1α,25-Dihydroxyvitamin D₃ upregulates FGF23 gene expression in bone: the final link in a renal-gastrointestinal-skeletal axis that controls phosphate transport

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Kolek, Olga I., Eric R. Hines, Marci D. Jones, Loren K. LeSueur, Maciej A. Lipko, Pawel R. Kiela, James F. Collins, Mark R. Haussler, and Fayez K. Ghishan. 1α,25-Dihydroxyvitamin D₃ upregulates FGF23 gene expression in bone: the final link in a renal-gastrointestinal-skeletal axis that controls phosphate transport. Am J Physiol Gastrointest Liver Physiol 289: G1036–G1042, 2005.—1α,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃] and elicits fibroblast growth factor (FGF)23 is a phosphaturic hormone that decreases circulating 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] and elicits hypophosphatemia, both of which contribute to rickets/osteomalacia. It has been shown recently that serum FGF23 increases after treatment with renal 1,25(OH)₂D₃ hormone, suggesting that 1,25(OH)₂D₃ negatively feedback controls its levels by inducing FGF23. To establish the tissue of origin and the molecular mechanism by which 1,25(OH)₂D₃ increases circulating FGF23, we administered 1,25(OH)₂D₃ to C57BL/6 mice. Within 24 h, these mice displayed a dramatic elevation in serum immunoreactive FGF23, and the expression of FGF23 mRNA in bone was significantly upregulated by 1,25(OH)₂D₃, but there was no effect in several other tissues. Furthermore, we treated rat UMR-106 osteoblast-like cells with 1,25(OH)₂D₃, and real-time PCR analysis revealed a dose- and time-dependent stimulation of FGF23 mRNA concentrations. The maximum increase in FGF23 mRNA was 1.024-fold at 10⁻⁷ M 1,25(OH)₂D₃ after 24-h treatment, but statistically significant differences were observed as early as 4 h after 1,25(OH)₂D₃ treatment. In addition, using cotreatment with actinomycin D or cycloheximide, we observed that 1,25(OH)₂D₃ regulation of FGF23 gene expression occurs at the transcriptional level, likely via the nuclear vitamin D receptor, and is dependent on synthesis of an intermediary transactivator. These results indicate that bone is a major site of FGF23 expression and source of circulating FGF23 after 1,25(OH)₂D₃ administration or physiological upregulation. Our data also establish FGF23 induction by 1,25(OH)₂D₃ in osteoblasts as a feedback loop between these two hormones that completes a kidney-intestine-bone axis that mediates phosphate homeostasis.

INORGANIC PHOSPHORUS (Pₐ) is required for skeletal mineralization, energy transfer, cellular signaling, and regulation of protein function. The homeostasis of serum Pₐ levels is effected through a complex interplay between intestinal absorption, exchange with intracellular and bone storage pools, and renal tubular reabsorption. These processes are primarily regulated by parathyroid hormone (PTH) and 1α,25-dihydroxyvitamin D [1,25(OH)₂D₃] (5, 7, 19). Recently, a growing body of evidence suggests that other factors of bone origin participate in maintaining Pₐ homeostasis, such as fibroblast growth factor (FGF)23, matrix extracellular phosphoglycoprotein, and frizzled-related protein 4. These factors, known collectively as the phosphatonin, have been shown to be associated with hypophosphatemic diseases, including autosomal dominant hypophosphatemic rickets (ADHR), tumor-induced osteomalacia (TIO), and X-linked hypophosphatemic rickets (XLH) (1–4, 6, 8, 9, 12, 16–18, 20–22, 26–29, 31–33).

Among these factors, FGF23 is the only protein to date that has been linked to all three disorders. Missense mutations in FGF23, which likely prevent its cleavage and inactivation, are the cause of ADHR (1). Administration of an ADHR mutant form of FGF23 to mice inhibited sodium-phosphate cotransport activities in both the kidney (NaPi-2a) and small intestine (NaPi-2b), and suppressed 1,25(OH)₂D₃ (22). Ectopic overproduction of FGF23 overwhelms its processing and degradation, leading to TIO (26). Administration of recombinant FGF23 in normal and parathyroidectomized animals induced a decrease in serum phosphate levels, phosphaturia accompanied by a reduction in renal mRNA and protein levels for NaPi-2a, a decrease in renal mRNA for 25-hydroxyvitamin D-1α-hydroxylase, and an increase in 25-hydroxyvitamin D-24-hydroxylase, the cytochrome P-450 enzymes that generate and inactivate 1,25(OH)₂D₃ hormone, respectively (24, 26). Mice implanted with FGF23-expressing Chinese hamster ovary (CHO) cells showed more severe hypophosphatemia, osteomalacia, and decreased 1,25(OH)₂D₃ levels (26). Conversely, FGF23-null mice exhibited increased circulating 1,25(OH)₂D₃ despite hyperparathyroidism, hypercalcemia, and low PTH levels (25). Elevated circulating FGF23 levels have also been found in most, but not all, patients with XLH (12, 33).

FGF23 mRNA is expressed in a variety of human and mouse tissues, including the following: bone (calvaria, mandible, long bone, femoral heads), brain, thymus, small intestine, heart, lung, liver, kidney, thyroid/parathyroid, lymph node, skeletal muscle, spleen, skin, stomach, and testis (1, 14, 26, 32). Among these tissues, FGF23 mRNA expression is found to be highest in bone in both the human and mouse. Liu et al. (14) reported that Hyp mice express markedly increased FGF23 levels in the calvaria, mandible, and long bone compared with normal mice. The increase of FGF23 in Hyp mice was limited.
to bone with no observable increases of FGF23 in the bone marrow, kidney, lung, or liver (14).

Regulation of FGF23 production is still unclear. Recent studies have revealed that circulating FGF23 is regulated by serum P3, controlled by dietary P1, in five-sixth nephrectomized rats (23). Mirams et al. (15) showed upregulation of FGF23 mRNA expression by extracellular phosphate in osteoblast-like cells. In addition, the administration of 1,25(OH)2D3 increased serum FGF23 in both thyroparathyroidectomized rats and normal mice (23, 24). Furthermore, Ito et al. (11) have recently reported that 1,25(OH)2D3 enhanced FGF23 promoter activity and mRNA expression in human chronic myelogenous leukemia K562 cells. Consistent with the observation that FGF23 is potentially dependent on 1,25(OH)2D3, vitamin D receptor (VDR)-null mice have very low serum FGF23 and do not respond to 1,25(OH)2D3 administration (11, 23).

In this study, we examined the effect of 1,25(OH)2D3 on FGF23 mRNA expression levels in different mouse tissues to identify the major source of elevated circulating FGF23. We also investigated the molecular mechanism whereby 1,25(OH)2D3 induces FGF23 in an osteoblast-like cell line, UMR-106. Our data indicate that bone, likely the osteoblast or its precursor cell, is a major source of FGF23 in response to 1,25(OH)2D3. This establishes a reciprocal relationship between 1,25(OH)2D3 and FGF23, with phosphatemic 1,25(OH)2D3 hormone generated in the kidney, inducing skeletal endocrine cells to produce FGF23, which then feedback represses renal 1α-OHase to curtail 1,25(OH)2D3 biosynthesis as well as inhibits the renal reabsorption of phosphate to elicit phosphaturia.

MATERIALS AND METHODS

Experimental animals. Four- to five-week-old male C57BL/6 mice in groups of three to four were subcutaneously injected once a day for 2 days with either vehicle (1:4, ethanol-propylene glycol) or 6 μg/kg body wt 1,25(OH)2D3 (Sigma; St. Louis, MO) (30). Animals were supplied with food and water ad libitum. Mice were killed 24 h after the second 1,25(OH)2D3 injection by CO2 narcosis followed by decapitation. Trunk blood was collected, allowed to clot, centrifuged to obtain serum, and stored at −70°C. Tissues (calvaria, tibia, jejunum, liver, spleen, kidney, and brain) were removed, flash frozen in liquid nitrogen, and stored at −70°C. All methods used in this study were approved by the Institutional Animal Care and Use Committee of the University of Arizona.

Chemicals and reagents. Sodium pyruvate (100 mM), 100× anti-biotic-antimycotic, and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA). Dulbecco’s modified Eagle’s high-glucose medium and FBS were from Irvine Scientific (Santa Ana, CA). FGF23 and 18S Taqman primer/probe sets were purchased from Applied Biosystems (Foster City, CA). iScript cDNA Synthesis Kit and iQ Supermix were purchased from Ambion (Austin, TX). DNase I-treated RNA (250 ng) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s protocol (Ambion). The resulting RNA was evaluated by agarose gel electrophoresis, and concentrations were adjusted according to densitometric analysis of the 18S rRNA band. DNase I-treated RNA (250 ng) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s protocol. Subsequently, 20-μl PCRs were set up in 96-well plates containing 10 μl iQ Supermix, 1 μl TaqMan primer/probe set, 2 μl cDNA synthesis reaction (out of a 20-μl total volume), and 7 μl molecular grade water. Reactions were run and analyzed on a Bio-Rad iCycler iQ Real-Time PCR detection system. Cycling parameters were determined, and resulting data were analyzed according to ABI protocols. Briefly, data were analyzed using the comparative cycle threshold (Ct) method as a means of relative quantification, normalized to an endogenous reference (18S rRNA) and relative to a calibrator [normalized Ct value obtained from ethanol (EtOH)-treated UMR-106 cells or mice], and expressed as 2(−ΔΔCt) according to Applied Biosystems User Bulletin no. 2: Rev B Relative Quantitation Of Gene Expression. Applied Biosystems does not supply the sequence of the custom-designed FGF23 primer/probe sets; therefore, we independently designed and obtained PCR primers encompassing mouse and rat FGF23 gene coding regions. These independent primers yielded essentially equivalent results for FGF23 mRNA induction by 1,25(OH)2D3 in the mouse in vivo (see Fig. 2) and UMR-106 cells in vitro (see Fig. 4) (data not shown). This result eliminates the unlikely possibility of an error in the Applied Biosystems primer/probe construction causing the amplification of a gene unrelated to FGF23.

FGF23 ELISA kit. Serum FGF23 was determined using a FGF-23 ELISA kit (Kainos Laboratories; Tokyo, Japan).

Cell culture. Rat osteogenic sarcoma cells (UMR-106) were obtained from the American Type Culture Collection. UMR-106 cells were cultured in Dulbecco’s modified Eagle’s high-glucose media containing 10% FBS, 1 mM sodium pyruvate, and 1× antibiotic-antimycotic. Cell cultures were maintained at 37°C with 5% CO2. For analysis of endogenous FGF23 gene expression, cells were seeded on six-well plates at 0.3 × 105 cells/well and treated with 10−7 M 1,25(OH)2D3 or EtOH 48 h postseeding. Where indicated, 1 μg/ml actinomycin D (Calbiochem; San Diego, CA) or 10 μM cycloheximide (Sigma) was added 30 min before 1,25(OH)2D3 or EtOH treatment and continued throughout the treatment period. In each experiment, the results from three wells for six-well plates were averaged and considered as n = 1. There was no significant variance among the individual wells in each averaged group.

Statistical analysis. Statistical significance was determined by the Student’s t-test or ANOVA followed by Fisher’s protected least-significance difference test using StatView software package version 4.53 (SAS Institute; Cary, NC). All data are expressed as means ± SE.

RESULTS

1,25(OH)2D3 increases serum FGF23 in mice. We first reexamined and confirmed recent reports showing that 1,25(OH)2D3 increases circulating FGF23 in vivo (23, 24). As shown in Fig. 1, a dramatic elevation of serum FGF23 levels was observed...
in 1,25(OH)₂D₃-injected mice (n = 3, P < 0.01), with an approximate 80-fold enhancement in circulating FGF23 24 h after the second injection of 1,25(OH)₂D₃.

FGF23 expression is increased in bone of 1,25(OH)₂D₃-injected mice. We next investigated whether FGF23 mRNA expression is regulated by 1,25(OH)₂D₃ in vivo. We examined the expression of FGF23 mRNA in tissues of vehicle-injected mice using real-time PCR. Our findings are in agreement with prior reports (14) showing that FGF23 expression is highest in bone followed by the spleen, brain, jejunum, and liver, with nearly undetectable levels in the kidney (Fig. 2A; n = 3). Next, we compared FGF23 mRNA expression in tissues of mice injected with 1,25(OH)₂D₃ or vehicle. 1,25(OH)₂D₃ markedly increased FGF23 mRNA levels in the calvaria (57 ± 1.7-fold) and tibia (8 ± 0.7-fold) (Fig. 2B; n = 4, P < 0.0001). However, no significant increases in FGF23 mRNA were observed in several nonbone tissues derived from 1,25(OH)₂D₃-injected mice (Fig. 2C). Thus 1,25(OH)₂D₃ selectively upregulates expression of bone FGF23 mRNA in vivo.

1,25(OH)₂D₃ dose and time dependently increases endogenous FGF23 mRNA expression in UMR-106 cells. The in vivo findings presented in Fig. 2 and other reports suggest that FGF23 is expressed mainly in bone. FGF23 transcripts have also been found in primary human osteoblast-like bone cells (15) and immortalized osteoblast cell lines derived from wild type (TMOb-Ni) and Hyp (TMOb-Hyp) mice (14). We detected FGF23 expression by real-time PCR in the rat osteogenic sarcoma cell line UMR-106.

To determine whether 1,25(OH)₂D₃ had a similar effect on FGF23 mRNA expression in bone and in cultured osteoblasts, and to evaluate the dose dependency of that effect, UMR-106 cells were incubated for 24 h with the hormone at doses ranging from 10⁻⁹ to 10⁻⁷ M. As shown in Fig. 3, we found that 1,25(OH)₂D₃ increased FGF23 mRNA levels in a dose-dependent manner, and the maximum increase of FGF23 mRNA expression (1,024 ± 305-fold) was observed at 10⁻⁷ M 1,25(OH)₂D₃ (n = 3, P < 0.05). In addition, we examined the time course of 1,25(OH)₂D₃ effects on mRNA levels for FGF23 in osteoblast-like cells. As shown in Fig. 4, a significant increase in FGF23 mRNA expression (154 ± 80-fold) was observed as rapidly as 4 h after 10⁻⁷ M 1,25(OH)₂D₃ treatment and reached the maximum (1,122 ± 118-fold) within 24 h after hormone treatment (n = 3, P < 0.05). Therefore, 1,25(OH)₂D₃ induces FGF23 mRNA in UMR-106 osteoblasts within 4 h of
treatment, consistent with a transcriptional effect, but maximum FGF23 mRNA accumulation requires 24 h.

1,25(OH)2D3 regulates FGF23 mRNA expression at the transcriptional level, likely via synthesis of an intermediary transfactor. To elucidate the molecular mechanism involved in the increase of FGF23 mRNA in osteoblast-like cells after 1,25(OH)2D3 treatment, we examined whether regulation of FGF23 mRNA expression by 1,25(OH)2D3 occurs at the transcriptional level. Actinomycin D was added to the culture media before 1,25(OH)2D3 or EtOH treatment. As expected, actinomycin D completely abolished the 1,25(OH)2D3 effect on mRNA levels for FGF23 in UMR-106 cells (Fig. 5; n = 5, *P < 0.0001). Interestingly, as shown in Fig. 6, pretreatment with cycloheximide also prevented the 1,25(OH)2D3 effect on FGF23 mRNA expression in osteoblast-like cells (n = 4, *P < 0.0001). Thus the induction of FGF23 by 1,25(OH)2D3 in rat osteoblast-like cells is a transcriptional effect (Fig. 5), likely mediated by the nuclear VDR (13), but because ongoing protein synthesis is required for this action (Fig. 6), a rapidly turned over intermediary transfactor appears to participate in the mechanism.

DISCUSSION

It has been reported recently that a single injection of 1,25(OH)2D3 increased serum FGF23 in normal mice (24). Moreover, Saito et al. (23) showed that the 1,25(OH)2D3 effect on circulating FGF23 was dose dependent and independent of serum Pi in normal and thyroparathyroidectomized rats. In the present study, we investigated a contributing source of circulating FGF23 in 1,25(OH)2D3 injected mice and the molecular mechanisms by which 1,25(OH)2D3 regulates FGF23 mRNA expression in rat osteoblast-like cells.

We first showed that the levels of serum FGF23 in mice after 1,25(OH)2D3 injection were significantly elevated compared
with FGF23 concentrations after EtOH administration (Fig. 1). Next, we confirmed, by real-time PCR, that expression of FGF23 mRNA in bone is high relative to other mouse tissues previously reported to express FGF23 (Fig. 2A). Additionally, we examined mRNA levels for FGF23 in several mouse tissues, including bone (calvaria and tibia), jejunum, kidney, liver, spleen, and brain, after a subcutaneous injection of 1,25(OH)2D3. We observed that 1,25(OH)2D3 markedly increased FGF23 mRNA expression in bone (calvaria and tibia; Fig. 2B). The increase in FGF23 mRNA in 1,25(OH)2D3-injected mice was limited to the skeleton because levels of FGF23 mRNA expression in other tissues of 1,25(OH)2D3-treated mice were similar to levels of FGF23 found in vehicle-treated mice. Observations such as high FGF23 expression in bone, large total mass of the skeleton, and a bone-restricted increase in FGF23 mRNA levels after 1,25(OH)2D3 administration suggest that bone is the major site of FGF23 expression and is the predominant tissue of origin of circulating FGF23 in vehicle- and 1,25(OH)2D3-treated mice. Similar to the increased expression of FGF23 in bone of 1,25(OH)2D3-injected mice, FGF23 mRNA was greater in 1,25(OH)2D3-treated compared with EtOH-treated rat osteoblast-like cells. We determined that 10−7 M is the most effective concentration of 1,25(OH)2D3 in the regulation of endogenous FGF23 expression in UMR-106 cells (Fig. 3). Moreover, Ito et al. (11) have reported recently that 1,25(OH)2D3 dose dependently upregulates FGF23 promoter activity in K562 cells, and the maximum increase occurred with 10−7 M 1,25(OH)2D3. Because serum vitamin D binding protein is present in the culture medium, the available free 1,25(OH)2D3 concentration at 10−7 M total hormone is 10−9 M, close to the dissociation constant of the VDR. Thus 10−7 M 1,25(OH)2D3 is commonly used in all culture experiments and can only be considered slightly supraphysiological. We also determined that 1,25(OH)2D3 increases mRNA levels for FGF23 as rapidly as 4 h after treatment (Fig. 4), consistent with transcriptional regulation of FGF23 expression by the hormone. Consequently, to confirm this hypothesis, we cotreated UMR-106 cells with a DNA transcription inhibitor, actinomycin D, and 1,25(OH)2D3. As expected, actinomycin D abolished the effect of 1,25(OH)2D3 on FGF23 mRNA (Fig. 5). Surprisingly,
cotreatment UMR-106 cells with 1,25(OH)\textsubscript{2}D\textsubscript{3} and cycloheximide (an inhibitor of mRNA translation) also abolished the 1,25(OH)\textsubscript{2}D\textsubscript{3} effect on FGF23 mRNA expression (Fig. 6). Thus it is likely that a labile protein is required for 1,25(OH)\textsubscript{2}D\textsubscript{3} induction of FGF23 in bone cells. This hypothesized protein may be a cofactor in the 1,25(OH)\textsubscript{2}D\textsubscript{3}-VDR/retinol X receptor (RXR) nuclear receptor complex that activates transcription (13) at the level of the FGF23 promoter. Alternatively, the transcriptional action of 1,25(OH)\textsubscript{2}D\textsubscript{3}-VDR/RXR on FGF23 could be secondarily mediated by the activation of a primary transactivator, which, in turn, stimulates the FGF23 promoter. This second notion is consistent with the apparent lack of VDREs in the mouse FGF23 proximal promoter (11).

In conclusion, there exists a feedback loop among \( P_{\text{t}} \), 1,25(OH)\textsubscript{2}D\textsubscript{3}, and FGF23 as part of a kidney-intestine-bone hormonal axis that is hypothesized to play a key role in phosphate homeostasis. This axis is depicted schematically in Fig. 7. In this model, 1,25(OH)\textsubscript{2}D\textsubscript{3} is produced in the kidney by \( 1\alpha\)-OHase, an enzyme that is induced under low circulating phosphate situations to generate excess 1,25(OH)\textsubscript{2}D\textsubscript{3} to enhance phosphate absorption from the intestine, reabsorption from the kidney, and resorption from bone, thereby correcting hypophosphatemia (10). Calcium absorption from the small intestine is also enhanced by 1,25(OH)\textsubscript{2}D\textsubscript{3}, acting via VDR and the induction of calcium transport machinery (10). The resulting increased calcium and phosphate in the blood exceed their ion products and promote bone mineralization (Fig. 7). Importantly, to ensure phosphate homeostasis and prevent hyperphosphatemic spikes that would precipitate ectopic calciumification, enhanced 1,25(OH)\textsubscript{2}D\textsubscript{3} also induces FGF23 synthesis and release from bone, specifically, the osteoblast endocrine cell (Fig. 7). Increased FGF23 then, as illustrated in Fig. 7, would close the endocrine loop, both by eliciting phosphaturia via inhibition of Na\textsubscript{d}P\textsubscript{d}-2a in the kidney and by repressing renal 1\alpha\textsubscript{2}OHase, resulting in the correct adjustment in circulating PO\textsubscript{4}\textsuperscript{3}\textsuperscript{-} and 1,25(OH)\textsubscript{2}D\textsubscript{3}. The present data (Figs. 1–4) support the conclusion that 1,25(OH)\textsubscript{2}D\textsubscript{3}-induced FGF23 from bone constitutes the final link in a renal-gastrointestinal-skeletal axis that controls serum phosphate and active vitamin D levels.

Finally, as depicted in Fig. 7, low circulating calcium conditions stimulate renal 1\alpha\textsubscript{2}OHase through the alternative pathway of increased PTH secretion (10). 1,25(OH)\textsubscript{2}D\textsubscript{3} in turn activates both calcium and phosphate absorption from the intestine, with the former correcting hypocalcemia but the latter possibly eliciting hyperphosphatemia. Initially, this hyperphosphatemia could be compensated for by PTH-triggered phosphaturia, although this will be short lived because the corrected serum calcium will suppress PTH. We propose that in the long term, 1,25(OH)\textsubscript{2}D\textsubscript{3} induction of FGF23 provides protection against hyperphosphatemia associated with restoring serum calcium levels, which may explain why there is a need for two hypophosphatemic hormones, PTH and FGF23. Clearly the role of PTH is more significant in the calcium-vitamin D axis, whereas FGF23 mediates the phosphate-vitamin D axis.

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