis revealed that the zone of hypertrophic chondrocytes in the growth plate was markedly expanded in homozygotes (data not shown). According to these observations, our VDR KO mouse is not phenotypically different from previously established VDR KO strains (10, 30).

FGF23 action on phosphate metabolism in VDR KO mice. Five micrograms of recombinant human FGF23 protein were intravenously administered to VDR KO mice (5–6 wk of age) and age-matched wild-type mice by a rapid bolus injection. Subsequently, blood samples were sequentially collected at 2,

Table 1. Serum parameters and body weights of 6-wk-old male VDR KO mice and control littermates

	VDR+/+	VDR+/-	VDR-/-
Body wt, g	29.8 ± 1.0	27.4 ± 1.6	23.6 ± 1.0†
Phosphate, mg/dl	9.54 ± 0.19	9.18 ± 0.29	6.95 ± 0.44†
Calcium, mg/dl	9.18 ± 0.12	9.53 ± 0.12	6.03 ± 0.21†
1,25(OH) ₂ D, pg/ml	364.5 ± 45.4	289.2 ± 52.3	1206.2 ± 14.1†
PTH, pg/ml	38.6 ± 11.2	32.5 ± 7.3	1399.6 ± 194.8†
FGF23, pg/ml	130.7 ± 8.7	126.3 ± 9.9	ND

Values are means ± SE [wild-type (VDR+/+), *n* = 7; heterozygotes (VDR+/-), *n* = 6; and homozygote (VDR-/-), *n* = 5]. VDR, vitamin D receptor; KO, knockout; PTH, parathyroid hormone; ND, not detectable (<3 pg/ml). †*P* < 0.001 vs. wild-type.

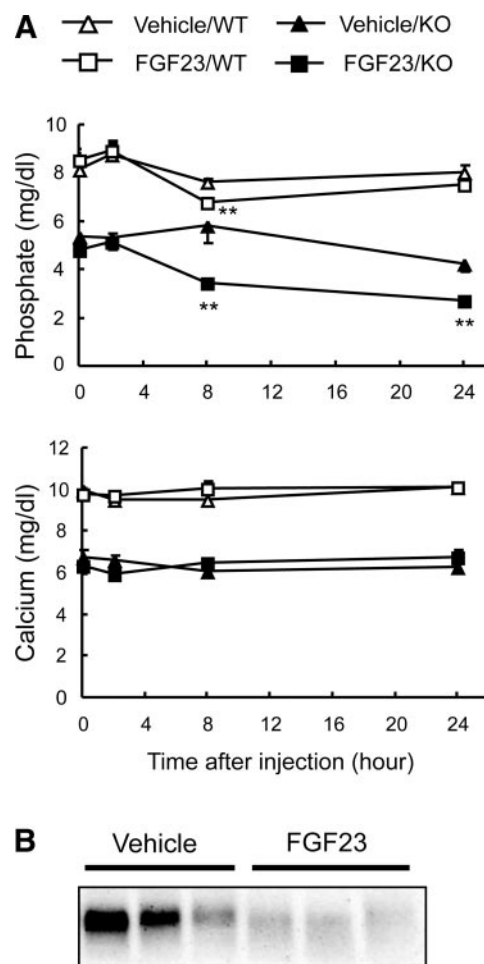


Fig. 2. A: time course of changes in serum phosphate and calcium levels in vehicle- or FGF23-treated wild-type mice (WT) and VDR null (KO) mice. Values are means ± SE for each group (vehicle-treated WT mice, *n* = 6; FGF23-treated WT mice, *n* = 6; vehicle-treated VDR KO mice, *n* = 6; FGF23-treated VDR KO mice, *n* = 6). ***P* < 0.01 vs. vehicle-treated group. B: Western blot analysis of sodium-phosphate cotransporter type IIa (NaPi2a) protein in the renal brush-border membrane (BBM) prepared from VDR null mice at 8 h after injection. Each lane represents an individual mouse BBM fraction.

8, and 24 h after the injection to monitor the changes in serum phosphate and calcium levels. As previously reported (17), serum phosphate levels decreased 8 h after the injection of recombinant FGF23 and recovered during the following 16 h in wild-type mice (Fig. 2A). Treatment with FGF23 aggravated hypophosphatemia in VDR KO mice 8 h after injection (Fig. 2A). This decrease in the phosphate level was still maintained at 24 h after the injection in VDR KO mice, whereas the significant reduction in the serum phosphate level induced by FGF23 disappeared in FGF23-treated wild-type mice. Administration of FGF23 did not alter serum calcium levels for up to 24 h in both wild-type and VDR KO mice (Fig. 2A). We then analyzed the expression of renal NaPi2a in VDR KO mice treated with FGF23. Immunoblot analysis of the NaPi2a protein in renal BBM after FGF23 administration demonstrated a clear reduction in NaPi2a protein abundance in VDR KO mice (Fig. 2B). Thus the FGF23-induced downregulation of renal NaPi2a was not impaired in the absence of VDR.

FGF23 action on vitamin D metabolism in VDR KO mice.

To evaluate the effect of FGF23 on vitamin D metabolism in VDR KO mice, 5 μ g of recombinant human FGF23 protein were intravenously administered to VDR KO or wild-type mice (5–6 wk of age) by a rapid bolus injection; subsequently, the animals were killed at 8, 12, and 24 h after the injection to collect blood and tissue samples. Administration of FGF23 significantly decreased the serum 1,25D level in wild-type mice, as previously reported (17). However, following FGF23 treatment, the serum 1,25D level did not show any change in VDR KO mice (Fig. 3). Next, we analyzed the mRNA levels of 1 α OHase and 24OHase in FGF23-treated VDR KO mice. At 8 h after the injection, administration of FGF23 to wild-type mice drastically decreased 1 α OHase mRNA abundance to 39% of that in vehicle-treated mice (Fig. 4). The basal expression level of 1 α OHase in the VDR KO strain was 18-fold higher than that in wild-type mice (Fig. 4). Administration of FGF23 clearly lowered the enhanced 1 α OHase mRNA expression in VDR KO mice to 23% of that in the vehicle-treated VDR KO group at 8 h after the injection, although the amount of 1 α OHase mRNA in FGF23-treated VDR KO mice was still higher (4-fold) than that in vehicle-treated wild-type mice. In contrast, 24OHase mRNAs in our VDR KO strain were undetectable (Fig. 4). Administration of FGF23 significantly increased the 24OHase mRNA level in wild-type mice at 8 h, as previously reported (17). However, in VDR KO mice, the 24OHase mRNA level was still undetectable even after FGF23 treatment (Fig. 4). We also employed real-time PCR to detect 24OHase mRNAs in VDR KO mice; however, only faint amplification was observed, and these amplification products could not be quantitatively estimated. Administration of FGF23 lowered the serum PTH level in wild-type mice (Fig. 3). A similar result was also obtained in our previous study regarding the early effects of FGF23 in normal mice (17). Because the decrease in the PTH level occurred simultaneously

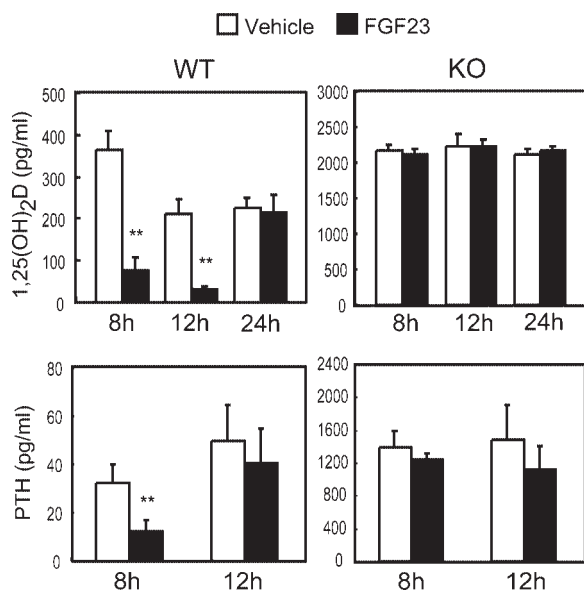


Fig. 3. Effects of FGF23 on serum concentrations of 1,25D and parathyroid hormone (PTH) in WT (left) and VDR KO mice (right). Values are means \pm SE (vehicle, phosphate-buffered saline; vehicle-treated wild-type mice, $n = 5$; FGF23-treated wild-type mice, $n = 6$; vehicle-treated VDR KO mice, $n = 6$; FGF23-treated VDR KO mice, $n = 7$). ** $P < 0.01$ vs. vehicle-treated group.

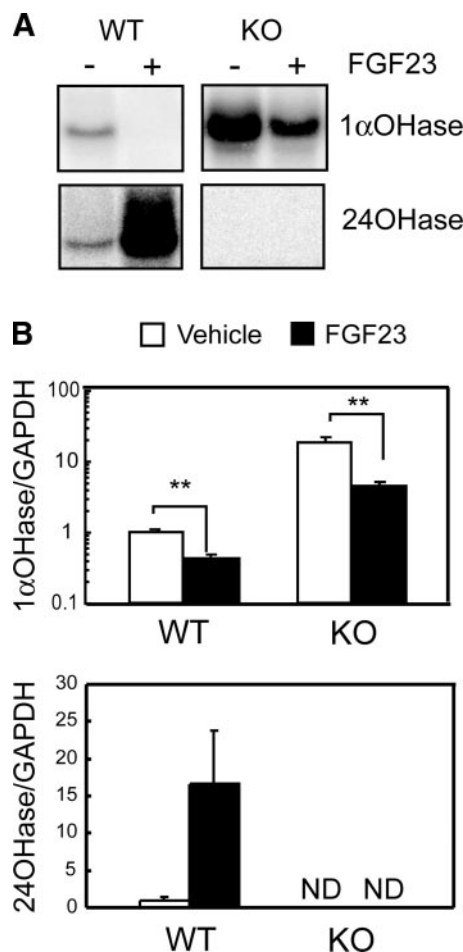


Fig. 4. Renal 1 α -hydroxylase (1 α OHase) and 24-hydroxylase (24OHase) mRNA abundance at 8 h after FGF23 administration in WT and VDR KO mice. A: Northern blot analysis of 1 α OHase and 24OHase mRNAs. B: quantitative assessment. The ratios of 1 α OHase or 24OHase to GAPDH are represented as the fold-increase over vehicle-treated WT mice. Values are means \pm SE (vehicle-treated wild-type mice, $n = 3$; FGF23-treated wild-type mice, $n = 3$; vehicle-treated VDR KO mice, $n = 3$; FGF23-treated VDR KO mice, $n = 3$). ND, not detectable. ** $P < 0.01$ vs. vehicle-treated group.

with the reduction in the serum phosphate level, it is possible that the reduced serum phosphate level might have transiently affected the serum PTH level in normal mice (23). However, because the PTH level in VDR KO mice did not respond to a further decrease in the serum phosphate level after FGF23 treatment, it is unlikely that phosphate is solely responsible for the FGF23-induced change in the PTH level. A question that needs to be addressed is whether FGF23 has a direct action on PTH secretion in the parathyroid gland.

Endogenous production of FGF23 in VDR KO mice. As shown in Table 1, FGF23 in circulation was not detectable in 6-wk-old VDR KO mice. Similar results were also obtained from 3-wk-old suckling homozygous pups that had just started to develop statistically significant hypocalcemia (wild-type, 9.44 ± 0.11 vs. homozygotes, 8.40 ± 0.25 ; $P < 0.01$). Because abnormal phenotypes of VDR KO mice could be rescued by feeding the animals a high-calcium diet (9), it is possible that undetectable FGF23 in circulation might have been caused by hypocalcemia. To elucidate this possibility, VDR KO mice and wild-type mice fed a high-calcium diet (2.0% calcium, 1.25%

phosphate, and 20% lactose) or control diet (0.5% calcium, 0.6% phosphate, and 20% lactose) were analyzed. After 1 wk of these diets, the serum calcium levels in VDR KO mice fed a high-calcium diet recovered to almost the same level as that in wild-type mice fed a control diet (Fig. 5). All wild-type mice evenly exhibited hypercalcemia and marked elevation of serum FGF23 concentrations after being fed a high-calcium diet for 1 wk (Fig. 5). Because blood urea nitrogen levels in these mice were not changed by this treatment, the possibility that the increase in serum FGF23 concentrations was caused by renal insufficiency was eliminated. In contrast, there were wide variations in the serum calcium and FGF23 levels in VDR KO mice fed with a high-calcium diet for 1 wk. Of the seven VDR KO mice fed a high-calcium diet for 1 wk, three still showed undetectable levels of FGF23, three mice demonstrated serum FGF23 levels ranging from 37.4 to 81.5 pg/ml, and one showed an extraordinarily high level of FGF23 (5,357.2 pg/ml). Serum calcium concentrations of these three groups of mice were 7.7–10.5, 8.8–11.1, and 13.0 mg/dl, respectively. Because serum FGF23 levels appeared to be induced according to the serum calcium levels, particularly by hypercalcemia, the duration of feeding the high-calcium diet was increased. After 4 wk, all VDR KO mice evenly developed hypercalcemia (12.1 ± 0.7 mg/ml), and significantly elevated serum FGF23 levels of $>2,000$ pg/ml were observed. These findings suggest

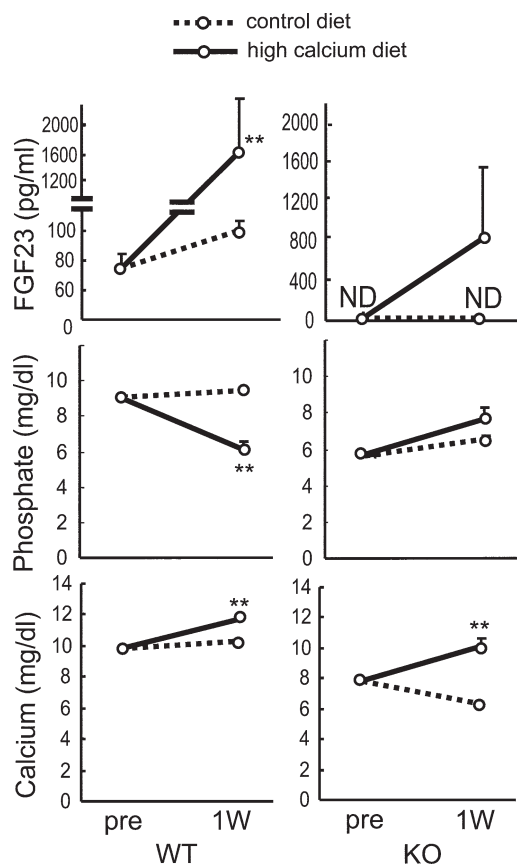


Fig. 5. Changes in serum concentrations of FGF23, phosphate, and calcium in WT and VDR KO mice before and after they were fed a high-calcium diet or control diet for 1 wk. Values are means \pm SE (WT mice fed a control diet, $n = 10$; WT mice fed a high-calcium diet, $n = 5$; KO mice fed a control diet, $n = 5$; and KO mice fed with high calcium diet, $n = 7$). ND refers to <3 pg/ml. ** $P < 0.01$ vs. control diet group.

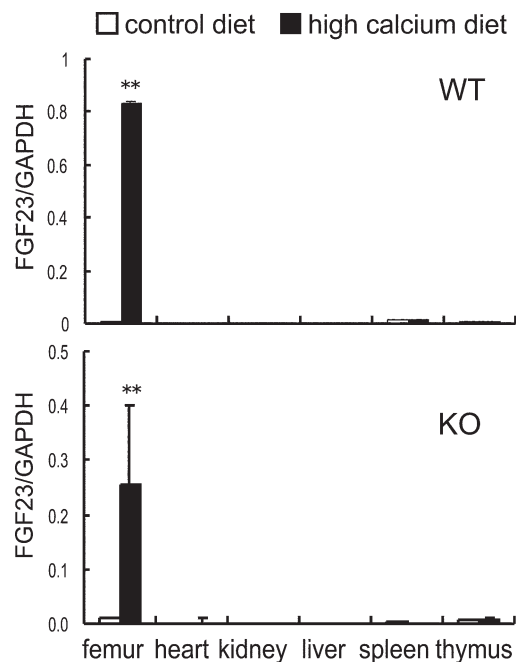


Fig. 6. Quantitative analysis of FGF23 mRNA levels in various tissues of WT and VDR KO mice fed a high-calcium diet for 4 wk. The quantities of FGF23 and GAPDH mRNAs were determined by real-time PCR, and their ratio was calculated. Values are means \pm SE for each group (WT mice fed a control diet, $n = 3$; WT mice fed a high-calcium diet, $n = 3$; VDR KO mice fed a control diet, $n = 3$; and VDR KO mice fed a high-calcium diet, $n = 3$). ** $P < 0.01$ vs. same tissue from mice fed a control diet.

that the serum calcium concentration is one of the determining factors of serum FGF23 levels. It is of interest to note that changes in FGF23 levels in response to calcium may alter serum phosphate levels. This enhanced FGF23 level observed after the high-calcium diet might partly contribute to the reduction in serum phosphate levels observed in wild-type mice (Fig. 5).

Subsequently, we attempted to identify the tissue responsible for the enhanced circulatory level of FGF23 in response to the high-calcium diet. The organs in which FGF23 expression was previously reported, namely, the femur, heart, kidney, liver, spleen, and thymus, were isolated from wild-type and VDR KO mice fed a high-calcium diet or a control diet for 4 wk. Additionally, FGF23 mRNA levels in each tissue were determined by real-time PCR. As shown in Fig. 6, a significant increase in FGF23 mRNA levels was observed in the femur isolated from both wild-type and VDR KO mice that were fed with the high-calcium diet.

On the other hand, the high-calcium diet used in the present study also contained a high level (1.25%) of phosphate. Therefore, we evaluated the sole effect of high phosphate levels in VDR KO and wild-type mice. Dietary loading of phosphate (1.2% phosphate, 0.5% calcium) for 1 wk led to a significant elevation of serum FGF23 levels in all wild-type mice without any variations (Fig. 7). In contrast, serum FGF23 levels in VDR KO mice fed the high-phosphate diet were not detected at all, although the serum phosphate level increased to the control level (Fig. 7). Additional feeding for 2 wk of a high-phosphate diet failed to induce detectable serum FGF23 levels in VDR KO mice (data not shown).

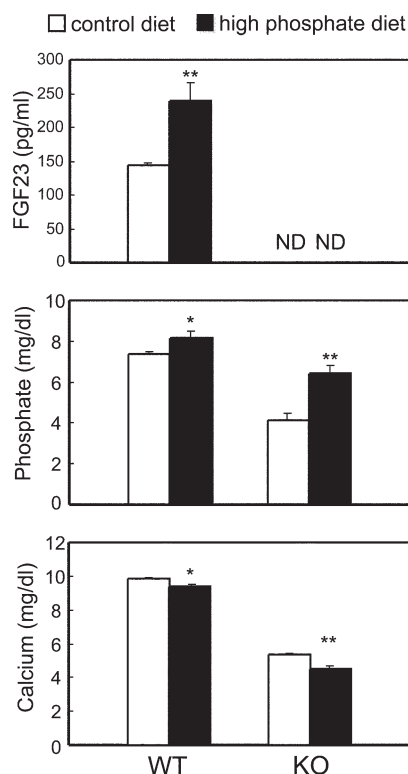


Fig. 7. Changes in serum concentrations of FGF23, phosphate, and calcium in WT and VDR KO mice after they were fed a high-phosphate diet or control diet for 1 wk. Values are means \pm SE for each group (WT mice fed a control diet, $n = 5$; WT mice fed a high-phosphate diet, $n = 5$; VDR KO mice fed a control diet, $n = 5$; and VDR KO mice fed a high-phosphate diet, $n = 5$). * $P < 0.05$, ** $P < 0.01$ vs. control diet group.

DISCUSSION

Previous studies demonstrated that FGF23 is a potent regulator of both vitamin D and phosphate metabolism (2, 3, 8, 16–21, 28). However, short-term *in vivo* experiments indicated that changes in serum 1,25D levels by FGF23 occurred before alterations in serum phosphate levels (17). In addition, clinical findings from a TIO patient demonstrated a rapid recovery of the serum 1,25D level before normalization of the serum phosphate level after surgery (29). Therefore, the rapid change in the serum 1,25D level in response to FGF23 may have some influence on the subsequent biological responses induced by FGF23, including the change in the serum phosphate concentration. To elucidate the direct effects of FGF23 and evaluate the contribution of the change in the 1,25D level to the biological effects induced by FGF23, we generated a VDR KO mouse line and used it to analyze the role of FGF23 in the present study.

Our VDR KO mice showed hypocalcemia, hypophosphatemia, rachitic skeletal phenotypes, and high serum concentrations of 1,25D and PTH, as reported in previously established VDR KO lines (10, 30). We previously demonstrated that a rapid bolus injection of recombinant FGF23 protein in normal mice caused a decrease in the serum phosphate level due to a reduction in the amount of renal NaPi2a protein after 8–9 h of the injection (17). Similarly, administration of recombinant FGF23 protein to VDR KO mice further aggravated the preexisting hypophosphatemia and was accom-

panied by a significant reduction in the amount of renal NaPi2a protein. These findings clearly demonstrate that FGF23 downregulates NaPi2a expression and lowers the serum phosphate level independently of VDR. Interestingly, this phosphate-lowering effect lasted longer in VDR KO mice than in wild-type mice (Fig. 2). This may be explained by the absence of 1,25D action in VDR KO mice because the decrease in the 1,25D level by FGF23 was transient and the serum 1,25D level recovered at 24 h after the FGF23 injection in wild-type mice. In addition, FGF23-induced downregulation of the NaPi2a protein is independent of PTH action, as previously demonstrated (17). Thus FGF23 regulates phosphate metabolism using a quite unique mechanism independently of the 1,25D/VDR system and PTH action.

It is well known that PTH and 1,25D are important regulators of renal 1α OHase mRNA expression (4, 7, 13, 25). The 1α OHase mRNA level in our VDR KO strain was significantly elevated, probably due to lack of negative feedback regulation by 1,25D and excess PTH (4, 13). Even in this situation, FGF23 evidently decreased 1α OHase mRNA levels in VDR KO mice without changing the serum PTH concentration. This result clearly indicates that FGF23 is a unique regulator of 1α OHase mRNA and functions independently of the 1,25D/VDR system. Furthermore, the PTH-independent effect may account for the previous observation that FGF23 could reduce serum 1,25D concentrations in parathyroidectomized rats (17). On the other hand, a series of *in vivo* studies have shown that FGF23 could stimulate renal 24OHase expression in wild-type mice (2, 3, 8, 17, 19). However, the basal expression level of 24OHase was extremely low in this VDR KO strain. In addition to Northern blot analysis, we attempted to quantify the expression level by real-time PCR; however, it remained undetectable even after the FGF23 treatment. It is well known that 24OHase mRNA expression is predominantly regulated by the 1,25D/VDR system via two positive vitamin D response elements in the promoter region (5). The absence of VDR-dependent regulation appeared to be very critical to investigating the effect of FGF23 on 24OHase gene expression. Our findings indicate that FGF23 failed to induce a drastic increase in 24OHase expression in VDR KO mice; however, whether the FGF23-inducible increase in 24OHase mRNA involves a VDR-dependent mechanism remains unclear from the findings of the present study. Serum 1,25D concentrations transiently decreased after FGF23 treatment in wild-type mice but did not in VDR KO mice (Fig. 3). Despite the significant reduction in 1α OHase expression levels by FGF23, the level in VDR KO mice still remained much higher than that in wild-type mice. Induction of 24OHase expression was completely absent in the VDR KO mice, whereas it was strongly induced in the wild-type mice. Taken together, insufficient reduction in 1α OHase and lack of 24OHase induction were not sufficient to lower the serum 1,25D concentration in FGF23-treated VDR KO mice. The physiological significance of VDR-independent regulation of the serum 1,25D level by FGF23 still remains unclear, and further studies are required to address this issue.

In this study, FGF23 administration transiently decreased serum PTH levels in wild-type mice, as described previously (17), but not in VDR KO mice. These results may indicate an unknown function of FGF23 in VDR-dependent regulation of PTH production. Hypocalcemia and the absence of 1,25D/VDR-dependent PTH suppression cause extraordinary eleva-

tion of serum PTH levels in VDR KO mice. FGF23 is probably less effective in overcoming these strong stimulations in the regulation of PTH production. On the other hand, Bai et al. (2) reported that FGF23 transgenic mice developed hyperparathyroidism. These mice first developed a hypocalcemic condition with reduced serum 1,25D concentrations accompanied by a decrease in renal $1\alpha\text{OHase}$ mRNA abundance. Subsequently, the serum PTH levels increased to compensate for the hypocalcemia by stimulating $1\alpha\text{OHase}$ expression with advancing age (2). These observations are considered to be similar to the conditions of VDR KO mice in which hypophosphatemia and lack of 1,25D action are potent triggers for PTH production. Therefore, it is explicable that the excess FGF23 in transgenic mice was no longer effective in the regulation of PTH under the dominant hypocalcemic condition as well as in VDR KO mice. It is likely that increased endogenous PTH competed with FGF23 for the regulation of $1\alpha\text{OHase}$ expression in FGF23 transgenic mice and vice versa in VDR KO mice treated with FGF23. Our findings are still preliminary to clarify the physiological relationship between FGF23 and PTH; however, they may provide an insight into the involvement of FGF23 in the network regulation of mineral metabolism.

The complete disappearance of FGF23 from the circulation in VDR KO mice and our previous observation that 1,25D administration elicited an increase in serum FGF23 in normal mice (17) suggest that basal expression of FGF23 appears to be regulated by the 1,25D/VDR system. In addition, in a recent preliminary study, Liu et al. (12) assigned a vitamin D response element in the promoter region of the mouse *Fgf23* gene. In addition to the possible VDR-dependent regulation of FGF23, we found that being fed a high-calcium diet for 1 wk led to hypercalcemia and an increase in the serum FGF23 concentration in the wild-type mice even and also induced increases in serum calcium levels and FGF23 concentrations in the VDR KO mice with wide variations. There appeared to be a positive correlation between the serum calcium and FGF23 levels in VDR KO mice. Being fed a high-calcium diet for 4 wk resulted in hypercalcemia and elevated serum FGF23 concentrations, which did not show any variations, in all VDR KO mice. These findings exemplified the relationship between serum calcium and FGF23 concentrations and suggest that calcium is one of the regulatory factors of FGF23 production. The calcium-dependent regulation of FGF23 may play a remarkable role in humoral hypercalcemia of malignancy (22) and primary hyperparathyroidism (27), in which positive correlations between serum FGF23 and calcium levels have recently been reported.

It is noteworthy that the calcium-induced increase in the serum FGF23 level was accompanied by strongly enhanced expression of FGF23 mRNA in bone. Although the main tissue supplying FGF23 in normal circulation has not yet been confirmed, recent studies strongly suggested that bone is a dominant tissue source for serum FGF23 in patients with fibrous dysplasia (15) and hypophosphatemic (*Hyp*) mice (11). The present study provides evidence indicating the importance of bone as a source of FGF23 and the VDR-independent regulation of FGF23 expression in bone. Further studies are necessary to elucidate the manner in which dietary calcium can stimulate FGF23 production and to identify the cells that are involved in this process.

We also evaluated the possible contribution of dietary phosphate to FGF23 production. The present study revealed that

continuous phosphate loading by being fed a high-phosphate diet caused a significant increase in serum FGF23 in wild-type mice. This result indicates that FGF23 participates in feedback regulation of phosphate homeostasis. However, the serum FGF23 level remained undetectable in VDR KO mice even when they were fed a high-phosphate diet. In this study, the serum calcium level of VDR KO mice was not rescued by feeding the animals a high-phosphate diet. In addition, calcium loading increased the serum FGF23 level more drastically than phosphate loading in wild-type mice. Based on these observations, it appears that the calcium level is one of the fundamental determinants of FGF23 production and, in some cases, may be more important than phosphate and 1,25D. Because FGF23 administration did not affect the serum calcium level in normal mice (17), the role of FGF23 in calcium metabolism has not been investigated in great detail thus far. This study emphasizes that FGF23 is closely related to calcium metabolism as well as bone metabolism, and this may lead to the determination of a new physiological role for FGF23 in further studies.

Taken together, we conclude that FGF23 regulates renal expressions of NaPi2a and $1\alpha\text{OHase}$ in a VDR-independent manner. In addition, we found that calcium is a potent stimulator of FGF23 production via a VDR-independent pathway. These findings ensure that FGF23 is a unique molecule regulating mineral metabolism and that the control of FGF23 expression is also integrated into multiple feedback loops to maintain mineral homeostasis.

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