FGF-23 Is a Potent Regulator of Vitamin D Metabolism and Phosphate Homeostasis

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ABSTRACT: We analyzed the effects of an FGF-23 injection in vivo. FGF-23 caused a reduction in serum 1,25-dihydroxyvitamin D by altering the expressions of key enzymes for the vitamin D metabolism followed by hypophosphatemia. This study indicates that FGF-23 is a potent regulator of the vitamin D and phosphate metabolism.

Introduction: The pathophysiological contribution of FGF-23 in hypophosphatemic diseases was supported by animal studies in which the long-term administration of recombinant fibroblast growth factor-23 reproduced hypophosphatemic rickets with a low serum 1,25-dihydroxyvitamin D [1,25(OH)2D] level. However, there is no clear understanding of how FGF-23 causes these changes.

Materials and Methods: To elucidate the molecular mechanisms of the FGF-23 function, we investigated the short-term effects of a single administration of recombinant FGF-23 in normal and parathyroidectomized animals.

Results: An injection of recombinant FGF-23 caused a reduction in serum phosphate and 1,25(OH)2D levels. A decrease in serum phosphate was first observed 9 h after the injection and was accompanied with a reduction in renal mRNA and protein levels for the type IIa sodium-phosphate cotransporter (NaPi-2a). There was no increase in the parathyroid hormone (PTH) level throughout the experiment, and hypophosphatemia was reproduced by FGF-23 in parathyroidectomized rats. Before this hypophosphatemic effect, the serum 1,25(OH)2D level had already descended at 3 h and reached the nadir 9 h after the administration. FGF-23 reduced renal mRNA for 25-hydroxyvitamin D-1α-hydroxylase and increased that for 25-hydroxyvitamin D-24-hydroxylase starting at 1 h. In addition, an injection of calcitriol into normal mice increased the serum FGF-23 level within 4 h.

Conclusions: FGF-23 regulates NaPi-2a independently of PTH and the serum 1,25(OH)2D level by controlling renal expressions of key enzymes of the vitamin D metabolism. In conclusion, FGF-23 is a potent regulator of phosphate and vitamin D homeostasis.

INTRODUCTION

TUMOR-INDUCED OSTEOMALACIA (TIO), autosomal dominant hypophosphatemic rickets (ADHR), and X-linked hypophosphatemic rickets (XLH) share common clinical features, such as hypophosphatemia, inappropriately low serum 1,25-dihydroxyvitamin D [1,25(OH)2D] levels for hypophosphatemia, and rickets/osteomalacia.1,2 It has been postulated that an abnormally excessive action of an unidentified humoral phosphaturic factor sometimes referred to as “phosphatonin” may be a common pathogenic mechanism of these disorders.1,2 Recently, FGF-23 was identified as a causative factor of TIO3-5 as well as a gene responsible for ADHR6-8 In addition, a recent demonstration of a high serum level of FGF-23 in patients with XLH suggested the involvement of FGF-23 in the development of this hypophosphatemic disease as well.9,10 The pathogenic role of FGF-23 has been shown by the finding that continuous administration of recombinant FGF-23 reproduced hypophosphatemia, inappropriately low 1,25(OH)2D, and rickets/osteomalacia in animals.5,11 However, it remains unclear how FGF-23 causes hypophosphatemia and an abnormal vitamin D metabolism.

The direct effect of FGF-23 on the phosphate uptake of renal epithelial cells has been reported in several experiments.5,12,13 Yamashita et al.13 reported that the rapid change in phosphate transport activity by FGF-23 was mediated by FGF receptor 3c and the p38 MAP kinase cascade. However, the absence of a direct action of FGF-23 on phosphate uptake5 and conflicting observations concerning
the requirement of heparin for FGF-23 effects\(^{(12)}\) have also been demonstrated in similar in vitro studies. Therefore, it is still unclear how these in vitro studies reflect the biological effects of FGF-23 in vivo.

In this study, we analyzed biological events induced by a single administration of purified recombinant FGF-23 protein in vivo and investigated the molecular mechanisms induced by FGF-23, resulting in impaired phosphate and vitamin D metabolism.

**MATERIALS AND METHODS**

**Experimental animals**

Male BALB/c mice at 5 weeks of age were purchased (SLC) and housed for 1 week before the following experiments. A purified recombinant FGF-23 protein or the same volume of vehicle was administered into the tail vein through a rapid bolus. At 1, 3, 5, 9, 13, and 24 h after administration, a pair of vehicle- and FGF-23–treated groups (n = 6 each) were anesthetized using ether to collect blood samples from the heart. They were then killed, and the kidneys were removed. In some mice, urine samples were also collected using metabolic cages (Sugiyamagen) for 12 h after the injection. To examine the effects of FGF-23 on fasting animals, access to food was prohibited for 24 h before some experiments. To evaluate the effect of the parathyroid hormone (PTH), 8-week-old male Sprague-Dawley rats (Charles River) were subjected to a parathyroidectomy (PTX) with an intact thyroid gland or a sham operation while anesthetized with a combination of pentobarbital (35 mg/kg) and ether. Ten days after the operation, the blood samples were collected from the tail artery, and serum calcium and phosphate levels were determined to confirm the effects of PTX. Only animals exhibiting a serum Ca level of less than 8.0 mg/dl and a phosphate level in excess of 11.0 mg/dl were used. Thirteen days after the surgery, a purified recombinant FGF-23 protein or vehicle was administered into the tail vein of the PTX– or sham-operated rats (n = 6 each) through a rapid bolus, respectively, and the blood samples were collected from the tail artery 12 h after the injection. All animals were fed standard rodent chow containing 1.1% P and 1.0% Ca (CLEA) and tap water ad libitum. All studies using animals were reviewed and approved by the institutional animal care and use committee at the Pharmaceutical Research Laboratories, KIRIN Brewery Co., Ltd.

**Recombinant FGF-23 protein**

The purified recombinant FGF-23 was prepared as described previously.\(^{(7)}\) Briefly, the Chinese hamster ovary (CHO) cells expressing full-length human FGF-23 were cultured for 3 days, and the conditioned medium was collected. The recombinant human FGF-23 protein was purified from the conditioned medium by a combination of chromatography using SP-sepharose FF (Amersham Bioscience), Ni-NTA Superflow (QIAGEN), and anti FGF-23 monoclonal antibody-conjugated sepharose. The purified protein was resolved in a 20 mM sodium phosphate buffer (pH 6.7) and 0.3 M NaCl and stored at −20°C. The protein concentration was determined by measuring the absorbance at 280 nm with the molar extinction coefficient, ε = 18,610 M\(^{-1}\)cm\(^{-1}\). Approximately 0.13 mg of recombinant FGF-23 was recovered from 1 liter of conditioned media.

**Measurement of serum parameters**

Serum phosphate and calcium concentrations were determined using the P-test and Ca-test, respectively (Wako). The serum PTH level was determined by Mouse Intact PTH RIA Kit (Immutopics). The serum 1,25(OH)\(_{2}\)D level was measured with a RIA Kit (Immunodiagnostic Systems).

**Preparation of brush border membrane and Western blotting**

The brush border membrane (BBM) was prepared by the method previously reported.\(^{(14)}\) The pooled renal cortex in the same group was homogenized, and the supernatant was subjected to the precipitation of BBM. The protein concentration of the suspended BBM fraction was determined by Bradford’s standard method. To analyze the NaPi-2a content in the BBM fraction, 20 μg of BBM protein was separated by SDS-PAGE and subjected to Western blot analysis using an anti-NaPi-2a polyclonal antibody that was affinity-purified from the rabbit antisera raised by the synthetic peptide corresponding to the C-terminal sequence of NaPi-2a (LALPAHHNATRL). The signals were detected by an ECL system (Amersham Bioscience).

**Probes and Northern blotting**

The DNA fragments used as probes for all experiments were prepared from murine kidney or bone cDNA by PCR with the following primer pairs: 25-hydroxyvitamin D-1α-hydroxylase (1αOHase); cacagacagactcgtgtag and cccatgtgcggtcacttgct, 25-hydroxyvitamin D-24-hydroxylase (24OHase); ccgtgattcctgatgatgatc, ccagacagctcctctta and caggctgctgggaatatctc, type I sodium-dependent phosphate cotransporter; gtaaagaaccctgtgtattcc and cttgcctgtgatgatgatc, and GAPDH; ccagacagctcctctta and caggctgctgggaatatctc. Total RNA was isolated from the frozen tissues using an ISOGEN reagent (Nippongene). Twenty micrograms of RNA samples was electrophoresed and transferred to Hybond N\(^+\) (Amersham Bioscience). A radiolabeled probe was prepared using a Megaprime labeling system (Amersham Bioscience). The membrane was hybridized with a \(^{32}P\)-labeled probe in a PerfectHyb reagent (Toyobo) overnight at 65°C. The blot was washed with a solution of 0.1× SSC and 0.5% SDS for 30 minutes at 65°C. The signals were visualized by the BAS system (Fuji).

**Measurement of mouse FGF-23 concentration**

Various doses of calcitriol (0.2, 2, or 20 ng/head; Wako) or vehicle was administered to 10-week-old male BALB/c mice intraperitoneally. Four hours after the injection, the blood samples were collected from an orbital cavity. The obtained sera were subjected to the measurement of serum phosphate and FGF-23 levels using an ELISA system for human full-length FGF-23,\(^{(9)}\) whose antibodies recognize...
mouse FGF-23 as well as human FGF-23. The measured values were calculated from a standard curve using recombinant human FGF-23 protein.

Statistical analyses
Statistical significance was evaluated either by the Student's t-test or a one-way ANOVA followed by Dunnet's method for the comparison of multiple means. An unadjusted p value less than 0.05 was considered to be significant.

RESULTS

Time course of changes in serum parameters
To investigate the time-course of effects of FGF-23 on mineral metabolism, 5 μg of purified human full-length FGF-23 protein was administrated intravenously into normal BALB/c mice, and the blood samples and the kidneys were collected at 1, 5, 9, 13, and 24 h after the bolus injection. As shown in Fig. 1A, the serum phosphate levels of FGF-23-injected mice were significantly lower compared with those of vehicle-treated animals from 9 to 13 h after the injection. The serum phosphate of these groups did not differ at 24 h after the injection. The administration did not affect serum calcium levels at any points examined.

Before the changes in serum phosphate level, serum 1,25(OH)2D started to decrease in the FGF-23-injected mice at 3 h after the injection, and this reduction in the 1,25(OH)2D level was maintained for at least 10 more hours. Again, the serum 1,25(OH)2D was not different between the two groups 24 h after the injection. In FGF-23-treated mice, a slight but significant decrease in serum PTH levels occurred at 9 and 13 h after injection, when the serum phosphate levels dropped.

To examine the dose–response relationship of the FGF-23 effects on these changes, serum phosphate and 1,25(OH)2D levels were examined 9 h after the treatment with various amounts of FGF-23 or vehicle. As shown in Fig. 1B, a reduction in serum 1,25(OH)2D was observed in the lowest dose examined (0.18 μg), and serum 1,25(OH)2D decreased in a dose-dependent manner. In contrast, a decrease in the serum phosphate level was observed only at two higher doses (4.5 and 9.0 μg). Thus, FGF-23 could induce a rapid reduction in serum 1,25(OH)2D before the decrease in the serum phosphate levels. However, the diminution of 1,25(OH)2D induced by the lower dose of FGF-23 did not seem to be sufficient for the development of hypophosphatemia.

Effect of FGF-23 on renal phosphate reabsorption
To address the mechanism for the decrease in the serum phosphate level, we first examined the effect of FGF-23 in fasting animals. These animals were not permitted to eat for 24 h to avoid interference of intestinal phosphate absorption. The serum phosphate levels of fasting mice were also significantly reduced compared with those of vehicle-treated animals at 8 h after the injection of 4.5 μg of FGF-23 (vehicle: 7.65 ± 0.15 versus FGF-23: 6.22 ± 0.19 mg/dl, p < 0.001, n = 6 each). A reduction in serum 1,25(OH)2D levels by FGF-23 treatment was also observed in the same samples (vehicle: 120.84 versus FGF-23: 28.29 pg/ml, pooled sera, n = 6 each). These findings indicate that the effects of FGF-23 on serum phosphate and 1,25(OH)2D are independent of intestinal phos-
phate absorption and suggest an important role of the kidney as a target organ of FGF-23 in the regulation of the phosphate and vitamin D metabolism.

It has been demonstrated that the type IIa sodium-phosphate cotransporter (NaPi-2a) in the renal proximal tubules plays a pivotal role in renal phosphate reabsorption. Therefore, we next examined the effect of FGF-23 on this molecule. The kidneys obtained from the same groups in the study described above were pooled and used to prepare the BBM fractions. As shown in Fig. 2A, the amount of NaPi-2a protein was clearly diminished at 9 and 13 h after the injection of FGF-23, when serum phosphate also decreased in FGF-23–treated mice. Treatment of FGF-23 also reduced the renal mRNA level of NaPi-2a at 9 h (Fig. 2B), whereas neither the mRNA levels of the type I sodium-dependent phosphate cotransporter nor the type III sodium-dependent phosphate cotransporter (pit-1) in the bone changed (Fig. 2B). Consistent with the decreased expression of NaPi-2a, the fractional excretion of phosphate for 12 h in mice injected with FGF-23 was higher than that of the vehicle-treated ones (vehicle: 22.6 ± 3.2% versus FGF-23: 34.3 ± 4.9%, p < 0.05, n = 4 each). Thus, the simultaneous reduction in serum phosphate levels and NaPi-2a amount in the BBM indicate that FGF-23 reduces serum phosphate by inhibiting renal phosphate reabsorption through NaPi-2a.

Regulation of renal vitamin D metabolizing enzymes by FGF-23

To elucidate the molecular mechanism for the reduction in the serum 1,25(OH)2D level, we analyzed the renal expressions of 1αOHase and 24OHase genes. Northern blot analysis revealed that FGF-23 decreased the 1αOHase expression and increased the 24OHase expression starting as early as 1 h after the administration (Fig. 3A). The quantitative analysis standardized by the expression levels of GAPDH indicated that FGF-23 almost halved the 1αOHase expression and increased the 24OHase expression by 2.5-fold (Fig. 3B). These alterations lasted more than 9 h after the administration. Because 24OHase is also involved in the degradation of 1,25(OH)2D, these rapid changes in mRNA levels in both vitamin D–metabolizing enzymes can be the causative mechanism for the significant reduction in serum 1,25(OH)2D induced by FGF-23.

**PTH-independent action of FGF-23**

Although PTH is known to be a potent suppressor of NaPi-2a protein expression in the BBM fraction, serum PTH levels were not elevated throughout the examined period in this study, as shown in Fig. 1A. These results suggest that the reduction in NaPi-2a protein observed here was not caused by PTH action. To confirm that the biological activity of FGF-23 is independent of PTH, we evaluated the action of FGF-23 in PTX animals. Sprague-Dawley (SD) rats were subjected to parathyroidectomy, and the rats exhibiting significant hypocalcemia and hyperphosphatemia were selected as the PTX ones for the following study (Ca, sham-operated: 10.30 ± 0.18 versus PTX: 6.87 ± 0.17 mg/dl, p < 0.001, Pi, sham-operated: 9.03 ± 0.11 versus PTX: 13.42 ± 0.30 mg/dl, p < 0.001, n = 12 each). In sham-operated rats, recombinant human FGF-23 reduced serum phosphate and 1,25(OH)2D 12 h after the bolus injection of FGF-23 (45 μg/head; Fig. 4). The administration of recombinant FGF-23 to PTX rats significantly improved hyperphosphatemia toward the normal range and further decreased 1,25(OH)2D levels (Fig. 4). These results indicate that the effects of FGF-23 on phosphate and vitamin D metabolism do not require PTH action.

**Possible mutual regulatory mechanism between FGF-23 and 1,25(OH)2D**

This study revealed that FGF-23 can induce rapid and significant reduction in serum 1,25(OH)2D levels, indicating that FGF-23 is a novel hormonal factor playing a critical role in the regulation of the vitamin D metabolism in mammals. It is well known that 1,25(OH)2D has a feedback regulatory mechanism on its own serum level through in-
creasing the expression of renal 24OHase and decreasing that of 1αOHase. The results shown above indicate that FGF-23 is deeply involved in the vitamin D metabolism. To investigate the further involvement of FGF-23 in the regulation of the vitamin D metabolism, we examined whether the administration of 1,25(OH)2D could cause changes in serum FGF-23 levels using the ELISA system. This ELISA was originally developed for human FGF-23 and turned out to be applicable to rodent FGF-23. As shown in Fig. 5, a significant elevation of serum FGF-23 levels was observed after 4 h in calcitriol-treated mice. It is noteworthy that even 2 or 20 ng of calcitriol, which did not change the serum phosphate levels, could evoke the elevation of the serum FGF-23 concentration, consistent with the possibility that the serum FGF-23 level could be regulated by 1,25(OH)2D.

FIG. 3. Effects of FGF-23 on renal expression of vitamin D–metabolizing enzymes. (A) Time course of changes in renal mRNA levels for 1αOHase and 24OHase after treatment with FGF-23 or vehicle. (B) Quantitative analysis of the relative abundance of 1αOHase and 24OHase mRNA to GAPDH mRNA at 1 h after injection. Results are means ± SEM. Statistical analysis was done by Student’s t-test against the vehicle-treated group (p < 0.01, n = 5).

DISCUSSION

Previous studies revealed the involvement of FGF-23 in the development of hypophosphatemic rickets/osteomalacia, and several reports showed the biological function of FGF-23 in vitro. However, it has remained unclear how FGF-23 induces these effects in vivo. To address these questions, we investigated the effects of FGF-23 in vivo with time-course experiments.

Concerning the regulation of serum phosphate by FGF-23, we first identified that hypophosphatemia was induced after 9 h of FGF-23 administration. The serum phosphate level is mainly regulated by proximal tubular phosphate reabsorption, and NaPi-2a has been identified as a physiological key molecule that determines renal phosphate reabsorption. We have shown here that FGF-23 decreased both serum phosphate and the amount of NaPi-2a protein in the BBM with the same time course. The renal mRNA levels of NaPi-2a also decreased at 9 h. Thus, these results indicate that impaired phosphate reabsorption by FGF-23 is at least in part caused by the reduction in NaPi-2a protein in

FIG. 4. Effects of FGF-23 in PTX rats. Serum phosphate and 1,25(OH)2D levels at 12 h after injection of FGF-23 or vehicle are shown. Results are means ± SEM. Statistical analysis was done by Dunnet’s method. p < 0.01.
BBM of renal proximal tubules. A nether potent regulator of the decrease in NaPi-2a in BBM is PTH.\textsuperscript{(17,18)} It is possible that PTH might work in the downstream of FGF-23 because PTH can reduce the NaPi-2a protein within 2 h,\textsuperscript{(11)} whereas FGF-23 required more than 5 or 9 h. However, serum PTH levels after the treatment with FGF-23 did not increase, and the FGF-23 action was maintained even in the PTX animals. Therefore, FGF-23 seems to use a PTH-independent pathway to modulate renal phosphate reabsorption. On the other hand, the amount of NaPi-2a protein seemed to be slightly increased at 1 h after the injection of FGF-23 (Fig. 2A). This change was temporary, but it was reproduced in another experiment. It is still unclear whether the rapid increase in NaPi-2a protein is a specific event by the administration of FGF-23 protein. Further studies are required to investigate how FGF-23 modulates the expression of NaPi-2a protein in BBM.

On the other hand, it is possible that FGF-23 also modulates the serum phosphate level by a mechanism that is independent of NaPi-2a. Dietary phosphate loading has been reported to cause the downregulation of NaPi-2a protein.\textsuperscript{(17)} In our preliminary study, a single injection of FGF-23 could decrease the serum phosphate level even in mice fed a high-phosphate diet (1.2% phosphate) for 7 days (vehicle: 11.04 ± 0.55 versus FGF-23: 7.08 ± 0.37 mg/dl, \(p < 0.001\), \(n = 5\) each). This result suggests that FGF-23 can regulate phosphate metabolism through an unidentified mechanism that is independent of NaPi-2a. One possibility is that FGF-23 also reduces the recently identified NaPi-2c-dependent renal phosphate reabsorption.\textsuperscript{(20)} A nether possibility is that FGF-23 controls extrarenal mechanisms to regulate serum phosphate. Because hypophosphatemic effects by FGF-23 were reproduced in fasting animals, intestinal phosphate absorption is not likely to be the target of FGF-23 action. In addition, FGF-23 injection did not change the mRNA expression for the type III sodium-dependent phosphate cotransporter, pit-1, in bone at 9 h. However, it remains unclear whether pit-1 is the primary phosphate transporter in bone, although it has been reported that pit-1 is abundantly expressed in osteoblasts and is suggested to play an important role in the phosphate transport in bone.\textsuperscript{(21)}

Before the reduction in serum phosphate concentration, FGF-23 induced a significant reduction in serum 1,25(OH)\(_2\)D within 3 h. Such a rapid reduction in the serum 1,25(OH)\(_2\)D level seems to be caused by a preceding decrease and increase in the expression levels of \textit{1aOHase} and \textit{24OHase} genes, respectively. These changes were maintained even with low serum 1,25(OH)\(_2\)D and phosphate levels, which, in general, adversely tend to increase \textit{1aOHase} expression and decrease \textit{24OHase} expression. Therefore, one of the primary functions of FGF-23 seems to be to change the expression levels of these enzymes. The presence of such a rapid regulation of serum 1,25(OH)\(_2\)D levels by FGF-23 was supported by the fact that serum 1,25(OH)\(_2\)D levels increased soon after the resection of a FGF-23–expressing responsible tumor in a patient with TIO.\textsuperscript{(9)} Furthermore, recently developed FGF-23 K O mice showing high serum 1,25(OH)\(_2\)D levels show that FGF-23 is an indispensable factor to control serum 1,25(OH)\(_2\)D levels.\textsuperscript{(22)} It is well known that 1,25(OH)\(_2\)D has a negative feedback mechanism, in which increased 1,25(OH)\(_2\)D regulates \textit{1aOHase} and \textit{24OHase} gene expressions directly through a vitamin D receptor.\textsuperscript{(23,24)} On the contrary, our study suggests a negative feedback pathway involving FGF-23 because the administration of 1,25(OH)\(_2\)D induced the elevation of serum FGF-23 levels. Taken together, these lines of evidence indicate that FGF-23 is a unique and potent downregulator of serum 1,25(OH)\(_2\)D levels.

It still remains unclear whether a reduction in 1,25(OH)\(_2\)D by FGF-23 is required for a later decrease in serum phosphate. The reduction in 1,25(OH)\(_2\)D by FGF-23 occurred in a dose-dependent manner and required less FGF-23 than that required for the induction of hypophosphatemia. Because injections of lower doses, such as 0.18 or 0.9 \(\mu g\)/head of FGF-23 showed a nearly maximum reduction in 1,25(OH)\(_2\)D but did not affect the serum phosphate levels, the biological activity of FGF-23 needed to reduce serum phosphate levels seems to be different from that needed to decrease serum 1,25(OH)\(_2\)D levels. One possible explanation is that FGF-23 may have multiple receptor systems with different affinity for the ligand. Alternatively, there may be an unidentified physiological system to maintain the serum phosphate level against the action of FGF-23 to induce hypophosphatemia, and this system is overcome only by treatment with a high dose of FGF-23. To address these issues, further research, such as the identification of the FGF-23 receptor(s) or the establishment of an in vitro assay system(s) reflecting in vivo events, is necessary.

In conclusion, FGF-23 is a unique molecule regulating both phosphate and vitamin D metabolism through novel mechanisms. Further understanding of the biological activity and physiological role of FGF-23 will provide new insights into the homeostatic control of mineral metabolism under normal and abnormal conditions.
ROLE OF FGF-23 IN PHOSPHATE AND VITAMIN D METABOLISM

ACKNOWLEDGMENTS

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and from the Ministry of Health, Labor, and Welfare of Japan.

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Received in original form May 29, 2003; in revised form August 5, 2003; accepted October 10, 2003.