Fibroblast growth factor-23 abolishes 1,25-dihydroxyvitamin D₃-enhanced duodenal calcium transport in male mice

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Khuituan P, Teerapornpuntakit J, Wongdee K, Suntoonsaratoo P, Konthapakdee N, Sangsaksri J, Sripong C, Krishnamra N, Charoenphandhu N. Fibroblast growth factor-23 abolishes 1,25-dihydroxyvitamin D₃-enhanced duodenal calcium transport in male mice. Am J Physiol Endocrinol Metab 302: E903–E913, 2012. First published January 24, 2012; doi:10.1152/ajpendo.00620.2011.—Despite being widely recognized as the important bone-derived phosphaturic hormone, whether fibroblast growth factor (FGF)-23 modulated intestinal calcium absorption remained elusive. Since FGF-23 could reduce the circulating level of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], FGF-23 probably compromised the 1,25(OH)₂D₃-induced intestinal calcium absorption. FGF-23 may also exert an inhibitory action directly through FGF receptors (FGFR) in the intestinal cells. Herein, we demonstrated by Ussing chamber technique that male mice administered 1 µg/kg 1,25(OH)₂D₃ sc daily for 3 days exhibited increased duodenal calcium absorption, which was abolished by concurrent intravenous injection of recombinant mouse FGF-23. This FGF-23 administration had no effect on the background epithelial electrical properties, i.e., short-circuit current, transepithelial potential difference, and resistance. Immunohistochemical evidence of protein expressions of FGFR isoforms 1–4 in mouse duodenal epithelial cells suggested a possible direct effect of FGF-23 on the intestine. This was supported by the findings that FGF-23 directly added to the serosal compartment of the Ussing chamber and completely abolished the 1,25(OH)₂D₃-induced calcium absorption in the duodenal tissues taken from the 1,25(OH)₂D₃-treated mice. However, direct FGF-23 exposure did not decrease the duodenal calcium absorption without 1,25(OH)₂D₃ preinjection. The observed FGF-23 action was mediated by MAPK/ERK, p38 MAPK, and PKC. Quantitative real-time PCR further showed that FGF-23 diminished the circulating level of 1,25-dihydroxyvitamin D₃ and parathyroid hormone (PTH) (21, 47).

FGF-23 is part of the bone-kidney-parathyroid endocrine axis, in which 1,25(OH)₂D₃ induces FGF-23 secretion from bone. FGF-23 subsequently suppresses the productions of 1,25(OH)₂D₃ and PTH in the kidney and parathyroid gland, respectively (29). Some hereditary and acquired diseases, e.g., autosomal dominant hypophosphatemic rickets/osteomalacia, tumor-induced osteomalacia, and X-linked hypophosphatemic rickets, result from abnormally high circulating FGF-23 activity. Dysregulation of FGF-23 action is also evident in various pathological conditions, such as chronic metabolic acidosis and chronic kidney disease (11, 20).

Once secreted from osteoblasts and osteocytes, FGF-23 exerts its phosphaturic action in the renal proximal tubular cells via the FGF receptor (FGFR)/Klotho coreceptor complex, thereby downregulating Na⁺-dependent phosphate transporter (NPT)-2a and NPT-2c expression (14). Its intracellular signaling in renal epithelial cells is conveyed through a number of pathways, e.g., mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), p38 MAPK, phosphoinositide 3-kinase (PI3K)/Akt, and protein kinase C (PKC) (12, 13, 43). FGF-23 also downregulates renal 25-hydroxyvitamin D₁α-hydroxylase (1-OHase; also known as Cyp27b1) and upregulates 24-hydroxylase (24-OHase, Cyp24a1), which are important enzymes for production and inactivation of 1,25(OH)₂D₃, respectively, thereby reducing circulating levels of 1,25(OH)₂D₃ (35, 42). Since 1,25(OH)₂D₃ is the cardinal regulator of intestinal calcium absorption, and since there is a strong link between 1,25(OH)₂D₃ and calcium-phosphate homeostasis (for reviews see Refs. 10 and 30), it is probable that FGF-23 not only regulates phosphate metabolism but may also inhibit the 1,25(OH)₂D₃-induced intestinal calcium transport. In other words, besides being a phosphatonin, FGF-23 may also act as a calcium-regulating hormone to modulate intestinal calcium absorption. In addition to its indirect action by lowering plasma 1,25(OH)₂D₃ levels, we hypothesized that FGF-23 could directly regulate calcium transport in intestinal epithelial cells, which have been reported to express FGFR mRNA (16).

In humans and rodents, 1,25(OH)₂D₃ enhances transcellular active calcium transport especially in duodenal villous epithelial cells (5, 22). This transport mechanism is a three-step, metabolically energized process consisting of (1) apical calcium uptake via the transient receptor potential cation channel subfamily V (TRPV)-5 and -6, (2) cytoplasmic translocation by calcium-binding protein calbindin-D₉k, and (3) basolateral extrusion by plasma membrane Ca²⁺/ATPase (PMCA)-1b (8, 22). A small proportion of the cytoplasmic calcium may be extruded via the basolateral Na⁺/Ca²⁺ exchanger (NCX)-1, the

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activity of which is ~5-fold lower than that of PMCA_{1b} (15). The expressions of the major calcium-transporting proteins are strongly upregulated by 1,25(OH)_{2}D_{3} (10). Therefore, it is reasonable to postulate that FGF-23 can suppress the 1,25(OH)_{2}D_{3}-induced upregulation of the intestinal calcium transporter gene expression.

Thus, the objectives of the present study were 1) to demonstrate that FGF-23 administration could abolish the increase in duodenal calcium transport in 1,25(OH)_{2}D_{3}-treated mice; 2) to investigate whether duodenal tissue directly responded to FGF-23; 3) to determine possible signaling pathways of FGF-23 in the mouse duodenum by using various pharmacological antagonists; and 4) to demonstrate the expression of calcium transporter genes in the duodenal epithelial cells of 1,25(OH)_{2}D_{3}- and FGF-23-treated mice.

**MATERIALS AND METHODS**

**Animals.** Male ICR mice (Mus musculus; 7 wk old, weighing 30–40 g) were obtained from the National Laboratory Animal Centre, Thailand. Mice were acclimatized for 7 days before start of the

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**Fig. 1. A–D: diagrams show the experimental design.** 1,25(OH)_{2}D_{3}, 1,25-dihydroxyvitamin D_{3}; FGF, fibroblast growth factor. For more details, please see MATERIALS AND METHODS.
experiment. They were placed in solid bottom, open-top plastic cages, fed standard chow containing 10% wt/wt calcium, 0.9% wt/wt phosphorus, and 4,000 IU/kg vitamin D (CP, Bangkok, Thailand), and reverse osmosis water ad libitum under a 12:12-h light-dark cycle. The room had a temperature of 20–25°C, humidity of 50–60%, and average illumination of 150–200 lux in the daytime. Body weight and food intake were recorded daily. This study was approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Mahidol University, Thailand. All animals were cared for in accordance with the principles and guidelines of the American Physiological Society, “Guiding Principles in the Care and Use of Animals.”

**Experimental design.** In the first series of experiments (Fig. 1A), mice were injected with 1 μg/kg 1,25(OH)2D3 sc (catalog no. 71820; Cayman Chemical, Ann Arbor, MI), 1 μg/kg 1,25(OH)2D3 sc plus various doses of recombinant mouse FGF-23 (35, 70, 140, or 280 μg/kg ivvia the tail vein; catalog no. 2629-FG; R&D Systems, Minneapolis, MN) at 72, 48, and 24 h prior to determination of duodenal calcium flux and epithelial electrical properties. The doses of FGF-23 and 1,25(OH)2D3 used were as reported previously (25, 33). The vehicle-treated mice (control) were injected with 3 ml/kg 9:1 propylene glycol-ethanol sc [for 1,25(OH)2D3 preparation] and 1 ml/kg 0.9% NaCl iv (for FGF-23 preparation). The 1,25(OH)2D3-treated mice were also injected with the vehicle of FGF-23. In one experiment, mice were injected with 140 μg/kg FGF-23 alone.

To determine whether murine intestine could directly respond to FGF-23, duodenal tissues taken from normal mice were subjected to immunohistochemical analyses of FGFR and Klotho expression. FGF-23 mRNA expression was also investigated in various tissues, e.g., small and large intestine, tibia, femur, spleen, liver, kidney, brain, and lung, to reveal the possible sources of circulating FGF-23 in mice. Thereafter, duodenal calcium flux and epithelial electrical properties were determined by Ussing chamber technique in FGF-23-exposed duodenal tissues. Recombinant mouse FGF-23 was added directly into the mucosal or serosal sides of the chamber to obtain a final concentration of 10, 20, or 40 ng/ml (38, 43). In this Ussing chamber experiment (Fig. 1B), duodenal tissues were obtained from vehicle-treated (3 ml/kg 9:1 propylene glycol-ethanol sc; control group) or 1 μg/kg sc 1,25(OH)2D3-treated (at 72, 48, and 24 h prior to tissue collection).

Although subcutaneous 1,25(OH)2D3 administration efficiently increases circulating 1,25(OH)2D3 levels (40), a number of studies also used the intraperitoneal route for systemic 1,25(OH)2D3 administration (1, 9). Therefore, in some experiments (Fig. 1C), mice were injected intraperitoneally with 1 μg/kg 1,25(OH)2D3 or its vehicle at 52, 28, and 4 h prior to tissue collection to confirm that FGF-23 could also suppress the action of 1,25(OH)2D3 administered via the intraperitoneal route. Finally, calcium flux was measured in 10, 20, or 40 ng/ml FGF-23-exposed duodenal tissues from untreated normal mice to determine whether the direct effect of FGF-23 on mouse duodenum could be observed without pretreatment with 1,25(OH)2D3 (timeline diagram not shown).

In the last series of experiments (Fig. 1D), all mice were injected with 1 μg/kg 1,25(OH)2D3 sc at 72, 48, and 24 h before tissue collection. Calcium flux and electrical parameters were determined in the duodenal tissues directly exposed on the serosal side to 20 ng/ml recombinant mouse FGF-23. Some tissues were also exposed to various signal transduction inhibitors of effective concentrations (3, 4, 12, 13, 44), as listed in Table 1. The studied mediators, e.g., MAPK/ERK,Src,Jun Kinase (JNK), protein kinase C (PKC), are involved in FGFR signaling (12, 13, 44). FGF-23 and/or signal transduction inhibitors were present in the Ussing chamber throughout the 10-min equilibration and 70-min 45Ca exposure periods. A preliminary dose-response study of each inhibitor was also performed (data not shown). As for the calcium transporter expression study by quantitative real-time PCR (qRT-PCR) (Fig. 1D), duodenal epithelial cells were collected at 0, 3, 6, 12, and 24 h after a single injection of 2 μg/kg 1,25(OH)2D3.
exposure area of 0.3 cm² and bathed on both sides with 3 ml of normal mucosa. The tissue was then mounted in a Ussing chamber with an from the pylorus) was removed and cut longitudinally to expose the 1.5-cm median laparotomy was performed, duodenal tissue (0 – 5 cm/HS9262 g/kg 1,25(OH)2D3 sc plus 280

Cytoplasmic calcium transport
Calbindin-D9k
AF136283
5'-TGGCTGTCTCTGCTGACTCT-3'
5'-GGGGAAAACTTGACTGAATCAG-3'

Basolateral calcium extrusion
Plasma membrane Ca²⁺-ATPase-1b (PMCA1b)
NM_026482
5'-AATCCGTAGGGTAAAGGGG-3'
5'-CGGTGAGCAATCACATTCTCA-3'

FGF-23
Fibroblast growth factor-23 (FGF-23)
NM_022657
5'-ACATAGCTCTGCTGACTCT-3'
5'-TGGTTAGTGAATACAGATTCCC-3'

Housekeeping gene
Hypoxanthine guanine phosphoribosyl transferase-1 (HPRT1)
NM_013556
5'-GAGCTTAGTTAGGGAGACTG-3'
5'-ATCCAGCAGGTACGAAAA-3'

Table 2. *Mus musculus* primers used in the PCR experiments

<table>
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<tr>
<th>Gene Name (Abbreviated)</th>
<th>Accession No.</th>
<th>Primer (Forward/Reverse)</th>
<th>Product Length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical calcium entry</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transient receptor potential vanilloid family Ca²⁺ channel-5 (TRPV5)</td>
<td>NM_00100757</td>
<td>5'-TGCTACTGTCATATGAGGG-3'</td>
<td>200</td>
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<tr>
<td>Transient receptor potential vanilloid family Ca²⁺ channel-6 (TRPV6)</td>
<td>NM_022413</td>
<td>5'-GGGAACTTGACTGAATCAG-3'</td>
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<tr>
<td>Cytoplasmic calcium transport</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Calbindin-D9k</td>
<td></td>
<td>AF136283</td>
<td>180</td>
</tr>
<tr>
<td>Basolateral calcium extrusion</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Plasma membrane Ca²⁺-ATPase-1b (PMCA1b)</td>
<td>NM_026482</td>
<td>5'-AATCCGTAGGGTAAAGGGG-3'</td>
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<td>FGF-23</td>
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<td>NM_022657</td>
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<tr>
<td>Fibroblast growth factor-23 (FGF-23)</td>
<td></td>
<td>5'-ACATAGCTCTGCTGACTCT-3'</td>
<td></td>
</tr>
<tr>
<td>Housekeeping gene</td>
<td></td>
<td>NM_013556</td>
<td>123</td>
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To obtain $R_{H-C}$, six samples (100 µl per sample) were collected from the cold side at 30, 40, 50, 60, 70, and 80 min after the hot side was filled with 44Ca-containing solution. The same volumes were also aliquoted from the hot side. 44Ca radioactivity in each sample was analyzed by liquid scintillation spectrophotometer (model Tri-Carb 3100; Packard, Meriden, CT). Linear regression was then performed [cold-side radioactivity (cpm) vs. time (h)], and the slope of the regression line was $R_{H-C}$. Total calcium concentration in bathing solution was analyzed by an atomic absorption spectrometer (model SpectraAA-300; Varian Techtron).

Since the duodenal voltage (PD)-dependent calcium transport was very small and could be considered negligible (data not shown), all experiments were performed under an open-circuit condition. In the absence of transepithelial calcium gradient (i.e., bathing solution in both hemichambers contained the same calcium concentration of 1.25 mmol/l), the measured transepithelial calcium flux represented the

$R_{H-C} = \frac{R_{H-C}}{S_H \times A}$

where $R_{H-C}$ is the rate of tracer appearance in the cold side (cpm/h); $S_H$ is the specific activity in the hot side (cpm/mmol); $A$ is the surface area of the tissue (cm²); $C_H$ is the mean radioactivity in the hot side (cpm); and $C_T$, is the total calcium content in the hot side (nmol).

\[ \text{SpectrAA-300; Varian Techtron).} \]

**Fig. 2.** Unidirectional calcium influx in 1.25(OH)₂D₃-treated and 1.25(OH)₂D₃ + FGF-23-treated mice. Vehicle-treated mice were injected with 3 ml/kg 9:1 propylene glycol-ethanol sc [for 1.25(OH)₂D₃ preparation] and 1 ml/kg 0.9% NaCl iv (for FGF-23 preparation). Mice treated with 1.25(OH)₂D₃ alone (0 µg/kg FGF-23) were also injected with FGF-23 vehicle. In one experiment, mice were injected with 140 µg/kg FGF-23 iv alone without 1.25(OH)₂D₃ injection. Numbers in parentheses represent nos. of experimental animals. *$P < 0.05$ vs. vehicle-treated group; ++$P < 0.01,$ +++$P < 0.001$ vs. mice treated with 1.25(OH)₂D₃ alone (black bar).
metabolically energized active calcium transport in mucosal-to-serosal (MS) direction (i.e., unidirectional calcium influx). Because the serosal-to-mucosal (SM) calcium fluxes of 1,25(OH)2D3-treated mice (7.01 ± 1.11 nmol·h−1·cm−2; 1 μg·kg−1·day−1 sc for 3 days) were comparable to that of the vehicle-treated mice (5.85 ± 0.54 nmol·h−1·cm−2; unpaired Student’s t-test; n = 5 per group, P = 0.1885), the SM calcium flux measurement was not performed in the present study. In addition, our preliminary study in 1,25(OH)2D3-treated mice also demonstrated that the SM duodenal calcium flux (7.01 ± 1.11 nmol·h−1·cm−2) was smaller than the MS calcium flux (17.91 ± 1.17 nmol·h−1·cm−2; unpaired Student’s t-test; n = 5 per group, P < 0.001). Nevertheless, this measurable SM flux suggested that the unidirectional influx measured in our study was the sum of an active transcellular and a paracellular flux. A solvent drag component of unknown magnitude may be present in the measured unidirectional MS flux.

**Tissue preparation and immunohistochemistry.** Mouse duodenal tissues (~5 cm) were dissected and rinsed off the luminal content, while the kidney and liver were also collected as positive controls for α- and β-Klotho expression, respectively. After removal of the adhering connective tissues, the tissues were preserved overnight at 4°C in 0.1 mol/l phosphate-buffered saline (PBS) containing 4% wt/vol paraformaldehyde (Merck, Darmstadt, Germany). Thereafter, they were dehydrated and cleared by graded ethanol and xylene, respectively. After being embedded in paraffin, the specimens were cut cross-sectionally into 5-μm sections. Deparaffinized sections were cross-sectionally into 5-m sections. Deparaffinized sections were incubated at 37°C for 30 min in antigen retrieval solution (0.01 mg/ml proteinase K, 50 mmol/l Tris-HCl, pH 8.0, and 5 mmol/l EDTA). Thereafter, the sections were incubated for 1 h with 3% vol/vol H2O2 to inhibit endogenous peroxidase activity. Nonspecific bindings were blocked by 2-h incubation with 4% bovine serum albumin, 5% normal goat serum, and 0.1% vol/vol Tween-20 in PBS. To label the target proteins, the sections were incubated at 4°C overnight with rabbit polyclonal primary antibodies against FGFR-1 (1:200, catalog no. sc-121), FGFR-2 (1:230, catalog no. sc-122), FGFR-3 (1:200, catalog no. sc-123), FGFR-4 (1:200, catalog no. sc-9006), or goat polyclonal primary antibodies against α-Klotho (1:20, catalog no. sc-22220) or β-Klotho (1:20, catalog no. sc-74343; all purchased from Santa Cruz Biotechnology, Santa Cruz, CA). After being washed with 0.1% vol/vol Tween-20 in PBS, the sections were incubated for 60 min at room temperature with 1:500 horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG (for α- and β-Klotho only; catalog no. 2020, Santa Cruz Biotechnology) or 1:500 biotinylated goat anti-rabbit IgG (catalog no. sc-2040, Santa Cruz Biotechnology), followed by a 60-min incubation with streptavidin-conjugated HRP solution (Invitrogen, Carlsbad, CA) or 3,3′-diaminobenzidine chromogen (Pierce, Rockford, IL). As for negative controls, the sections were incubated with blocking solution in the absence of primary antibodies (data not shown). Finally, all sections were counterstained with hematoxylin and examined under a light microscope (model BX51; Olympus, Tokyo, Japan).

**Total RNA preparation, quantitative real-time PCR and sequencing.** Total RNA was collected from intestinal mucosal scrapings or homogenates from other organs (for FGF-23 expression study), such as tibia, femur, spleen, liver, intestine (duodenum, jejunum, ileum, cecum, and colon), kidney, brain, and lung in normal untreated mice (n = 6). C: FGF-23 mRNA expression in duodenal epithelial cells of mice injected with a single dose of 2 μg/kg 1,25(OH)2D3 sc, as demonstrated by qRT-PCR. Specimens were collected at 0, 3, 6, 12, and 24 h after 1,25(OH)2D3 injection. Values are means ± SE of FGF-23/HPRT1 expression ratio. Fold change value is also presented on each bar; reference value (0 h) is normalized to 1.00, + , gene upregulation. C: FGF-23 mRNA expression in duodenal epithelial cells of mice injected with a single dose of 2 μg/kg 1,25(OH)2D3 sc or 2 μg/kg 1,25(OH)2D3 sc + 280 μg/kg FGF-23 iv. Specimens were collected at 3, 6, and 12 h after injection. 1,25(OH)2D3 data in B were reused in C. Numbers in parentheses represent nos. of experimental animals. \(^* P < 0.05\) vs. corresponding 1,25(OH)2D3-treated group.

**Fig. 3.** A: representative images show mRNA expression of FGF-23 and HPRT1 (housekeeping gene) in bones (tibia and femur), spleen, liver, intestine (duodenum, jejunum, ileum, cecum, and colon), kidney, brain, and lung in normal untreated mice (n = 6). B: time-dependent FGF-23 mRNA expression in duodenal epithelial cells of mice injected with a single dose of 2 μg/kg 1,25(OH)2D3 sc, as demonstrated by qRT-PCR. Specimens were collected at 0, 3, 6, 12, and 24 h after 1,25(OH)2D3 injection. Values are means ± SE of FGF-23/HPRT1 expression ratio. Fold change value is also presented on each bar; reference value (0 h) is normalized to 1.00, + , gene upregulation. C: FGF-23 mRNA expression in duodenal epithelial cells of mice injected with a single dose of 2 μg/kg 1,25(OH)2D3 sc or 2 μg/kg 1,25(OH)2D3 sc + 280 μg/kg FGF-23 iv. Specimens were collected at 3, 6, and 12 h after injection. 1,25(OH)2D3 data in B were reused in C. Numbers in parentheses represent nos. of experimental animals. \(^* P < 0.05\) vs. corresponding 1,25(OH)2D3-treated group.
Duodenal epithelial cells abundantly expressed FGF-23 mRNA and FGFR proteins. Although it was previously believed that circulating FGF-23 was secreted predominantly by osteoblasts and osteocytes, FGF-23 expression has also been reported in other tissues such as lung, thymus, and intestine (45). We found that both small and large intestine, i.e., duodenum, jejunum, ileum, cecum, and colon, strongly expressed FGF-23 mRNA (Fig. 3A). The FGF-23 expression was also observed in bone (tibia and femur; positive controls), spleen, liver, kidney, brain, and lung (Fig. 3A). Interestingly, as determined by qRT-PCR, a single-dose injection of 2 μg/kg 1,25(OH)2D3 sc markedly enhanced the duodenal expression of FGF-23.

The FGF-23 mRNA expression within 3 h, and the effect lasted at least 24 h after the single-dose injection (Fig. 3B). Moreover, FGF-23 administration to 1,25(OH)2D3-treated mice further upregulated the duodenal FGF-23 mRNA expression in duodenal villi as determined by immunohistochemical technique (magnification ×1,000; scale bars, 20 μm). All FGFR isoforms (brownish) were abundantly expressed in duodenal villi, where signals were observed in the cytoplasm, apical membrane (black arrows), and basolateral membrane (red-brown arrows) of villous epithelial cells (E). Cells in the lamina propria (L) modestly expressed FGFRs. In contrast, duodenal epithelial cells expressed neither α-Klotho nor β-Klotho, both of which were abundantly expressed in kidney and liver (positive controls), respectively. Nuclei were stained blue.

RESULTS

Intravenous FGF-23 administration abolished 1,25(OH)2D3-enhanced duodenal calcium absorption. As shown in Fig. 2, the 3-day injection of 1 μg/kg 1,25(OH)2D3 sc significantly enhanced duodenal calcium transport (unidirectional calcium influx; MS direction) ~1.7-fold compared with vehicle treatment. Although 140 μg/kg FGF-23 injection alone did not decrease the duodenal calcium transport, 140 and 280 μg/kg FGF-23 injected via the tail vein concurrently with 1 μg/kg 1,25(OH)2D3 sc for 3 days totally abolished the 1,25(OH)2D3-enhanced calcium transport. Lower doses of FGF-23 had no such effect (Fig. 2). FGF-23 administration did not alter the epithelial electrical properties, i.e., Isc, PD, and TER, in 1,25(OH)2D3-treated mice (Table 3). The results thus suggested that FGF-23 was a calcium-regulating hormone that antagonized 1,25(OH)2D3 action in the mouse duodenum.

In addition to regulating calcium transport, FGF-23 expression in the duodenum was antagonized by 1,25(OH)2D3 sc, PD, and TER, in 1,25(OH)2D3-treated mice (Table 3). The results thus suggested that FGF-23 was a calcium-regulating hormone that antagonized 1,25(OH)2D3 action in the mouse duodenum.

Furthermore, immunohistochemical analysis was performed to visualize the duodenal expression of FGFR and coreceptor Klotho proteins. As illustrated in Fig. 4A, FGFRs 1–4 were expressed in the apical and basolateral membrane and in the cytoplasm of duodenal villous cells. However, α- and β-Klotho proteins, which were abundantly expressed in the kidney and liver (positive controls), respectively, were not expressed in the duodenum (positive controls), respectively, were not expressed in the duodenum.
duodenal sections (Fig. 4B), which gave signals no different from that in the negative control section (without primary antibody; data not shown).

Direct FGF-23 exposure abolished 1,25(OH)2D3-enhanced duodenal calcium absorption. To investigate whether FGF-23 directly exerted its effect on the duodenal epithelial cells, the duodenal tissue taken from 1,25(OH)2D3-treated mice was directly exposed to FGF-23 in the Ussing chamber. Similar to the results in Fig. 2, both subcutaneous and intraperitoneal injection of 1 μg/kg 1,25(OH)2D3 for 3 days (without iv injection of normal saline, a vehicle of FGF-23) significantly stimulated the duodenal calcium transport compared with the corresponding vehicle-treated groups (Figs. 5 and 6). As expected, direct exposure on the serosal side, but not the mucosal side, to 10–40 ng/ml recombinant mouse FGF-23 exerted its effect on the duodenal epithelial cells, the duodenal calcium absorption.

FGF-23 prevented 1,25(OH)2D3-induced upregulation of duodenal calcium transporter genes. Prior to the investigation of FGF-23 effect on the 1,25(OH)2D3-induced duodenal calcium transporter genes, the time-dependent 1,25(OH)2D3 action was first determined to find out the appropriate time points for tissue collection from 1,25(OH)2D3 plus FGF-23-treated mice. As depicted in Fig. 8, A–D, a single-dose injection of 2 μg/kg 1,25(OH)2D3 sc transiently upregulated TRPV5, TRPV6, calbindin-D9k, and PMCA1b mRNA expression in duodenal epithelial cells. Peak responses of TRPV5, TRPV6, and PMCA1b expression were observed at 6 h after injection, whereas that of calbindin-D9k was seen at 12 h (Fig. 8, A–D). FGF-23 significantly decreased the 1,25(OH)2D3-induced upregulation of TRPV5 and TRPV6 at 6 or 12 h after injection, respectively (Fig. 8, E and F). Moreover, the 1,25(OH)2D3-induced calbindin-D9k expression was diminished by the intravenous FGF-23 administration at both time points (Fig. 8G). FGF-23 had no effect on the 1,25(OH)2D3-induced PMCA1b expression (Fig. 8H).

Fig. 5. Unidirectional calcium influx in duodenal tissues directly exposed on the mucosal (M) or serosal (S) sides to FGF-23. Mice were injected with 3 doses of 1 μg/kg 1,25(OH)2D3 sc at 72, 48, and 24 h before tissue collection. Vehicle-treated mice were injected with 3 ml/kg 9:1 propylene glycol-ethanol lip for 1,25(OH)2D3 preparation. In some experiments, untreated normal mice were used to demonstrate whether the effect of FGF-23 could be observed without 1,25(OH)2D3 preinjection. After being mounted in an Ussing chamber, tissue was directly exposed to 10, 20, or 40 ng/ml recombinant mouse FGF-23 in bathing solution. Numbers in parentheses represent nos. of experimental animals. NS, not statistically significant. **P < 0.01 vs. vehicle-treated group; ††P < 0.01 vs. duodenal tissues without FGF-23 exposure. 1,25(OH)2D3-treated mice, 0 ng/ml FGF-23, black bar.

DISCUSSION

As a multifunctional calcium/phosphate-regulating hormone, 1,25(OH)2D3 exerts a plethora of genomic and nongenomic actions in the small intestine to increase calcium and phosphate...
FGF-23 REGULATES INTESTINAL CALCIUM ABSORPTION

Consistent with previous reports of FGFR expression in the small intestine, i.e., the duodenum and jejunum (16, 39), the present immunohistochemical analyses showed FGFR 1–4 protein expression in the basolateral membrane of duodenal villous epithelial cells, thus confirming that these 1,25(OH)₂D₃-responsive duodenal cells acted as targets of FGF-23. Indeed, FGFR proteins were also observed in other duodenal cell compartments, i.e., cytoplasm and apical membrane, but their function is not well understood. Cytoplasmic FGFR proteins were previously reported in human mammary epithelial cells and might be important for the intracellular signaling of other FGFs, such as FGF-1 or -2, that could be produced intracellularly or internalized into the cells (49). In addition, the expressions of receptor tyrosine kinases [e.g., FGFR and epidermal growth factor (EGF) receptors] in the apical membrane of small intestinal enterocytes have been reported before, and the intraluminal secretion of their ligands (e.g., basic FGFs and EGF) probably participated in intestinal cell proliferation and healing (27, 28, 37). However, despite the presence of FGFRs absorption, thus providing adequate calcium and phosphate at an optimal ratio for bone mineralization (10, 30). Since the phospha-
ton FGF-23 negatively regulates 1,25(OH)₂D₃ actions through various mechanisms, such as 1-OHase downregulation and 24-
OHase upregulation (35), one may hypothesize that FGF-23 also modulates 1,25(OH)₂D₃-dependent calcium metabolism. In the present study, we have provided empirical evidence, for the first time, that FGF-23 did exert a calcium-regulatory action in the mouse duodenum by counteracting the 1,25(OH)₂D₃-enhanced calcium absorption. Although part of this FGF-23 action certainly resulted from the FGF-23-induced upregulation of 24-OHase expression in the kidney and the subsequent reduction in circulating 1,25(OH)₂D₃ levels (35), FGF-23 was also found to antagonize the calcium regulatory action of 1,25(OH)₂D₃ at the target organ (i.e., duodenum).

Normal mice were treated with neither 1,25(OH)₂D₃ nor its vehicle. FGF-23 was directly added in Ussing chamber. M and S, mucosal and serosal exposure, respectively. One-way ANOVA with Dunnett’s posttest was used to compare 1,25(OH)₂D₃-treated groups with vehicle-treated group. In normal mice, 0 ng/ml 1,25(OH)₂D₃-treated mice of 1 signaling inhibitors. Mice were injected with 3 doses of 1 μg/kg 1,25(OH)₂D₃ sc at 72, 48, and 24 h prior to electrical parameter measurement. Normal mice were treated with neither 1,25(OH)₂D₃ nor its vehicle. FGF-23 was directly added in Ussing chamber. M and S, mucosal and serosal exposure, respectively. One-way ANOVA with Dunnett’s posttest was used to compare 1,25(OH)₂D₃-treated groups with vehicle-treated group. In normal mice, 0 ng/ml FGF-23 was the control group for statistical analysis.

![Graph showing unidirectional calcium influx in FGF-23-exposed duodenal tissues in the presence of various signaling inhibitors](image)

**Table 4. Electrical parameters of FGF-23-exposed duodenal epithelium**

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>n</th>
<th>PD, mV</th>
<th>Isc, μA cm⁻²</th>
<th>TER, Ω cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-treated mice</td>
<td>11</td>
<td>2.80 ± 0.38</td>
<td>14.55 ± 3.19</td>
<td>242.50 ± 35.75</td>
</tr>
<tr>
<td>1,25(OH)₂D₃-treated mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 ng/ml FGF-23</td>
<td>6</td>
<td>2.71 ± 0.69</td>
<td>16.28 ± 5.48</td>
<td>216.00 ± 44.42</td>
</tr>
<tr>
<td>10 ng/ml FGF-23 (M)</td>
<td>6</td>
<td>1.95 ± 0.37</td>
<td>16.67 ± 6.21</td>
<td>149.90 ± 25.70</td>
</tr>
<tr>
<td>40 ng/ml FGF-23 (M)</td>
<td>10</td>
<td>1.96 ± 0.49</td>
<td>13.40 ± 3.97</td>
<td>176.40 ± 24.96</td>
</tr>
<tr>
<td>10 ng/ml FGF-23 (S)</td>
<td>9</td>
<td>2.00 ± 0.30</td>
<td>12.96 ± 3.88</td>
<td>247.70 ± 53.52</td>
</tr>
<tr>
<td>20 ng/ml FGF-23 (S)</td>
<td>8</td>
<td>1.67 ± 0.29</td>
<td>12.47 ± 1.99</td>
<td>160.30 ± 33.17</td>
</tr>
<tr>
<td>40 ng/ml FGF-23 (S)</td>
<td>12</td>
<td>1.77 ± 0.13</td>
<td>12.18 ± 2.03</td>
<td>206.10 ± 38.65</td>
</tr>
<tr>
<td>Normal mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 ng/ml FGF-23</td>
<td>8</td>
<td>2.17 ± 0.24</td>
<td>9.83 ± 1.32</td>
<td>241.70 ± 38.65</td>
</tr>
<tr>
<td>10 ng/ml FGF-23 (S)</td>
<td>7</td>
<td>1.91 ± 0.44</td>
<td>13.59 ± 2.95</td>
<td>196.10 ± 67.03</td>
</tr>
<tr>
<td>20 ng/ml FGF-23 (S)</td>
<td>8</td>
<td>1.65 ± 0.23</td>
<td>7.43 ± 1.31</td>
<td>260.50 ± 48.94</td>
</tr>
<tr>
<td>40 ng/ml FGF-23 (S)</td>
<td>7</td>
<td>1.23 ± 0.23</td>
<td>7.14 ± 1.65</td>
<td>205.60 ± 28.17</td>
</tr>
</tbody>
</table>

Values are means ± SE. The apical side was negative with respect to the basolateral side. Vehicle-treated mice were injected with 3 ml/kg 9:1 propylene glycol-ethanol sc, and 1,25(OH)₂D₃-treated mice were injected with 1 μg/kg 1,25(OH)₂D₃ sc at 72, 48, and 24 h prior to electrical parameter measurement. Normal mice were treated with neither 1,25(OH)₂D₃ nor its vehicle. FGF-23 was directly added in Ussing chamber. M and S, mucosal and serosal exposure, respectively. One-way ANOVA with Dunnett’s posttest was used to compare 1,25(OH)₂D₃-treated groups with vehicle-treated group. In normal mice, 0 ng/ml FGF-23 was the control group for statistical analysis.
in the enterocytic apical membrane, the inhibitory action of FGF-23 on 1,25(OH)2D3-induced duodenal calcium absorption was likely to result from plasma FGF-23, since direct FGF-23 exposure on the mucosal side was without effect (Fig. 5).

In the present study, abolition of 1,25(OH)2D3-enhanced duodenal calcium absorption by direct exposure to FGF-23 in the serosal solution implied that FGF-23 could exert its inhibitory effect directly on duodenal epithelial cells. Under physiological conditions, the FGF-23 that acted on the mouse duodenum could have come from bone (endocrine) and duodenal epithelial cells themselves (paracrine/autocrine). We found that the stimulatory action of 1,25(OH)2D3 on FGF-23 mRNA expression in duodenal epithelial cells was similar to its effect on osteoblasts and osteocytes, in which 1,25(OH)2D3 stimulated FGF-23 mRNA expression, protein synthesis, and secretion (23,45). However, 1,25(OH)2D3 may not be the only regulator of FGF-23 production. Recently, Shimada et al. (35) reported that feeding vitamin D receptor (VDR) knockout mice a 2% wt/wt high-calcium diet for a week resulted in elevated serum total calcium levels and a 16-fold increase in the serum levels of FGF-23. Hence, in addition to serum phosphate and 1,25(OH)2D3, calcium may be another determinant of FGF-23 production in a VDR-independent manner (35). On the basis of the present antagonistic effect of FGF-23 on the 1,25(OH)2D3-dependent intestinal calcium transport, we further postulated that FGF-23 was part of the negative feedback loop of the kidney-intestine-bone axis. Specifically, once the kidney produced 1,25(OH)2D3 to enhance intestinal calcium absorption, an increase in circulating calcium in turn stimulated FGF-23 production and secretion from bone, and perhaps from the intestinal epithelial cells, to counterbalance 1,25(OH)2D3 action. The intestine-derived FGF-23 production might indeed help fine-tune the 1,25(OH)2D3 action at the target organ, thus preventing excessive intestinal absorption of calcium and/or phosphate. FGF-23 knockout mice, therefore, manifested both hypercalcemia and hyperphosphatemia (34,48).

The signal transduction of FGF-23 in the duodenal epithelial cells was initiated by binding of FGF-23 to FGFRs in the basolateral membrane; however, the precise FGFR subtypes responsible for such FGF-23 action need further study. Indeed, FGF-23 could activate the c-splice isoforms of FGFR 1–3 and FGFR 4, as determined by surface plasmon resonance and mitogenesis techniques (46). Whether the obligatory coreceptor Klotho is also required to initiate intestinal FGFR signaling remains unknown. Since the duodenum did not express Klotho proteins, it is possible that the duodenal FGF-23/FGFR signaling was Klotho independent, similar to the inhibitory effect of FGF-23 on osteoblast differentiation and bone mineralization (29,41). Alternatively, duodenal FGFR might use the circulating form of Klotho, which is produced by cleavage of the transmembrane Klotho just above the plasma membrane of Klotho-producing cells, such as renal tubular cells (24). Evidence supporting the role of Klotho in the duodenal response to FGF-23 was further provided by the report that Klotho knockout mice manifested increases in intestinal calcium absorption and duodenal mRNA expression of TRPV6 and calbindin-D9k but not PMCA1b (2). In other words, the present study was a reversed scenario that, in the presence of FGF-23 (and Klotho), the 1,25(OH)2D3-induced increases in intestinal calcium absorption, and the expression of TRPV6 and calbindin-D9k, but not PMCA1b, were diminished. The subsequent intracellular...
signal transduction in the duodenal epithelial cells was found to be mediated by MAPK/ERK and p38 MAPK, similar to those reported previously in renal proximal and distal tubular cells, MC3T3.E1 osteoblast-like cells, parathyroid cells, and neonatal rat ventricular cardiomyocytes (12, 13, 32). PKC also participated in the duodenal FGF-23 signal transduction. Other signaling pathways, e.g., JAK2 or PI3K, were not involved in the present inhibitory action of FGF-23.

Consistent with previous reports (10, 36), at the molecular level, a single subcutaneous injection of 1,25(OH)2D3 transiently upregulated the mRNA expression of the key duodenal calcium transporter genes, namely TRPV5, TRPV6, and calbindin-D9k, which was inhibited by a concurrent intravenous injection of FGF-23. However, the 1,25(OH)2D3-induced expression of PMCA1b mRNA encoding the key basolateral calcium transporter was not abolished by FGF-23. Similarly, NCX1 mRNA expression, which was also enhanced by 1,25(OH)2D3 action, was not affected by FGF-23 (N. Charoenphandhu and J. Teerapornpuntakit, unpublished observation). Thus, FGF-23 appeared to predominantly regulate the genes for apical calcium entry and cytoplasmic calcium translocation, but not the basolateral calcium extrusion. However, further investigation is required to elucidate the underlying mechanism of the FGF-23-induced suppression of 1,25(OH)2D3 effect in duodenal epithelial cells.

In conclusion, we have provided corroborative evidence, for the first time, that FGF-23 has a calcium-regulatory role of counterbalancing 1,25(OH)2D3-enhanced duodenal calcium absorption. To exert this inhibitory action, FGF-23 binds to FGFRs in the basolateral membrane of duodenal epithelial cells, thereby activating MAPK/ERK, p38 MAPK, and PKC cascades. Although the detailed molecular mechanism is unknown, FGF-23 eventually prevented the 1,25(OH)2D3-induced upregulation of TRPV5, TRPV6, and calbindin-D9k, and diminished the stimulatory effect of 1,25(OH)2D3 on duodenal calcium absorption. Thus, FGF-23 appears to be an important negative feedback regulator of the vitamin D endocrine system in the kidney-intestine-bone axis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


FGF-23 regulates intestinal calcium absorption

E913


