Elevated levels of fibroblast growth factor 23 (FGF23), a bone-derived phosphaturic hormone, are associated with a number of pathologic conditions including chronic kidney disease, cardiac hypertrophy, and congestive heart failure. Currently, there are no specific treatments available to lower plasma FGF23 levels. We have recently reported that genetic plasminogen activator inhibitor–1 (PAI-1) deficiency provided a significant reduction in circulating FGF23 levels while simultaneously prolonging the life span of Klotho-deficient mice. We extend our investigations into the effect of PAI-1 on FGF23 homeostasis. Transgenic overexpression of PAI-1 resulted in threefold increase in FGF23 levels compared to wild-type littermates. Moreover, pharmacological modulation of PAI-1 activity with the small-molecule PAI-1 antagonist TM5441 significantly reduced FGF23 levels in PAI-1 transgenic and Klotho-deficient mice. In addition, TM5441 treatment or PAI-1 deficiency significantly accelerated the clearance of endogenous FGF23 and recombinant human FGF23 from circulation in mice with acute kidney injury. On the basis of these observations, we studied the effects of plasminogen activators (PAs), tissue-type PA (tPA) and urokinase-type PA (uPA), on FGF23. We demonstrate that both PAs directly cleave FGF23; however, it is not known whether the PA-generated FGF23 peptides retain or acquire functions that affect binding and/or signaling properties of intact FGF23. PAI-1 inhibits the PA-dependent cleavage of FGF23, and TM5441 inhibition of PAI-1 restores the proteolysis of FGF23. Furthermore, top-down proteomic analysis indicates that tPA cleaves FGF23 at multiple arginines including the proconvertase sensitivity site R176. In summary, our results indicate that PAI-1 prevents the PA-driven proteolysis of FGF23 and PAI-1 inhibition provides a novel therapeutic approach to prevent the pathologic consequences of increased FGF23.
of IGFBP3 (insulin-like growth factor binding protein 3) (25) indicates that both tPA and uPA regulate the metabolism of peptides and hormones affecting many physiological and pathophysiological processes independent from their ability to activate plasminogen. Epidemiological data link elevated FGF23 levels with LVH (4, 10) and an increased risk of death in patients with CKD (26, 27). Therefore, therapeutic agents that reduce FGF23 levels in plasma may provide important clinical benefits. These observations led us to hypothesize that PAs and PAI-1 directly regulate FGF23 homeostasis. Here, we describe the effects of selective pharmacological inhibition of PAI-1 by TM5441 on FGF23 metabolism in vitro and in vivo.

**RESULTS**

**PAI-1 inhibition affects FGF23 levels in kl/kl and Pai-1stab mice**

We first investigated the role of PAI-1 in FGF23 homeostasis by administering an orally active PAI-1 antagonist, TM5441, mixed into the standard chow diet to Pai-1stab, transgenic mice overexpressing a functionally stable variant of human PAI-1 under the control of mouse endothelin-1 promoter (28, 29), and kl/kl mice. We tested various doses of TM5441 in Pai-1stab and kl/kl mice and found that doses as low as 10 mg/kg per day are effective in promoting significant and reproducible inhibition of plasma PAI-1 activity in these animals. Plasma levels of FGF23 were determined using specific enzyme-linked immunosorbent assay (ELISA) kits that detect both iFGF23 and cleaved levels of FGF23 in circulation. We observed that Pai-1stab mice have threefold elevated levels of cFGF23 (n = 14, P < 0.001) and TM5441 treatment reduced it by ~50% (n = 9, P < 0.01) and returned plasma levels of FGF23 that are not significantly different from those observed in wild-type (WT) mice (Fig. 1A). In contrast, iFGF23 levels in Pai-1stab mice (with and without TM5441 treatment) were similar to those observed in WT mice, indicating tight control over iFGF23 synthesis. We also measured cFGF23 and iFGF23 levels in TM5441-treated and PAI-1-deficient kl/kl (kl/klpai-1−/−) mice. Specifically, 4-week-old kl/kl mice (n = 8) received TM5441 (10 mg/kg per day) mixed in standard chow diet. Pharmacological inhibition and genetic deficiency of PAI-1 activity in kl/kl mice yielded 66% (P = 0.0003) and 98% (n = 10, P = 0.0001) reductions in cFGF23 levels, respectively, compared to those of untreated kl/kl mice (n = 14), but remained significantly elevated over WT control mice (Fig. 1B). Likewise, iFGF23 levels were reduced by 75% with TM5441 treatment (n = 8, P = 0.004) and by 96% with genetic PAI-1 deficiency (n = 8, P < 0.001) in kl/kl mice. These findings indicate improved clearance of FGF23 from circulation with either PAI-1 deficiency or pharmacologic inhibition and further confirm our initial observation in kl/kl mice that PAI-1 plays a critical role in FGF23 homeostasis (17).

**Modulating PAI-1 activity improves FGF23 homeostasis in acute kidney injury**

Patients with acute kidney injury (AKI) have markedly increased circulating levels of FGF23 that are associated with increased risk of end-stage renal disease (ESRD) and mortality (30, 31). Next, we investigated whether PAI-1 contributes to the elevated levels of FGF23 in AKI. We induced AKI by intraperitoneal injection of folic acid (FA; 240 mg/kg) into mice, as described previously (32). FGF23 levels are significantly elevated as early as 1 hour after FA injection and reach 18-fold higher levels in 24 hours (33). Plasma samples were collected for PAI-1 and FGF23 measurements 24 hours after FA injection into animals. Plasma PAI-1 levels increased by 10-fold (n = 13, P = 0.0001) in mice with AKI (Fig. 2A). As expected, FGF23 levels increased by 30-fold in mice with AKI compared to those in vehicle-treated animals (Fig. 2B). The group of mice that received the chow diet containing TM5441 at 10 mg/kg per day for 7 days before the FA treatment displayed 20% reduction in iFGF23 levels (n = 15, P = 0.007). Similarly, PAI-1−/− mice treated with FA (Fig. 2B) displayed a 45% reduction in iFGF23 compared to WT mice with AKI (n = 9, P = 0.005). At baseline, PAI-1−/− mice have lower levels of FGF23 than WT mice (P = not significant). Together, these results indicate that PAI-1 affects FGF23 homeostasis in mice with AKI.

To further investigate the effect of PAI-1 on clearance of FGF23 from circulation, we injected recombinant human FGF23 (rhFGF23) via the
tail vein into FA-treated mice that received chow with and without TMS441 (10 mg/kg per day). PAI-1 inhibition preceded the FA treatment by 7 days and continued during the development of AKI. We collected plasma samples at 1, 3, 5, 15, 30, 60, and 120 min after rhFGF23 injection. Using an ELISA method that detects only the C terminus of human FGF23, we determined plasma levels of rhFGF23. As shown in Fig. 2C and D, rhFGF23 elimination from the plasma appears to be a biphasic process. Analysis of the FGF23 clearance data in Fig. 2C by two-phase decay nonlinear best-fit method using GraphPad Prism showed that the $T_{1/2}$ (half-life) of FGF23 is shortened with PAI-1 inhibition from 5.4 to 1.2 min and from 45 to 11.6 min in fast and slow phases of clearance, respectively ($P = 0.0001$). This corresponds to a 4.5-fold acceleration of the fast phase and 3.9-fold acceleration of the slow phase. These results further confirm the effects of PAI-1 on FGF23 homeostasis in AKI.

**Phosphate and parathyroid hormone levels in AKI and kl/kl mice**

In the folate-induced AKI model, serum phosphate levels and plasma parathyroid hormone (PTH) levels increase markedly, whereas calcium levels remain unchanged and FGF23 levels increase independently of signaling by PTH, vitamin D$_3$, and dietary phosphate (33). In kl/kl mice, we have previously reported that PAI-1 deficiency significantly reduces FGF23 and vitamin D$_3$ levels but has negligible effects on phosphate and calcium levels (17). Here, we investigated whether the PAI-1 inhibition by TMS441 affects serum phosphate levels in the FA-induced AKI model and kl/kl mice. We observed that neither TM5441 treatment nor PAI-1 deficiency affects the high levels of phosphate in FA-treated and kl/kl mice. Furthermore, neither PAI-1 inhibition nor PAI-1 deficiency has any effect on the elevated PTH levels observed in the FA-induced AKI. In kl/kl mice, PTH levels were the
same as those in WT mice (Fig. 3B). These findings indicate that FGF23 is incapable of regulating phosphate clearance in these mouse models, which likely reflects the deficiency of functional receptor heterodimer (FGFR and Klotho) in kl/kl mice and in mice with AKI.

In vitro proteolysis of FGF23 by PAs and plasmin

Our results from kl/kl and Pai-1stab mice suggest that one or more serine proteases inhibited by PAI-1, most likely tPA and/or uPA, are involved in the metabolism of FGF23. To test this hypothesis, we investigated the effects of tPA and uPA on FGF23 in vitro using recombinant human proteins. Under reaction conditions optimized for tPA or uPA activity, we incubated FGF23 with tPA or uPA at 37°C and resolved the resulting peptide fragments on silver-stained SDS-PAGE (polyacrylamide gel electrophoresis) gels. When incubated alone up to 6 hours at 37°C, we did not detect any spontaneous proteolysis or degradation of the recombinant proteins used in this study including tPA, uPA, PAI-1, and FGF23 (Fig. 4A). This indicates that any new peptide fragments detected on SDS-PAGE likely come from the proteolysis of FGF23, and it may provide additional catabolism of FGF23 downstream from the PAs.

Identification of tPA cleavage sites of FGF23

To determine which peptide bonds of FGF23 are cleaved by tPA specifically, we analyzed tPA-generated peptide fragments with a liquid chromatography–mass spectrometry (LC-MS)–based top-down proteomics approach (Fig. 5). Reactions containing FGF23 incubated with or without tPA for 4 hours at 37°C were subjected to reversed-phase
LC-MS to detect specific FGF23 fragments, and their amino acid identity was determined to locate the specific positions of proteolytic cleavage. The largest peptide fragment of FGF23 detected in the tPA-containing reactions had a mass of 17,267 Da (Fig. 5A), corresponding to amino acids 25 to 175 with an intact disulfide bond between cysteine residues 96 and 113 (Fig. 5B). No fragments of FGF23 were detected in the FGF23 alone reactions. Six other peptide fragments of FGF23 were detected in the tPA-catalyzed reactions as well, and a peptide map was generated summarizing these fragments (Fig. 5C), with numbers indicating which amino acids were present at each end. The table generated from this summary shows the specific tPA-dependent FGF23 cleavage sites (Fig. 5D). Amino acid numbers on the left side of the table, and standard amino acid single-letter code on the right side, indicate the positions within full-length human FGF23 where cleavage occurs, represented by the "^" symbol. The flanking four residues on each side of the cleavage site are also included for reference.

**TMS441 prevents PAI-1 inhibition of tPA-catalyzed FGF23 proteolysis**

Because PAI-1 is the main physiological inhibitor of tPA and uPA, we next tested whether or not FGF23 proteolysis by the PAs in vitro could occur in the presence of PAI-1. This mimics physiological conditions where increased circulating levels of PAI-1 are present, such as those seen in CKD, systemic inflammation, or aging (18, 34, 35). Again using purified human recombinant proteins, reactions containing FGF23, tPA, and PAI-1 were performed for 4 hours at 37°C. Incubation of FGF23 with tPA expectedly produced lower-molecular weight bands of approximately 17 and 13 kDa, which were completely absent if PAI-1 was present in the reaction (Fig. 5, lanes 4 and 5). Finally, addition of the novel small-molecule PAI-1 antagonist TM5441 to...
the reaction restored the proteolytic activity of tPA on FGF23 in the presence of PAI-1, as evidenced by the presence of the 17- and 13-kDa bands (Fig. 5, lane 6).

**DISCUSSION**

The findings reported in this study expand our understanding of the regulation of the complex and highly dynamic homeostasis of the phosphaturic hormone FGF23. Previous investigations of FGF23 proteolysis have revealed that inactivating proteolysis can occur at an RXRR motif located at FGF23 residues R176 and R179, a process that is thought to be mediated by SPCs (7). Here, we demonstrate for the first time that extracellular proteases of the plasminogen activation system, tPA and uPA, display proteolytic activity against FGF23 in vitro and both produce reaction products that have identical mobility on SDS-PAGE, suggesting that tPA and uPA most likely cleave FGF23 at the same sites. Although both proteases recognize similar target sequences and cleave substrates at arginine residues, the optimum target consensus sequences for each differ substantially (36). In time course comparisons of FGF23 proteolysis by tPA and uPA, tPA appears to exhibit greater catalytic efficiency in FGF23 proteolysis than does uPA.

Previous reports have identified the susceptibility of FGF23 to proteolytic cleavage by furin, which is confined to the same RXRR motif. In contrast, we report here for the first time that tPA cleavage of FGF23 occurs at multiple RX motifs, although R176 seems to be the preferred cleavage site to both tPA and furin. Because of the R179Q mutation present in the rhFGF23 protein used in this study, we did not detect any peptides generated by cleavage at R179. Although the largest peptide product that we found in these experiments was a 17-kDa fragment produced from cleavage at the R176 site (Fig. 5A), MS analysis of the tPA-generated peptides of FGF23 revealed several other novel tPA peptide targets. Some of these novel sites lie in additional RXR motifs present in FGF23, such as those seen at residues R140 and R228 (Fig. 6D). Most of these novel proteolytic cleavage sites are present in the C-terminal portion of FGF23. In contrast, we detected an intact disulfide bond in the N-terminal fragment, and it appears likely that this disulfide bond stabilizes the conformation of the N-terminal region that renders it resistant to proteolysis. One novel proteolytic site is located just distal to the disulfide bonding cysteine residue C113 (Fig. 6, B and D). Future investigations are warranted to examine the possible contribution of the disulfide bond to the metabolic stability of the N-terminal fragment and its potential effects on FGF23 signaling through the FGFR-klotho receptor complex.

Many studies have verified that posttranslational modification determines whether FGF23 polypeptide is secreted as an intact biologically active molecule or subjected to intracellular proteolysis (37). FGF23 is O-glycosylated at the residue T176 by the enzyme ppGalNAc-T3 (polypeptide N-acetylgalactosaminyltransferase 3), and this modification blocks its proteolysis and is required for the release of iFGF23 (38). Furthermore, a secretory kinase, FAM20C, phosphorylates FGF23 on S180 within the furin recognition motif R176XXR179/S180, and this phosphorylation blocks the O-glycosylation of FGF23 and renders it susceptible to proteolysis by furin (14, 39). It seems that these dynamic posttranslational processes including phosphorylation, glycosylation, and proteolysis play a critical role in the intracellular regulation of FGF23 homeostasis. In light of these findings, it is important to note that both tPA and uPA can cleave glycosylated FGF23, because glycosylated rhFGF23 was used in this study. This contention is supported by the fact that PAI-1 inhibition decreased FGF23 levels in kl/kl and Pai-1−/− transgenic mice, both of which likely have glycosylated FGF23, because the activity of the enzyme ppGalNAc-T3 has not been genetically altered in these animals.

Reductions in elevated plasma levels of FGF23 mediated by either TM5441 inhibition or genetic deficiency of PAI-1 in four different murine models including kl/kl, Pai-1−/−, WT, and Pai-1−/− mice with AKI serve as an important proof of concept. These findings have therapeutic implications for conditions and diseases associated with high levels of FGF23, including LVH, CKD, AKI, and ADHR. TM5441 inhibition of PAI-1 activity resulted in reduced levels of the total FGF23 in plasma measured by the C-term FGF23 ELISA, an assay that detects both iFGF23 and cFGF23, through recognition of the C-terminal fragment generated by cleavage at residue R176 (peptide 176–251) or R179 (peptide 179–251) of iFGF23. Because we identified novel cleavage sites of FGF23 by tPA within these peptides, namely, R196 and R228 (Fig. 6D), epitopes used by the capture and detection antibodies of the ELISA kit are most likely altered by the tPA proteolysis. Thus, the C-term FGF23 ELISA assay used in this study likely does not recognize advanced PA-digested peptides of C-terminal fragment of FGF23 and underestimates the TMS441-induced decreases in total FGF23, which further supports the conclusion that the inhibition of PAI-1 activity reduces circulating FGF23 levels.

This study demonstrates that the small-molecule PAI-1 antagonist TM5441 permits FGF23 proteolysis in the presence of PAI-1. We have observed that PAI-1 deficiency and TM5441 treatment decreased plasma FGF23 levels by 98% (17) and 66% (this study) in kl/kl mice, respectively. Although the resultant FGF23 levels are significantly higher than those of WT mice, modulating PAI-1 activity by either TM5441 treatment or genetic deficiency caused important physiological changes that extended the life span of kl/kl mice by fourfold. In addition, we detected that PAI-1−/− deficient kl/kl mice exhibit normalized plasma levels of creatinine, senescence messaging secretory factors, and reduced vitamin D3 (17). Many of the diseases and conditions associated with elevated FGF23 levels also display elevated PAI-1 levels, such as CKD (19, 40–42). Thus, pharmacological inhibition of PAI-1 might be useful in this setting to reduce FGF23 levels and possibly to restore homeostasis to other targets of PA extracellular proteolysis, including IGFBP3 (25). It has also been previously demonstrated that PAI-1 can
inhibit furin (43), another serine protease implicated in FGF23 proteolysis (14). However, because furin is thought to be mainly intracellular, it likely has no or minimal effect on extracellular processing of FGF23. Together, it seems likely that furin serves as the intracellular mechanism of regulating FGF23 processing, whereas the PAs play a critical role in the extracellular proteolysis of FGF23. PAI-1 has the potential to regulate both intracellular and extracellular homeostasis of FGF23 by inhibiting furin in the Golgi space and tPA and uPA in the extracellular space, respectively. Although PAI-1 inhibition accelerates FGF23 clearance and tPA cleaves FGF23 in multiple sites, including the furin cleavage site R176 that inactivates FGF23, it is not known whether the tPA-generated FGF23 peptides retain or acquire the capacity to bind to FGFR and act as a receptor agonist or as an antagonist to iFGF23. Although we speculate that the tPA-mediated proteolysis of FGF23 inactivates the protein, it is certainly possible that proteolysis may generate fragments with important functional properties.

Coding sequence mutations in FGF23 cause ADHR in humans. In this rare disorder, the furin cleavage site is mutated, leading to IFGF23 accumulation, unrelenting renal phosphate excretion, and bone disease. In this circumstance, the PA system is likely to be the only protease/ protease inhibitor system that can play a role in FGF23 homeostasis. Thus, inhibition of PAI-1 activity in these patients may be desirable. More commonly, patients with CKD have elevated FGF23 levels, even though the genetic mutations causing the above disorders do not exist. This suggests that elevated FGF23 levels are likely caused by augmented FGF23 expression or impaired clearance of FGF23 due to possible alterations in the balance between the activities of proteases and protease inhibitors. Although pharmacological inhibition of PAI-1 will not directly affect FGF23 expression, this approach will likely increase elimination of FGF23 from the circulation.

Our findings indicate that either genetic deficiency or pharmacological inhibition of PAI-1 can reduce circulating FGF23 levels by improving its proteolytic clearance but does not improve mineral homeostasis in kl/kl mice and mice with AKI, both of which lack functional FGF2. In addition to its roles in phosphate excretion and reducing the vitamin D$_2$ levels in blood, emerging evidence implicates FGF23 in LVH. The majority of patients with CKD and ESRD die from CVD that features LVH (4, 26, 44). FGF23 has been shown to induce LVH through FGFR but independent of klotho, the kidney-specific co-receptor of FGF23, via the PLC-γ (phospholipase C-γ)–calcineurin–NFAT (nuclear factor of activated T cell) pathway (4). Because the common treatment to prevent CVD is generally less effective in patients with CKD and ESRD, the potential value of PAI-1 inhibitors in cardiovascular mortality in these patient groups merits further investigation.

Phosphaturic mesenchymal tumors are rare, difficult to locate, and often linked to tumor-induced osteomalacia (TIO). Excessively high levels of FGF23, secreted by mesenchymal tumors, have been established as the causative factor for TIO, in which patients present with hypophosphatemia due to increased renal phosphate wasting, low vitamin D$_2$ levels, and reduced bone density (45–47). Although a number of different therapeutic approaches are currently being explored to treat this rare disorder, our observations indicate that pharmacological inhibition of PAI-1 may provide some therapeutic benefit in patients with TIO due to elevated FGF23.

In the FA-induced AKI model, FGF23 levels rise sharply due to increased FGF23 synthesis primarily in bone and possibly in other tissues as well. We present evidence that increased PAI-1 levels contribute to increased plasma FGF23 levels in mice with AKI. We observed that genetic PAI-1 deficiency reduces FGF23 levels in kl/kl mice and mice with AKI more than PAI inhibition. This finding suggests that PAI-1 itself may influence a receptor-dependent feedback loop that actually governs FGF23 production. This hypothesis is supported by our findings that PAI-1 inhibition was more effective in lowering plasma FGF23 levels in Pai-1stab transgenic mice, which likely have impaired clearance of FGF23 but not pathologically induced FGF23 synthesis. Together, the present study indicates that inhibition of PAI-1 activity significantly augments the clearance of endogenous or exogenously delivered FGF23 in mice. These findings suggest that selective PAI-1 inhibition may provide a multifaceted therapeutic benefit in humans with elevated FGF23 levels.

**MATERIALS AND METHODS**

**Study design**

The purpose of this study was to explore the effects of serine proteases, tPA and uPA, and the SERPIN PAI-1 of the plasminogen activation system on the clearance of FGF23, an endocrine hormone that primarily affects kidneys to regulate phosphate homeostasis. On the basis of our previous observation that PAI-1 deficiency reduced the extremely high levels of FGF23 in kl/kl mice, we hypothesized that PAs and PAI-1 directly regulate FGF23 homeostasis and this will result in altering FGF23 signaling. We tested this hypothesis using several in vivo models that have elevated levels of FGF23 and PAI-1 including kl/kl mice, PAI-1 transgenic mice, mice with AKI, and mice that were tail vein–injected with rhFGF23. We modulated PAI-1 activity either by administering a small-molecule PAI-1 antagonist, TMS441, or by genetic deficiency of PAI-1. Using recombinant human proteins, we further tested in vitro the proteolysis of FGF23 by tPA and uPA in the presence and absence of PAI-1 and additionally identified the peptide bonds of FGF23 cleaved by tPA. To avoid variations due to circadian rhythm, plasma samples were collected at the same time of the day. The end points of the study were measuring plasma levels of cFGF23, iFGF23, and PAI-1. We used a sample size varying from 6 to 14 mice in study groups to achieve a 90% power and a significance at P < 0.05 using the PS software to calculate power and sample size available from http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize.

**Mice**

All mice used in this study were housed in the Northwestern University Feinberg School of Medicine, and all the procedures were approved by the Institutional Animal Care and Use Committee of Northwestern University. kl/kl mice were obtained from M. Kuro-o and were of mixed background. Breeder pairs of all other mice strains were obtained from The Jackson Laboratory and of the C57BL/6J background. Study groups were 10- to 12-week-old mice except for kl/kl mice. Because of their short life span, kl/kl mice were 4 weeks old when admitted into the study.

**Recombinant proteins and quantitation of circulating factors**

Purified recombinant human two-chain tPA (catalog no. HTPA-TC), uPA (catalog no. UPA-LMW), and active PAI-1 (catalog no. CPAI) were obtained from Molecular Innovations. Purified recombinant human FGF23 (rhFGF23) protein containing R179Q mutation (native FGF23 is not available commercially) was obtained from R&D Systems (catalog no. 2604-FG-025). Plasma levels of FGF23 were determined using the following ELISA kits from ImmunoSays Inc.: Mouse/Rat FGF23 (C-Term) ELISA Kit (catalog no. 60-6300), Mouse/Rat FGF23...
(Intact) ELISA Kit (catalog no. 60-6800), and Human FGF23 (C-Term) ELISA Kit (catalog no. 60-6100). The Mouse PTH 1-84 ELISA Kit was purchased from Immutopics (catalog no. 60-2305). Total PAI-1 antigen levels in mouse plasma samples were measured using the ELISA kit from Molecular Innovations (catalog no. MPAIKT-TOT). Serum phosphate levels were measured using a kit from Stanbio Laboratories (catalog no. 0830-125).

Pharmacological inhibition of PAI-1

The PAI-1 antagonist TM5441 was obtained from T. Miyata. A 2.5 mM stock solution was prepared in dimethyl sulfoxide (DMSO) and used for the in vitro studies. Rodent diet containing the inhibitor was prepared by mixing a homogeneous suspension of TM5441 in 0.5% carboxymethyl cellulose into powder standard rodent diet (Harlan Teklad LM-485 number 7012), dried at room temperature, and stored at 4°C until use. Our preliminary tests have shown that the effective inhibitor doses were 10 mg/kg per day for kl/kl mice and 100 mg/kg per day for Pai-1stab mice. The reason for the higher dose of TM5441 required for Pai-1stab is likely to be the levels of PAI-1 stab in plasma and tissues as well as the transgenic expression of functionally stabilized human PAI-1 in Pai-1stab mice.

FGF23 clearance under normal physiological and AKI conditions

We analyzed the elimination of FGF23 from circulation and induced AKI, as described previously (33). Briefly, we reconstituted rhFGF23 in sterile saline solution and injected into the mice via the tail vein at a dose of 40 μg/kg 24 hours after they were treated with either vehicle or FA (240 mg/kg). Plasma samples were collected at 1, 3, 5, 15, 30, 60, and 120 min after injection of rhFGF23. Plasma samples were diluted 100-fold, and iFGF23 and cFGF23 levels were measured by ELISA method in duplicate of each sample. Results were analyzed by using nonlinear curve fitting statistical analysis function of GraphPad Prism.

In vitro protease reactions and Western blotting

rhFGF23 (1 to 1.5 μg) was incubated at 37°C for the indicated time points with either recombinant human TPA (1.1 μg) or uPA (1.1 μg). As needed, tPA or uPA was inhibited with 1.7 μg of recombinant active human PAI-1. Reactions were carried out in 25-μl final volume containing 50 mM tris-HCl (pH 8.0), 150 mM NaCl, 0.2 mM CHAPS, 0.1% PEG-8000 (polyethylene glycol, molecular weight 8000), and 1% DMSO. To terminate the reaction, samples were incubated at 95°C for 5 min in the presence of 1× NuPAGE LDS sample buffer and 1× NuPAGE sample reducing agent. Gels were silver-stained using the Pierce Silver Stain Kit from Thermo Scientific according to the manufacturer’s protocol. The anti–6xHis-tag antibody was obtained from Abcam, and the FGF23 antibody was obtained from Santa Cruz Biotechnology. Secondary horseradish peroxidase (HRP)–conjugated goat anti-rabbit antibodies were obtained from Molecular Innovations. NuPAGE 4-12% Bis-Tris Gels, PVDF iBlot Gel Transfer Stacks, 4× NuPAGE LDS sample buffer, and 10× NuPAGE sample reducing agent were from Life Technologies. Luminata Forte Western HRP substrate detection reagent was from EMD Millipore.

Determination of FGF23 fragments and sample preparation

Proteins [2.2 μg of tPA and 2 μg of FGF23 alone or in combination, incubated for 4 hours at 37°C in a 50-μl final volume of tris-buffered saline (pH 8.0)] were precipitated using the methanol-chloroform-water method (48). After precipitation, the sample was resuspended in 35 μl of 95% water, 5% acetonitrile + 0.1% formic acid. For MS, samples (5 μl) were injected onto a trap column [150 μm inside diameter (ID) × 3 cm] coupled with a nanobore analytical column (75 μm ID × 15 cm). The trap and analytical columns were packed with polymeric reversed-phase (PLRP-S, Phenomenex) medium (5 μm, 1000 Å pore size). Samples were separated using a linear gradient of solvent A and solvent B (5% water, 95% acetonitrile, and 0.1% formic acid) over 60 min. MS data were obtained on a 12T Velos FT Ultra (Thermo Fisher Scientific) mass spectrometer fitted with a custom nanospray ionization source. Intact MS data were obtained at a resolving power of 170,000 [mass charge ratio (m/z) 400]. The top 2 m/z species were isolated within the Velos ion trap and fragmented using CID. The data were initially processed with the cRAWler algorithm to create PUF files for searching with ProSightPC. The data were searched against a custom database containing only tissue plasminogen activator (UniProt: P00750) and fibroblast growth factor 23 (UniProt: Q9GZV9, modified usingXM: e1603259 13 September 2017)

Statistical analysis

Nonlinear curve fit analysis was used to evaluate the biphasic clearance of FGF23 from the circulation. We report the data group means ± SD. Results were considered statistically significant if P < 0.05. T test and ANOVA were used.

REFERENCES AND NOTES


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