The fibroblast growth factor receptor mediates the increased FGF23 expression in acute and chronic uremia

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**FGF23** is produced by osteocytes and osteoblasts, binds to its receptor, the fibroblast growth factor receptor 1 (FGFR1)-klotho heterodimer, in the kidney to cause a phosphaturia and decrease the synthesis of 1,25(OH)₂ vitamin D (25, 10). In addition, FGF23 acts on the FGFR1-klotho in the parathyroid to decrease parathyroid hormone (PTH) gene expression and parathyroid cell proliferation (2). In chronic kidney disease (CKD), there are extremely high levels of serum FGF23, which is one of the markers of the increased mortality in these patients (13). FGF23 also acts independently of klotho through FGFR1 and the calcineurin pathway in the heart and parathyroid (6, 8, 21). The synthesis and secretion of FGF23 by osteocytes are increased by a number of systemic factors (26). The fibroblast growth factor receptor mediates the effect of 1,25(OH)₂ vitamin D, which binds to a vitamin D-response element (VDRE) in the FGF23 promoter (15). One of the Nurr1 elements at the −200- to −399-bp region of the mouse FGF23 promoter is part of a VDRE in the FGF23 promoter (14). This vitamin D receptor (VDR)/Nurr1-element mediates the effect of 1,25(OH)₂ vitamin D and is a cis-regulatory module anchored by adjacent ETS1, a transcription factor that cooperates with VDR (4, 24), and VDRE/Nurr1 sites (14). Calcium, phosphorus retention in the milieu of CKD, metabolic acidosis, estrogens, leptin, and cleaved klotho have all been shown to increase serum FGF23 levels (16, 23, 27).

There are many local factors in the bone matrix that also regulate FGF23. These include the enzymes and transporters that determine the concentrations of phosphate and pyrophosphate in the bone matrix, namely, ANK1, ENPP1, ENTPD5, and TNAP (26). DMP1 binds to Phex in the osteocyte and inhibits FGF23 transcription (9). Mutations in the genes coding for these local factors lead to changes in FGF23 expression and subsequent coordinate changes in serum phosphate and 1,25(OH)₂ vitamin D (3, 26). FGFR signaling in the osteocyte stimulates FGF23 expression (29). Activating mutations in FGFR1 lead to an increase in serum FGF23 as in osteoglophonic dysplasia (28). The FGF2 gene produces 18-kDa low-molecular weight (LMW) FGF2 and 22- to 34-kDa high-molecular weight FGF2 isoforms created by alternative initiation codons. LMW-FGF2 binds to the cell surface FGFRs and heparin-sulfate proteoglycans (22). They stimulate FGF23 promoter activity in osteoblasts through membrane FGFR-mediated PLCγ and MAPK activation of NFAT and ETS1. HMW-FGF2 isoforms have an N-terminal nuclear localization sequence that leads to nuclear localization and activation of intracellular FGFR1/CREB/CRE signaling pathways (11). FGFR1, FGFR2, both LMW and HMW, and FGFR7 are ligands for FGFR1. PTH increases FGF2 and FGFR1 and FGF2 mRNA levels in neonatal mouse calvarial organ cultures, suggesting that some effects of PTH on bone remodeling may be mediated by regulation of FGFR2 and FGFR expression in osteoblastic cells (12). In CKD, the high serum PTH levels increase bone remodeling, which may release FGFs from the bone matrix to act on the osteocyte FGFRs and increase FGF23 expression. Activation of FGFRs by an activating antibody leads to an increase in serum FGF23, and inhibition of FGFRs both pharmacologically and by gene deletion leads to a decrease in FGF23 transcription in bone cells in vitro (29, 30). Conditional deletion of FGFR1 in osteocytes of Hyp mice reduces FGF23 expression (18).

It has recently been shown that there is an increase in serum FGF23 as early as 15 min after bilateral nephrectomy in rats that is independent of changes in serum calcium, phosphate,
and PTH (19). In acute kidney injury (AKI) in mouse models, there is a marked increase in serum FGF23 levels as early as 1 h after the induction of AKI by high-dose folic acid (5). The increased FGF23 precedes the increase in serum phosphate in this AKI model. We now report that the increase in FGF23 expression in folic acid-induced AKI as well as in adenine high phosphorus-induced CKD is prevented by the administration of a pharmacological FGFR inhibitor, an effect independent of changes in serum calcium, phosphate, and PTH.

**MATERIALS AND METHODS**

**Animals.** Male C57BL/6 mice at 11–13 (30 g) wk of age were fed a regular chow, and AKI was induced by a single intraperitoneal (ip) injection (300 μl) of folic acid (240 mg/kg in vehicle: 0.15 mol/l NaHCO3, pH 7.4, Sigma-Aldrich, St. Louis, MO). Control animals were injected with vehicle. CKD was induced in male Sprague-Dawley rats (120 –150 g) by a 0.75% adenine, high-phosphorus (1.5%) diet (Teklad; Harlan Laboratories, Madison, WI) given for 14 days. Controls received regular chow containing 0.7% phosphorus.

The FGFR inhibitor PD173074 from Sigma-Aldrich (19 mg/kg), solubilized in polyethylene glycol 300 (Sigma-Aldrich), 5% glucose, 2:1 or from LC Laboratories (10 mg/kg, Woburn, MA) solubilized in 96% ethanol, was given by oral gavage. PD173074 or vehicle was given to AKI mice 1 h before a single injection of folic acid or vehicle. The adenine high phosphorus-fed uremic rats received PD173074 or vehicle twice daily for the last 2 days of the 14-day diet. At the end of each experiment, blood was drawn and calvaria bone was excised. All animal experiments were approved by the Hadassah Hebrew University Animal Care and Use Committee.

**Serum biochemistry, PTH, and FGF23 measurements.** Serum was analyzed for serum calcium and blood urea nitrogen (BUN) using Quantichrom kits (BioAssay Systems, Hayward, CA). Serum phosphate was analyzed by a Stanbio Phosphorus Liqui-UV kit (Stanbio Laboratories, Boerne, TX). Serum PTH was measured using Mouse 1–84 or Rat Intact ELISA kits (Immutopics, San Clemente, CA). Mouse FGF23 was measured using either a FGF23 ELISA kit (Kainos, Tokyo, Japan) or an intact mouse/rat FGF23 (Immutopics).

**RNA extraction from rat calvaria.** Calvaria bones were removed and immediately snap-frozen in liquid nitrogen. RNA was extracted from half of each calvaria disc using TRIzol Reagent (Invitrogen, Carlsbad, CA). The tissue was homogenized with a bead beater. RNA integrity was confirmed by agarose gel.

**Quantitative RT-PCR analysis.** cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and quantitative (q) PCR analysis performed with PerfeCTa SYBR Green FastMix (Quanta, Gaithersburg, MD) in a 7900HT Fast Real-Time PCR System (Applied Biosystems).

**Primers.** Primers for qRT-PCR were designed with Primer Express 3.0 software (Applied Biosystems). Primers were as follows: mouse FGF23: Fw: 5′-CGGCTCTGTTGATAACG-3′ and Rev: 5′-GGCTTAGTGGATCCA-3′; mouse β-actin: Fw: 5′-CTCTGAGCACTTCA-3′ and Rev: 5′-GTTCTCTACCTTTCA-3′; Rat FGF23: Fw: 5′-TGGATCGTATCACTT-3′ and Rev: 5′-TGCTTCGGTGACAGGT-3′; and rat GAPDH: Fw: 5′-CGGCTCTGTTGATAACG-3′ and Rev: 5′-GGCTGAGTGGATCCA-3′.

**Fig. 1.** Serum blood urea nitrogen (BUN), phosphate, and parathyroid hormone (PTH) levels are increased in folic acid-induced acute kidney injury (AKI) with no effect of the FGF receptor (FGFR) inhibitor PD173074 in mice. A: mice received PD173074 or vehicle by oral gavage and 1 h later folic acid intraperitoneally (ip) or vehicle. At 6 h after folic acid, serum was analyzed for BUN (B), phosphate (C), calcium (D), and PTH (E). *P < 0.01 vs. vehicle or PD173074; n = 4–6.
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FGFR1 mediates the increase in FGF23 expression in folic acid-induced AKI in mice. To study the increase in FGF23 expression in AKI, we injected mice with folic acid and the selective pan-FGFR inhibitor PD173074 (29) (Fig. 1A). Folic acid led to the expected increase in serum urea and phosphate with no change in serum calcium at 6 h (Fig. 1, B–D). Serum PTH increased sevenfold, and this increase was not affected by the administration of PD173074 (Fig. 1E). Folic acid led to the expected increase in serum FGF23 (Fig. 2A). FGF23 mRNA levels were increased sevenfold by folic acid at 6 h (Fig. 2B). Pretreatment with PD173074 decreased basal serum FGF23 levels. Importantly, PD173074 prevented the folic acid-induced increase in serum FGF23 and mRNA levels (Fig. 2). Therefore, the FGFR is essential for the increased FGF23 expression by folic acid-induced AKI independently of serum calcium, phosphate, and PTH.

FGFR1 mediates the increase in FGF23 expression in adenine high phosphorus-induced CKD in rats. We then studied the effect of FGFR inhibition in experimental CKD. Rats were fed an adenine high-phosphorus diet for 2 wk and the FGFR inhibitor PD173074 administered by oral gavage twice daily for the last 2 days (Fig. 3A). The adenine diet resulted in renal failure with a small decrease in serum calcium and no change in serum phosphate (Fig. 3, B–D). PD173074 increased serum phosphate in the adenine-fed rats. Serum PTH was increased 17-fold by the adenine high-phosphorus diet, and this increase was not affected by treatment with PD173074 (Fig. 3E). FGF23 mRNA and serum FGF23 levels were increased 17- and 3.5-fold, respectively, by the adenine diet (Fig. 4). PD173074 given to the uremic rats corrected the high levels of FGF23 mRNA and serum FGF23 (Fig. 4). The normalized FGF23 levels in the PD173074-treated uremic rats may be responsible for the increase in serum phosphate in these rats (Fig. 3C). Therefore, the FGFR is essential for the increased FGF23 expression of both acute and chronic renal failure.

RESULTS

FGFR1 mediates the increase in FGF23 expression in folic acid-induced AKI in mice. To study the increase in FGF23 expression in AKI, we injected mice with folic acid and the selective pan-FGFR inhibitor PD173074 (29) (Fig. 1A). Folic acid led to the expected increase in serum urea and phosphate with no change in serum calcium at 6 h (Fig. 1, B–D). Serum PTH increased sevenfold, and this increase was not affected by the administration of PD173074 (Fig. 1E). Folic acid led to the expected increase in serum FGF23 (Fig. 2A). FGF23 mRNA levels were increased sevenfold by folic acid at 6 h (Fig. 2B). Pretreatment with PD173074 decreased basal serum FGF23 levels. Importantly, PD173074 prevented the folic acid-induced increase in serum FGF23 and mRNA levels (Fig. 2). Therefore, the FGFR is essential for the increased FGF23 expression by folic acid-induced AKI independently of serum calcium, phosphate, and PTH.

Discussion

We now show that the high levels of FGF23 in acute and chronic kidney failure are mediated by the FGFR. Inhibition of the FGFR by PD173074 prevented the increase in serum FGF23 in folic acid-induced AKI as well as in adenine high phosphorus-induced uremia. The changes in serum FGF23 levels were also evident at the level of FGF23 gene expression. Acute and chronic uremia increased calvarial FGF23 mRNA levels that were prevented by FGFR inhibition. Therefore, the FGFR has an important role in determining the high levels of FGF23 in both acute and chronic uremia by an action on FGF23 gene expression.

Parathyroidectomy of early CKD rats prevented and corrected the high FGF23 levels of uremia (17). Challenge with PTH increases FGF23 expression in vivo and in vitro in UMR106 osteoblast-like cells. This effect is mediated by the orphan nuclear transcription factor Nurrl, which binds to the FGF23 promotor and increases FGF23 after PTH/PKA activation (20). Both Nurrl and FGFR1 show a functional interaction in developing and mature dopaminergic neurons (1). In bone, PTH and the FGFR may act together through Nurrl to increase FGF23 gene expression.

Of interest, we show that the effect of the FGFR inhibitor was independent of changes in serum calcium, phosphate, and PTH. Mace et al. (19) found that rats after bilateral nephrectomy increased serum FGF23 at 15 min independently of serum calcium, phosphate, and PTH. Christov et al. (5) also had shown that the increased FGF23 in folic acid-induced AKI was independent of PTH, the PTH/PTH-related protein receptor (PPR), and VDR because mice with respective deletions in PTH, PPR, or VDR still increased FGF23 after folic acid. Therefore, despite the fact that 1,25(OH)2 vitamin D and PTH increase FGF23 levels and parathyroidectomy prevents the high FGF23 mRNA levels of experimental uremia, blockade of FGFR itself is sufficient to prevent the increase in FGF23 in uremia. These contrasting results suggest that there is an interdependence of the signaling of the PTH receptor, VDR, and FGFR on the FGF23 promoter. LMW FGF2 acts through NFAT and ETS1 and the HMW FGF2 through cAMP and CREB binding to a Cre in the FGF23 promoter. 1,25(OH)2 vitamin D upregulates ETS1, which cooperates with VDR (7, 24). An ETS1-VDRE/Nurrl1 composite conserved element has recently been defined in the FGF23 proximal promoter (14).
The proximity of these elements may allow interactions among the FGFR pathway through ETS1 and Cre, PPR, and PTH action through Nurr1 and VDR through the VDRE/ETS1. Stimulation with a variety of FGF ligands FGF-1, FGF-2, FGF-9, and FGF-18 significantly induced FGF23 transcription, which was blocked by PD173074 in UMR106 cells (29). LMW and HMW FGF-2 stimulated FGF-23 promoter activity in osteoblasts through membrane and nuclear FGFR signaling in MC3T3-E1 osteoblast precursor cells and SaOS-2 osteoblast cells (11). These in vitro studies show that FGFR ligands increase FGF23 transcription in different bone cells in culture and that inhibition of the FGFR prevents this effect. Inhibition of the FGFR also ameliorates the left ventricular hypertrophy of experimental uremia in rats (6). We show that FGFR activation mediates the high levels of FGF23 expression in both acute and chronic experimental uremia. Strategies aimed at decreasing FGFR-mediated effects on FGF23 production may benefit patients with CKD where the high levels of FGF23 are associated with mortality (13).

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DISCLOSURES
J. Silver is a member of an Amgen advisory board.

AUTHOR CONTRIBUTIONS

REFERENCES

Fig. 3. Adenine high phosphorus-induced renal failure increases serum PTH with no effect of PD173074 in rats. A: rats were fed a control or adenine diet for 14 days. PD173074 or vehicle was given by oral gavage for the last 2 days. B: serum creatinine. C: phosphate. D: calcium. E: PTH. *P < 0.01 compared with control diet. #P < 0.01 compared with adenine diet; n = 4–6.
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Fig. 4. The increase in FGF23 mRNA and serum levels after an adenine high-phosphorus diet is prevented by PD173074 in rats. Rats were fed a control diet or adenine high-phosphorus diet and PD173074 or vehicle given as in Fig. 3. A: calvarial FGF23 mRNA levels measured by qRT-PCR. B: serum FGF23.

*P < 0.01 compared with control diet. #P < 0.01 compared with adenine diet; n = 4–5.