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Phosphate-dependent FGF23 secretion is modulated by PiT2/SIc20a2

Nina Bon1,2, Giulia Frangi1,2, Sophie Sourice1,2, Jérôme Guicheux1,3, Sarah Beck-Cormier1,2, Laurent Beck1,2,*

ABSTRACT

Objective: The canonical role of the bone-derived fibroblast growth factor 23 (Fgf23) is to regulate the serum inorganic phosphate (Pi) level. As part of a feedback loop, serum Pi levels control Fgf23 secretion through undefined mechanisms. We recently showed in vivo that the two high-affinity Na+-Pi co-transporters PiT1/Slc20a1 and PiT2/Slc20a2 were required for mediating Pi-dependent signaling. Here, we addressed the contribution of PiT1 and PiT2 to the regulation of Fgf23 secretion.

Methods: To this aim, we used PiT2 KO and DMP1Cre; PiT1lox/lox fed Pi-modified diets, as well as ex vivo isolated long bone shafts. Fgf23 secretion and expression of Pi homeostasis-related genes were assessed.

Results: In vivo, PiT2 KO mice responded inappropriately to low-Pi diets, displaying abnormally normal serum levels of intact Fgf23. Despite the high Fgf23 level, serum Pi levels remained unaffected, an effect that may relate to lower Klotho expression in the kidney. Moreover, consistent with a role of PiT2 as a possible endocrine Pi sensor, the iFGF23/cFGF23 ratios were suppressed in PiT2 KO mice, irrespective of the Pi loads. While deletion of PiT1 in osteocytes using the DMP1-Cre mice was inefficient, adenovirus-mediated deletion of PiT1 in isolated long bone shafts suggested that PiT1 does not contribute to Pi-dependent regulation of Fgf23 secretion. In contrast, using isolated bone shafts from PiT2 KO mice, we showed that PiT2 was necessary for the appropriate Pi-dependent secretion of Fgf23, independently from possible endocrine regulatory loops.

Conclusions: Our data provide initial mechanistic insights underlying the Pi-dependent regulation of Fgf23 secretion in identifying PiT2 as a potential player in this process, at least in high Pi conditions. Targeting PiT2, therefore, could improve excess FGF23 in hyperphosphatemic conditions such as chronic kidney disease.

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Keywords FGF23 secretion; Phosphate sensing; PiT1/Slc20a1; PiT2/Slc20a2; Bone

1. INTRODUCTION

Fibroblast growth factor 23 (Fgf23) is a bone-derived hormone, which acts in concert with its co-receptor Klotho to regulate the serum inorganic phosphate (Pi) concentration and vitamin D metabolism [1,2]. Mutations in humans and mice preventing normal FGF23 bioactivity result in elevated serum Pi concentrations due to impaired urinary Pi excretion. Prolonged hyperphosphatemia can lead to life-threatening situations resulting from an inappropriate deposition of calcium-Pi crystals in cardiovascular tissue that occurs at high prevalence in diabetes, chronic kidney disease (CKD) and cardiovascular diseases (CVD) [3,4]. Conversely, elevated levels of biologically active FGF23 observed in inherited or acquired disorders of Pi homeostasis lead to renal Pi wasting, low serum Pi levels, and abnormal vitamin D metabolism [5,6]. The regulation of Fgf23 expression and/or secretion is controlled by numerous factors, including FGFR1 signaling [5,7], iron deficiency [8], and pro-inflammatory stimuli [9,10]. The intracellular cleavage of the biologically active intact form of FGF23 into N- and C-terminal fragments is also an important regulatory step. This regulation involves a balance between O-glycosylation of Thr178 by polypeptide N-acetylgalactosaminyltransferase 3 (GalNT3), providing a protection of furin-mediated cleavage, and phosphorylation of Ser180 by Family with sequence similarity 20, member C (Fam20C) that counteracts O-glycosylation [11]. Interestingly, the presence of regulatory feedback loops operating between FGF23 and Pi/vitamin D has been suggested [1,12]. In humans, serum FGF23 levels have been associated with the amount of dietary Pi [13,14], while intravenous infusion or acute duodenal Pi load were found to increase FGF23 levels in healthy humans [15]. Similarly, in vivo animal models have shown a relationship between dietary Pi loads and circulating Fgf23 levels [16,17], indicating that extracellular Pi induces the secretion of Fgf23. However, the mechanism by which Pi might regulate FGF23 production remains insufficiently defined and difficult to elucidate.

An interesting and possible mechanism is the direct regulation of FGF23 expression by Pi, in view of the recently accumulated data indicating that extracellular Pi acts as a signaling molecule [18,19]. While binding and/or cellular uptake of Pi by specialized Pi transporters are involved in
Brief Communication

the Pi signal processing in lower organisms, no Pi-receptor or ‘sensor’ has yet been identified in mammals [20]. Remarkably, we recently investigated the role of the two high-affinity mammalian Na\(^+\)-Pi co-
transporters PTT1/Slc20a1 and PTT2/Slc20a2 as Pi sensors in bone and showed that, in vitro, both PTTs were required for mediating Pi-
dependent signaling [21], raising the question of their role in the modulation of the Pi-dependent FGF23 secretion.

To gain insights into the mechanism by which Pi could regulate FGF23 production, we explored the contribution of PTT1 or PTT2 to the regu-
lation of Fgf23 expression and secretion by using in vivo and ex vivo murine models.

2. METHODS AND MATERIALS

2.1. Animals

The generation of PTT1fl/fl mice has been described previously [22]. Osteocyte-specific deletion of PTT1 was performed by crossing PTT1fl/fl
(129sv genetic background) to Dmp1-Cre mice, generated using the 9.6 kb Dmp1 promoter [23]. Slc20a2tm1a(EUCOMM)Wtsi mice (thereafter named PTT2KO mice) on the C57BL/6 genetic background were obtained from the European Mouse Mutant Archive (EMMA). The mutant allele contains an IRES: lacZ trapping cassette and a splicing site disrupting PTT2 gene expression [24]. Genomic DNA from tail was used for PCR geno-
typing. Animal care and maintenance were provided through the Uni-
versity of Nantes accredited animal facility at the Unité de Thérapeutique Expérimentale. Mice were housed under specific pathogen-free condi-
tions and were fed with RM1 diet for maintenance and with RM3 diet for breeding (SDS Special Diets Services, France). This study conformed to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. All procedures were approved by the Animal Care and Use Committee of the Région Pays de la Loire and conducted according to the French and European regulations on care and protection of laboratory animals (EC Directive 86/609, French Law 2001-486 issued on 6 June 2001) and the National Institutes of Health Animal Welfare (project #02286.02). The mice were weaned at post-natal day 21 (P21) to a normal Pi diet containing 0.7% calcium and 0.55% phosphorus. To induce Fgf23 regulation, the mice were fed at P28 with either a customized low-Pi
(<0.05% phosphorus) or normal-Pi (0.55% phosphorus) or high-Pi
(1.65% phosphorus) diet for 7 days (ssniff Spezialdiäten GmbH, Soest, Germany).

2.2. Serum and urine parameters

Serum phosphorus and calcium were analyzed using the Phosphorus
(1.65% phosphorus) diet for 7 days (ssniff Spezialdiäten GmbH, Soest, Germany).

2.4. Immunohistochemistry (IHC)

Paraffin embedded kidney sections were processed for IHC as previ-
ously described [22]. Primary antibody for Klotho (1:100, KM2076, TransGenic Inc.) was incubated overnight at 4 \(^\circ\)C. Secondary anti-
mouse biotinylated goat antibody (1:500, Dako) was used and stain-
ing was performed using 3,3-diaminobenzidine chromogen (Dako) for 5 min and counterstaining with Mayer’s hematoxylin. Stained sections were then mounted with Eukitt® and scanned using a Hamamatsu Nanozoomer HT (Hamamatsu Photonics KK) digital scanner at a 20×
magnification.

2.5. Long bone shafts preparation

Femur, tibia, and humerus bones from P35 mice were dissected free of the surrounding soft tissue and flushed. After stripping away the periosteum, the long bone shafts were trimmed into two pieces for weighing. One half of each bone type was cultured overnight at 37 \(^\circ\)C in 5% CO\(_2\) humidified incubator in a well of a 96-well plate with 100 \(\mu\)l of culture medium containing Dulbecco’s Modified Eagle’s Medium (DMEM) high glucose GlutaMAX™ (catalogue no 31966, ThermoFisher Scientific, Saint-Aubin, France) supplemented with 0.2% Bovine Serum Albumine (Sigma–Aldrich, St Louis, MO, USA), 10 mM HEPES, and 50 \(\mu\)g/ml gentamycin. To study Pi-dependent FGF23 secretion, the long bone shafts were washed three times with pre-warmed 0.9% NaCl solution and incubated during 24 h in stimulation medium. The stimulation medium consisted of phosphate-free high glucose Gluta-
MAX™ DMEM (catalogue no 11971-025, ThermoFisher Scientific) supplemented with 0.2% BSA, 10 mM HEPES, 50 \(\mu\)g/ml gentamycin and 0, 1, 3, 7, or 10 mM Na\(_2\)HPO\(_4\)/Na\(_2\)HPO\(_4\) pH 7.4. Intact and C-
terminal Fgf23 concentrations were assessed in the supernatants using ELISA kits according to the manufacturer’s protocol (Kainos Laboratories and Immuntopics, respectively) and normalized by bone mass.

2.6. Transduction

Adeno-CMV-iCre (catalogue no 1045 Vector Biolabs) was added to the media of PTT1fl/fl long bone shafts culture at a concentration of 10\(^7\) virus particles per mL of media. Control samples were treated with Ad-
CMV-GFP (catalogue no 1060 Vector Biolabs) at the same concen-
tration. The media were replaced 24 h after transduction, and, 72 h post-transduction, the long bone shaft were harvested and analyzed for PTT1 deletion.

2.7. Statistics

Data are expressed as mean ± S.E.M. GraphPad 5.0 software was used to perform Mann–Whitney tests or analysis of variance. A p value
of less than 0.05 was considered statistically significant. Unless otherwise stated, experiments were repeated at least three times (exact numbers are indicated in the figures).

3. RESULTS

3.1. Deletion of PTT2 in mice results in impaired Pi-dependent Fgf23 secretion

To investigate the role of PTT2 in the Pi-dependent regulation of Fgf23 secretion in vivo, we fed four-week-old PTT2 KO mice with low
Interestingly, when iFgf23/cFgf23 ratios were calculated for the no statistical difference between (Figure 1A) and of www.molecularmetabolism.com 199 was lower in was accompanied by a drastic reduction of the protein expression of 3.2. Deletion of PiT1 WT between iFgf23 levels in the normal-Pi diet condition (Figure 1C). Although the circulating iFgf23 levels were abnormally high, being similar to the decrease in serum Pi and urine Pi/creatinine ratio was consistent with the decrease in serum Pi levels (Figure 1B). However, feeding a high Pi diet failed to reveal an increase in serum Pi levels, an observation that was already made previously, which may relate to the existence of compensatory endocrine loops [27]. The urine Pi/creatinine ratio was with the decrease in serum Pi and urine Pi/creatinine levels (Figure 1B). Remarkably, in PiT2 KO mice fed low-Pi diet, the circulating iFgf23 levels were abnormally high, being similar to the iFgf23 levels in the normal-Pi diet condition (Figure 1C). Although the C-terminal Fgf23 (cFgf23) levels were not statistically different between WT and PiT2 KO mice, we could observe a similar trend (Figure 1D), suggesting that increased iFgf23 does not originate from a decrease in Fgf23 cleavage. In support of this, we observed no difference in iFgf23 levels in the normal-Pi diet condition (Figure 1D). Overall, the response of WT and mutant bones (Figure 1A) in isolated bone shafts, no change was observed in the Pi-dependent ERK1/2 phosphorylation with the MAPK inhibitor U0126. However, when blocking the Pi-dependent regulation of Fgf23 secretion by PiT1 in the Pi-dependent regulation of Fgf23 secretion, despite this efficient deletion of PiT1, results showed that following Pi challenge, secreted iFgf23 and cFgf23 levels were similar in WT and PiT1lox/lox; AdvCre KO mice. Consistently, the expression of Fgf23 in the diaphysis was similar in all conditions tested as were the expression of Galt3 and Fam20C and the Galt3/Fam20C expression ratio (not shown). These data showed that despite an efficient deletion of PiT1 in bone, the role of PiT1 in the Pi-dependent regulation of Fgf23 could not be demonstrated, suggesting that more appropriate in vivo tools are required to successfully address this question.

3.3. The Pi-dependent modulation of Fgf23 secretion by PiT2 is bone-autonomous

We used the ex vivo long bone shaft approach to determine whether the role of PiT2 in modulating the Pi-dependent Fgf23 secretion was bone-autonomous. RT-qPCR analysis confirmed the deletion of PiT2 in diaphyses, while PiT1 expression was higher in mutant than WT tibias under high-Pi diet condition (Figure 3A). In WT bones, while the expression of Fgf23 remained unchanged when extracellular Pi was changed (Figure 3B), the secretion of iFgf23 in the extracellular medium increased as a function of extracellular Pi concentration, as expected (Figure 3C). Interestingly, the secreted cFgf23 from PiT2 KO mice displayed an inverse profile than the secreted Fgf23 and appeared higher in mutant than in WT bones (Figure 3C). Accordingly, the Fgf23/cFgf23 ratios were blunted in PiT2 KO tibias (Figure 3D), as was observed in in vivo experiments (Figure 1F). The low iFgf23 secretion and Fgf23/cFgf23 ratios were consistent with the lower expression of Fgf23 in mutant bones (Figure 3B). Finally, no differences in Fam20C and Galt3 gene expression, nor in Galt3/Fam20C mRNA expression ratios, were evident between WT and mutant bones (not shown). Altogether, our data indicate that the expression of PiT2 is mandatory for a normal regulation of Fgf23 secretion following changes in extracellular Pi concentrations. Interestingly, we recently showed that PiT2 was necessary for the Pi-dependent activation of the ERK1/2 MAPK pathway [21], a finding that may be relevant with the activation of the ERK1/2 MAPK pathway through FGF1 that is known to stimulate Fgf23 secretion [29]. However, when blocking the Pi-dependent ERK1/2 phosphorylation with the MAPK inhibitor U0126 (Figure 3E) in isolated bone shafts, no change was observed in the Pi-dependent Fgf23 secretion, irrespective of the Pi load (Figure 3F). Moreover, similar expression of Fgf1 in WT and PiT2 KO mice (Figure 3G). These data suggested that mechanistic links other than the ERK1/2 pathway are likely at work between PiT2 and Fgf23 secretion.
Figure 1: Deletion of Pit2 in vivo leads to inappropriate serum Fgf23 levels in low-Pi condition. (A) RT-qPCR analysis of Pit2, Pit1 and Fgf23 mRNA levels in flushed tibias isolated from WT or Pit2 KO mice fed with low (0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. Pnn and GusB genes were used as internal controls. (B) Serum and urine Pi values from WT or Pit2 KO mice fed with low (0.05%), normal (0.55%), and high (1.65%) Pi diets for one week. (C-D) Serum intact Fgf23 (iFgf23) and C-terminal Fgf23 (cFgf23) levels (ELISA) from WT or Pit2 KO mice fed with low (0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. (E-F) Galnt3/Fam20C mRNA ratios as determined by RT-qPCR analysis (E) and iFgf23/cFgf23 ratios (F) from flushed tibias from WT or Pit2 KO mice fed with low (0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. Pnn and GusB genes were used as internal controls. (G) Serum PTH levels (Left) and serum and urine Ca values (Center and Right) from WT or Pit2 KO mice fed with low (0.05%), normal (0.55%) and high (1.65%) Pi diets for one week. Black arrowheads: αKlotho-positive staining. Top, low magnification; Bottom, high magnification, scale bar, 50 μm. Data are means ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001, NS: not significant.
4. DISCUSSION

This study presents the first direct evidence that PiT2 expression in vivo and ex vivo is essential for normal secretion of Fgf23 following dietary Pi changes. While iFgf23 levels were correlated to dietary Pi loads in vivo or extracellular Pi concentrations ex vivo in WT animals, the Pi-dependent regulation of Fgf23 secretion was blunted in PiT2 KO mice and isolated bones, as was clearly showed when calculating iFgf23/cFgf23 ratios. Since similar Pi-dependent regulation of Fgf23 secretion was observed in whole mutant animals and isolated bone shafts from PiT2 KO mice, the contribution of other organs or possible endocrine loops to this regulation appears unlikely. However, while this illustrates that Pi-dependent regulation of Fgf23 is dependent upon normal bone PiT2 expression and highlights PiT2 as an important putative Pi sensor, the underlying mechanism linking PiT2 to the secretion of Fgf23 remains to be deciphered. Notably, the similar expression of Fam20C and GaInt3 in WT and PiT2 KO mice was not consistent with the difference observed in the Fgf23/cFgf23 ratios, suggesting that the regulation of Fgf23 cleavage by PiT2 might involve Fam20C and GaInt3 independent mechanisms, as was suggested recently [30]. Moreover, although FGFR1-mediated stimulation of FGF23 secretion was illustrated through the activation of the ERK1/2 MAPK pathway [29], our data showed that the Pi-dependent activation of the ERK1/2 pathway was not involved in Fgf23 secretion from bone, further indicating that other mechanistic links are at work between PiT2 and Fgf23. One possibility is the involvement of PiT2-specific protein partners linking Pi signaling to Fgf23 secretion, as was shown recently for other PiT2-or PiT1-specific physiological functions [31,32]. Moreover, while our data clearly showed a role of PiT2 in the setting of Fgf23 secretion, most of the physiological parameters remained normal. This finding is consistent with the absence of identified PiT2 mutations in humans that would cause a disturbed Pi homeostasis and suggests the existence of mechanisms compensating the lack of PiT2. One of the possible compensatory mechanisms involves the negative regulation of αKlotho that we uncovered in the PiT2 KO kidneys, which may explain why mutant mice fed a low Pi diet and exhibiting abnormally normal Fgf23 levels retained normal serum and urine Pi levels. Other compensatory mechanisms, such as the existence of other Pi sensors, may also be involved. Along this line, although we could efficiently down-regulate PiT1 in isolated bone shafts and illustrated no role in Pi-dependent regulation of Fgf23 in this model, its actual role in vivo remains to be determined. As an illustration of the complexity of these regulations, the effect of Pi-modified diets on FGF23 secretion in humans still remains a matter of debate [33,34]. Much of the recent focus on FGF23 has been driven by human studies showing that even a mild loss of kidney function is associated with considerable elevations of serum FGF23 levels, preventing hyperphosphatemia early in the course of...
the disease. Hence, several studies were designed to restrict dietary Pi in CKD patients to decrease circulating FGF23 levels. Although most studies showed a reduction in Pi excretion, the effects on FGF23 levels have been mixed, with some studies showing mild reduction, particularly when measuring intact FGF23 [33], and other studies revealing no change in FGF23 levels [34]. The role of PiT2 and possible other players in the setting of high FGF23 concentration following the early phase of CKD characterized by hyperphosphatemia, remains to be determined. This is particularly important in view of the detrimental effects of FGF23 in the later stages of the disease, which has fueled the search of strategies to lower serum FGF23 levels or block its unwanted actions [33]. In view of the emerging cross-talk between inflammation, iron deficiency, and bone mineral metabolism in controlling FGF23 secretion and/or expression [8–10,12,35], the possible involvement of PiT2 in these processes should now be considered.

Figure 3: Ex vivo organ culture of long bone shafts from PiT2 KO mice illustrates the bone-autonomous Pi-dependent role of PiT2 in regulating the FGF23 secretion. (A) RT-qPCR analysis of PiT2 and PiT1 mRNA levels in flushed tibias isolated from WT or PiT2 KO mice and stimulated for 24 h in medium containing 0, 3, 7, or 10 mM Pi, as indicated. Pnn and GusB genes were used as internal controls. (B) RT-qPCR analysis of Fgf23 mRNA levels in flushed tibias isolated as in A. (C) Secreted levels of intact (Left) and C-terminal (Right) Fgf23 from flushed tibias isolated from WT or PiT2 KO and stimulated for 24 h in medium containing indicated Pi concentrations. (D) Western-blot analysis of ERK1/2 phosphorylation (P-ERK1/2) in flushed tibias from WT mice (C57BL/6 background) stimulated for 30 min with 0, 1 mM, or 10 mM extracellular Pi concentration, in the presence of DMSO or U0126, as indicated. Total ERK1/2 proteins were used as a loading control. (F) Secreted Fgf23 levels from flushed tibias from WT mice (C57BL/6 background) stimulated for 30 min with 0, 1 mM, or 10 mM extracellular Pi concentration, in the presence of DMSO or U0126, as indicated. (G) RT-qPCR analysis of Fgfr1 mRNA levels in diaphysis from WT or PiT2 KO mice fed with low (0.05%), normal (0.55%), and high (1.65%) Pi diets for one week. Pnn and GusB genes were used as internal controls. Data are means ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001, NS: not significant.
In summary, here we provide the first experimental evidence that PiT2 regulates synthesis and secretion of FGF23 in response to high Pi load in vivo and in bone organ cultures ex vivo. These findings may identify PiT2 as a target for novel therapies to improve the excessive FGF23 secretion in hyperphosphatemic disorders such as chronic kidney disease.

AUTHOR’S CONTRIBUTION

N.B., S.B.C., and L.B. designed experiments, N.B., G.F., S.B.C., and S.S. performed experiments, N.B., S.B.C., and L.B. analyzed data, N.B. and L.B. wrote the manuscript, N.B., J.G., S.B.C., and L.B. made adjustments to the final paper version. All authors reviewed the results and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2018.02.007.

REFERENCES


